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COVID-19 VACCINE:
TRADITIONAL VACCINE PLATFORMS
PART 1

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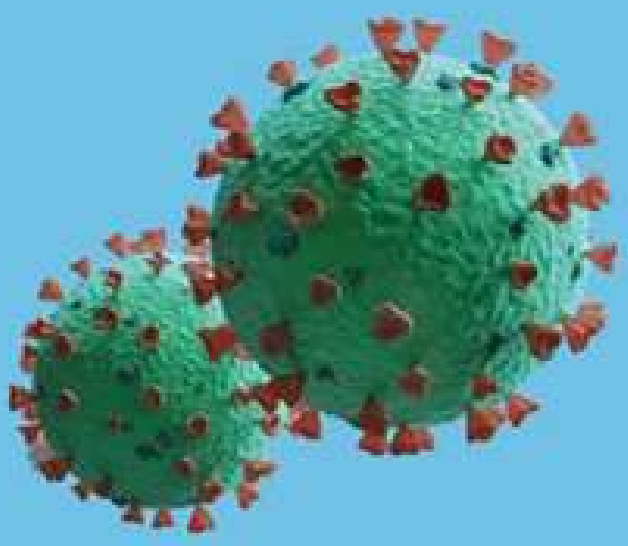

THE COVID-19 VACCINE

The work that will be presented below is an update and technical insight into the vaccine platforms developed for the prevention of COVID-19, complementing the first ebook "COVID-19: the vaccine to come" disseminated in May 2019.

Although much knowledge has been gained about the mechanism of damage caused by SARS-Cov-2 , the multi-organ pathologies of COVID-19, and effective therapies, many unanswered questions still remain, and the doubts and critical issues raised with concern by the scientific community about the large-scale use of vaccines and experimental drugs too quickly remain essentially unresolved.

The purpose of this paper is to provide an overview of vaccination platforms, as comprehensive as possible (but without claiming to be exhaustive) for dissemination purposes, divided into three areas:

- **Part One**: Traditional platforms (attenuated, inactivated virus, recombinant protein, and naoparticle vaccines) and vaccine adjuvants, with insights into virus genetics, immune system response to vaccines, and nanotoxicology

- **Part Two**: The innovative platforms (GMO viral vector and DNA/RNA vaccines) with an in-depth look at the experimental results of the two vaccines currently on the market with mRNA technology (vaccine "Pfizer" and "Moderna") particularly regarding the critical issues on the quality, efficacy, and safety of this new type of vaccines, as well as an update on the adenoviral vector vaccine, already reviewed in the first ebook

- **Part Three**: the accelerated, conditional, and emergency registration process, guidelines on GMO drugs, and nanotechnology applied to COVID-19 vaccines, with an in-depth look at guidelines for their industrial production (culture, isolation, purification of vaccine virus, and quality control required by competent authorities where available)

COVID-19 VACCINE: TRADITIONAL VACCINE PLATFORMS.

SUMMARY

INTRODUCTION

The development of SARS-Cov-2 vaccines has reached the conclusion of an important goal for the pharmaceutical industry with the December 2020 marketing authorization of the drug BNT162b2, coined under the trade name COMIRNATY, produced by the Pfizer-BioNTech company.

In fact, it is the first drug with GMO technology, and specifically with mRNA for preventive use against the agent that causes COVID-19, to be approved by the FDA and EMA regulatory agencies.

The importance is due to the fact that mRNA technology is very versatile, and together with that of adenoviral vectors, already discussed in detail in the previous ebook, it paves the way for the preventive and therapeutic application of GMO drugs, considered by industrial researchers to be among the most innovative tools in precision medicine. [1]

The coronavirus pandemic has been the subject of an unprecedented study by academic and industrial scientific researchers to understand the mechanisms of damage caused by SARS-Cov-2, the causative agent of COVID-19, and has seen a rush to test new drugs and vaccines by numerous companies in the biotech world.

As of Dec. 30, 2020, there are as many as **289** candidate vaccines of which **66** are in clinical phase [2].

As of December 28, 2020, the "Covid-19 - living NMA" initiative has collected 2358 treatment studies from the International Clinical Trials Registry Platform (ICTRP), of which 1317 are recruiting patients. [3]

The FDA is currently reviewing over 390 clinical trials for therapeutic treatments against COVID-19 under an expedited procedure, 8 treatments have been approved for emergency use, and 1 has received marketing authorization (remdesivir). [4] The EMA has 47 investigational drugs and 23 vaccines under evaluation. [5]

While the world of industrial research has greeted with great enthusiasm the very efficiently and rapidly achieved goal of commercializing the new prototype GMO vaccine [6], on the other hand

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[1] Ball P.
The lightning-fast quest for COVID vaccines - and what it means for other diseases.
Nature. 2021 Jan;589(7840):16-18. doi: 10.1038/d41586-020-03626-1. PMID: 33340018.
https://www.nature.com/articles/d41586-020-03626-1

[2] https://vac-lshtm.shinyapps.io/ncov_vaccine_landscape/ (12/30/2020)

https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines 3

COVID-NMA is an international research initiative supported by WHO and Cochrane.

https://www.who.int/publications/i/item/covid-19-landscape-of-experimental-treatments
https://www.covid-nma.com/dataviz/

Boutron I, Chaimani A, Meerpohl JJ, et al.
The COVID-NMA Project: Building an Evidence Ecosystem for the COVID-19 Pandemic.
Ann Intern Med. 2020;173(12):1015-1017. doi:10.7326/M20-5261
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7518109/

[4] https://www.fda.gov/drugs/coronavirus-covid-19-drugs/coronavirus-treatment-acceleration-program-ctap#dashboard

[5] https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines-covid-19#medicines-undergoing-evaluation-section

[6] https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-announce-vaccine-candidate-against

https://www.livescience.com/mrna-vaccines-future-vaccine-development.html
https://news.un.org/en/story/2020/12/1079322

there has been a growing vaccination distrust and hesitancy [7] that is opposing mass vaccination and multiple illegitimate imposition strategies, which blatantly violate all international declarations of the right to free choice and rights enshrined in the constitution, [8] without on the other hand being able to ensure that the goal of the containment and vaccination strategy, that is, to stop the transmission of infection, is achieved.[9]

DEEPENING: VIRUSES

It has been estimated that there are 10 -10^{31}[32] viruses in the Earth's atmosphere, an order of magnitude greater than the number of cells in our bodies. Consequently, every organism on the planet and probably every living cell constantly interacts with viruses, which are responsible for the greatest selective pressure for the evolution of all living species.

Despite their small size, viruses play an important role as obligate intracellular parasites, modulating the activity of their host cells for the purpose of replication and inducing adverse effects on both the infected cells and the whole organism.
The main emphasis of virology is focused on the identification and control of pathogenic viruses that invade humans, domestic animals, and plants, yet the origin, ~~and~~ organization of viruses, and their evolution are profound and fundamental questions for molecular virology.

In addition, sequencing of eukaryotic genomes has revealed that 5-10% of their DNA consists of coding genes, and a large fraction of the remainder is believed to be composed of mobile retrovirus-like elements (retro-transposons), which may have played a considerable role in the formation of these complex genomes.

Transposable elements in prokaryotes and eukaryotes
Genomic complexity and genetic variability - The role of transposons
Chromosome structure and transposable elements

Retroviruses and
retrotransposons

[7] Lazarus JV, Ratzan SC, Palayew A, et al.
A global survey of potential acceptance of a COVID-19 vaccine
[published online ahead of print, 2020 Oct 20]. Nat Med. 2020;1-4. doi:10.1038/s41591-020-1124-9
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7573523/

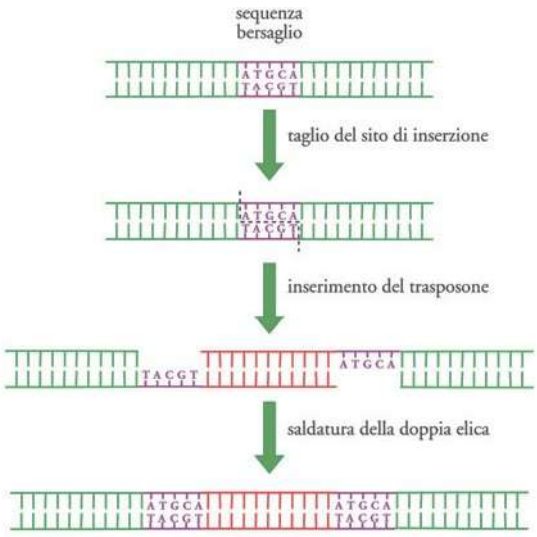
https://www.socialscienceinaction.org/resources/rapid-review-vaccine-hesitancy-and-building-confidence-in-covid-19-vaccination/
https://opendocs.ids.ac.uk/opendocs/bitstream/handle/20.500.12413/15794/SSHAP%20Rapid%20Review_Vaccine%20Hesitancy%20and%20Building%20Confidence%20in%20COVID-19%20Vaccination%20.pdf?sequence=4&isAllowed=y

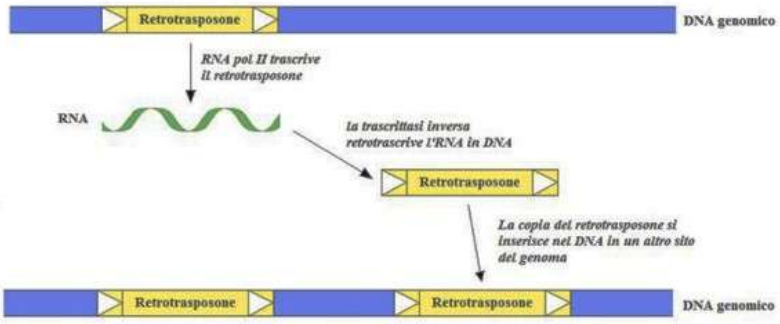
https://www.euronews.com/2020/10/16/coronavirus-only-around-1-3-of-french-respondents-would-take-covid-19-vaccine-euronews-pol

[8] Acosta, Juana I.,
Vaccines, Informed Consent, Effective Remedy and Integral Reparation: an International Human Rights Perspective,
131 Vniversitas , 19-64 (2015). http://dx.doi.org/10.11144/Javeriana.vj131.vier
http://www.scielo.org.co/pdf/vniv/n131/n131a02.pdf

http://www.assembly.coe.int/LifeRay/JUR/Pdf/TextesProvisoires/2020/20200702-CovidImpact-EN.pdf

[9] Bleier BS, Ramanathan M Jr, Lane AP.
COVID-19 Vaccines May Not Prevent Nasal SARS-CoV-2 Infection and Asymptomatic Transmission.
Otolaryngol Head Neck Surg. 2020 Dec 15:194599820982633. doi: 10.1177/0194599820982633. Epub ahead of print. PMID: 33320052.
https://journals.sagepub.com/doi/full/10.1177/0194599820982633





https://www.chimica-online.it/biologia/trasposoni.htm
Cut-and-sew transposition of transposonsMechanism of transposition of retrotransposons

Bacterial genomes do not have this extra genetic material, but the genomes of some bacteriophages bear a close resemblance to bacterial plasmids in their structure and the way they replicate, revealing that the relationship between viruses and other living organisms is perhaps more complex than previously thought. [10]

Finally, it is important to note that the study of metagenomics using new sequencing techniques [11] is enabling a deeper understanding of the virobiota and its fundamental function in the adaptive capacity and evolution of bacteria and higher organisms. [12]

The concept of hologenome evolution postulates that the holobiont (host + symbionts) with its hologenome (host genome + microbiome) is an evolving level of selection. Multicellular organisms can no longer be considered individuals according to the classical definition of the term, but every natural animal and plant is a holobiont consisting of the host and several symbiotic microbes and viruses.

A large number of studies have shown that these symbionts contribute to anatomy, physiology, development, innate and adaptive immunity, behavior, and finally also to genetic variation, origin, and evolution of species.[13]

[10] Chaitanya KV.
Structure and Organization of Virus Genomes.
Genome and Genomics. 2019;1-30. Published 2019 Nov 18. doi:10.1007/978-981-15-0702-1_1
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7119911/pdf/978-981-15-0702-1_Chapter_1.pdf

[11] Khan Mirzaei M, Xue J, Costa R, Ru J, Schulz S, Taranu ZE, Deng L.
Challenges of Studying the Human Virome - Relevant Emerging Technologies.
Trends Microbiol. 2020 Jul 1:S0966-842X(20)30162-1. doi: 10.1016/j.tim.2020.05.021.
https://www.cell.com/action/showPdf?pii=S0966-842X%2820%2930162-1

[12] Maurício Teixeira Lima, et al.
Virus and microbiota relationships in humans and other mammals: An evolutionary view,
Human Microbiome Journal, Volume 11, 2019, 100050, https://doi.org/10.1016/j.humic.2018.11.001.
http://www.sciencedirect.com/science/article/pii/S2452231718300356.

Pradeu T.
Mutualistic viruses and the heteronomy of life.
Stud Hist Philos Biol Biomed Sci. 2016;59:80-88. doi:10.1016/j.shpsc.2016.02.007
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7108282/

[13] Rosenberg E, Zilber-Rosenberg I.
Microbes Drive Evolution of Animals and Plants: the Hologenome Concept.
mBio. 2016;7(2):e01395. Published 2016 Mar 31. doi:10.1128/mBio.01395-15
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4817260/

Symbiotic relationships include several lifestyles, including **antagonistic** (or pathogenic, the most well-studied lifestyle for viruses), **commensal** (probably the most common lifestyle) and **mutualistic** (important beneficial partners).

Although antagonistic relationships are thought to lead to coevolution, this is not always clear in virus-host interactions, and the impacts on evolution can be complex. Commensalism implies a travel-only hitchhiking role for the selfish elements of viruses. Mutualistic relationships, on the other hand, have been described in detail over the past decade and reveal how important viruses are when considering host ecology.

Ultimately, symbiosis can lead to **symbiogenesis**, or speciation through fusion, and the presence of large amounts of viral sequences in the genomes of any living cell, from bacteria to humans, including some important functional genes [14], illustrates the significance of viral symbiogenesis in the evolution of all life on earth.[15]

DEFINITION AND ORIGIN OF

The definition of viruses is surprisingly controversial. [16]
This is largely due to the nature of the virus reproduction cycle apparently divided into two distinct phases:

- an intracellular phase during which the virus reprograms the infected cell to produce viral particles or virions (viral particle that can be purified and visualized. The nucleus of a virion comprises the virus nucleic acid (DNA or RNA) enclosed within a protein shell called a capsid),

- An extracellular phase: a phase during which virions escape infected cells and persist in the external environment (similar to plant seeds)

  Both stages, when considered separately, provide remarkably contrasting views of the nature and roles of viruses.

García-López R, Pérez-Brocal V, Moya A.
Beyond cells - The virome in the human holobiont.
Microb Cell. 2019;6(9):373-396. Published 2019 Jul 1. doi:10.15698/mic2019.09.689
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6717880/

[14] https://www.sciencefocus.com/the-human-body/virus-human-evolution/

[15] Roossinck MJ, Bazán ER.
Symbiosis: Viruses as Intimate Partners.
Annu Rev Virol. 2017 Sep 29;4(1):123-139. doi: 10.1146/annurev-virology-110615-042323. Epub 2017 Aug 8.
https://www.researchgate.net/publication/318999587_Symbiosis_Viruses_as_Intimate_Partners

Moelling K, Broecker F.
Viruses and Evolution - Viruses First? A Personal Perspective.
Front Microbiol. 2019;10:523. Published 2019 Mar 19. doi:10.3389/fmicb.2019.00523
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6433886/

[16] Nasir A, Romero-Severson E, Claverie JM.
Investigating the Concept and Origin of Viruses.
Trends Microbiol. 2020;28(12):959-967. doi:10.1016/j.tim.2020.08.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7609044/

Forterre P.
Defining life: the virus viewpoint.
Orig Life Evol Biosph. 2010;40(2):151-160. doi:10.1007/s11084-010-9194-1
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837877/

Kanduc D.
The comparative biochemistry of viruses and humans: an evolutionary path toward autoimmunity.
Biol Chem. 2019 Apr 24;400(5):629-638. doi: 10.1515/hsz-2018-0271. PMID: 30504522.
https://pubmed.ncbi.nlm.nih.gov/30504522/

For example, virions are metabolically inert infectious particles that do not meet any of the criteria we can use to define "life" or living organisms. [17]

Biologically, these recent definitions have been given:
A **living organism** can be defined as: "a collection of integrated organs (molecular apparatuses/structures) that produce individuals that evolve through natural selection" [18]
Life can be defined as a chemical self-maintenance system far from equilibrium that can process, transform and accumulate information acquired from the environment. [19]

However, as virions can be purified, counted, and visualized under the microscope, their physical and biochemical properties (e.g., size, shape, metabolic capacity, capsid) along with host/tissue specificity have become popular in the description, illustration, and naming of viruses (e.g., human immunodeficiency virus or SARS-Cov-2). These, in turn, have shaped our perceptions of viruses as inanimate nonliving biological objects that are, paradoxically, contagious.

Treating virions as viruses is a conceptual error that overlooks the dramatic changes that viruses introduce within infected cells. [20]
A virus-infected cell can be effectively turned into a "hot spot" for virion production and can virtually lose its identity (i.e., it now produces virions rather than two daughter cells) [21].

In some viral infections, large cell-like "virion factories" (viro-cells) are clearly visible. This remarkable transformation is due to virus-mediated manipulation and alteration of host metabolism and defenses [22].
The intracellular phase thus involves substantial viral activity and is often the target of antiviral drugs to combat infection (e.g., antivirals that target virus polymerases).

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[17] Forterre P.
Defining life: the virus viewpoint.
Orig Life Evol Biosph. 2010;40(2):151-160. doi:10.1007/s11084-010-9194-1
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837877/

[18] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837877/

[19] Vitas M, Dobovišek A.
Towards a General Definition of Life.
Orig Life Evol Biosph. 2019 Jun;49(1-2):77-88. doi: 10.1007/s11084-019-09578-5. Epub 2019 Jun 20.
https://pubmed.ncbi.nlm.nih.gov/31222432/

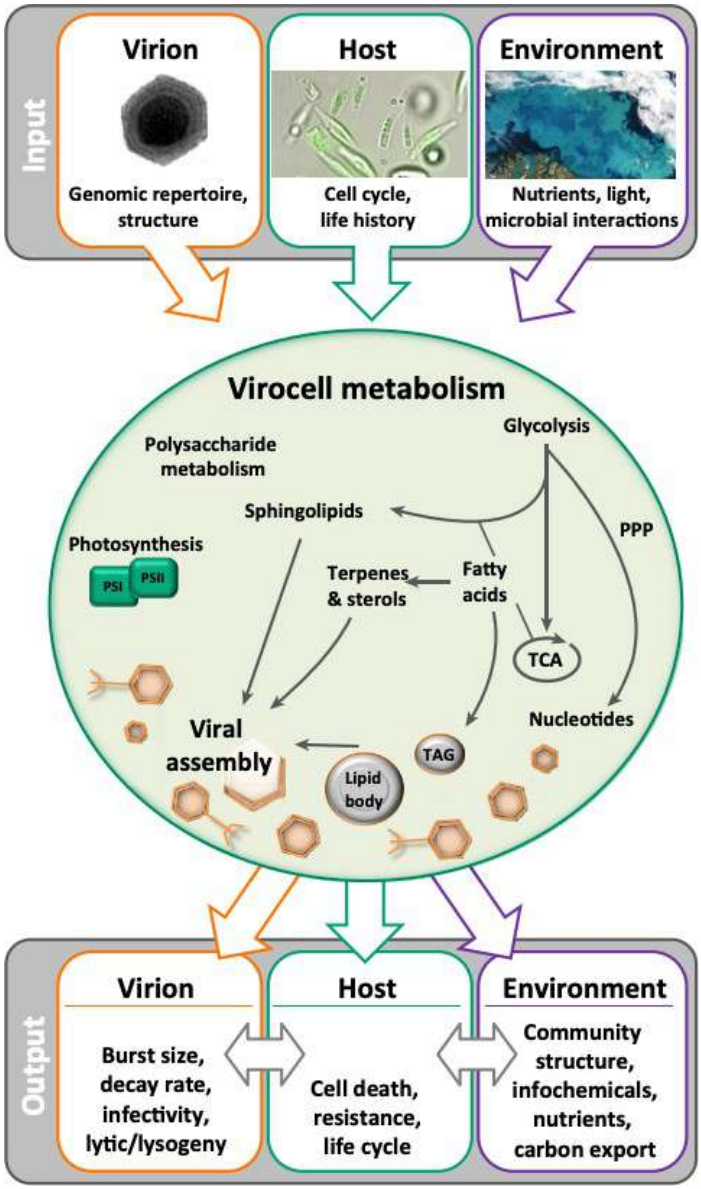
[20] Forterre P.
To be or not to be alive: How recent discoveries challenge the traditional definitions of viruses and life.
Stud Hist Philos Biol Biomed Sci. 2016 Oct;59:100-8. doi: 10.1016/j.shpsc.2016.02.013. Epub 2016 Mar 18. PMID: 26996409.
https://pubmed.ncbi.nlm.nih.gov/26996409/.

[21] Sakaguchi DS.
Genetic Manipulation and Selection of Mouse Mesenchymal Stem Cells for Delivery of Therapeutic Factors In Vivo.
Methods Mol Biol. 2019;1940:143-155. doi: 10.1007/978-1-4939-9086-3_10. PMID: 30788823.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7609044/

[22] Moniruzzaman M, Martinez-Gutierrez CA, Weinheimer AR, Aylward FO.
Dynamic genome evolution and complex virocell metabolism of globally-distributed giant viruses.
Nat Commun. 2020;11(1):1710. Published 2020 Apr 6. doi:10.1038/s41467-020-15507-2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7136201/

Rosenwasser S, Ziv C, Creveld SGV, Vardi A.
Virocell Metabolism: Metabolic Innovations During Host-Virus Interactions in the Ocean. Trends Microbiol. 2016 Oct;24(10):821-832. doi: 10.1016/j.tim.2016.06.006. Epub 2016 Jul 6.
https://www.cell.com/trends/microbiology/comments/S0966-842X(16)30069-5

Despite its immense role in establishing virus infection and existence within infected cells, the virion factory has unfortunately been referred to as the "eclipse" or "vegetative" phase to indicate the lack of distinctive signs of virus infection (e.g., lysis plaques and cell rupture) and ignored in virus definitions and descriptions.



https://www.cell.com/trends/microbiology/comments/S0966-842X(16)30069-5

Virocell metabolism is a unique metabolic state induced by viral infection.

Viral infection induces a significant metabolic shift in various host metabolic pathways such as photosynthesis, glycolysis, fatty acid metabolism, and nucleotide biosynthesis.

Viro-cell metabolism is determined by the host metabolic network, the viral gene repertoire, and dynamic environmental factors.

Viro-cell metabolism can substantially influence viral characteristics such as burst size and infectivity, host life cycle, and cell fate.

In addition, the metabolism of the viro-cell determines the chemical and microbial composition of the surrounding environment.

As suggested by Jean-Michel Claverie, the virion factory best represents the "virus self," and virions are simply means of disseminating genetic information in much the same way as human gametes and plant seeds [23].

In other words, we should depart from the established use of the word "virus" as a synonym for "virion." The term "virus" should refer to the process that encompasses all stages of the virus infection cycle [24]. In this context, questioning the origin of "viruses" takes on a completely different and much broader meaning than simply questioning the origin of viral particles.[25]

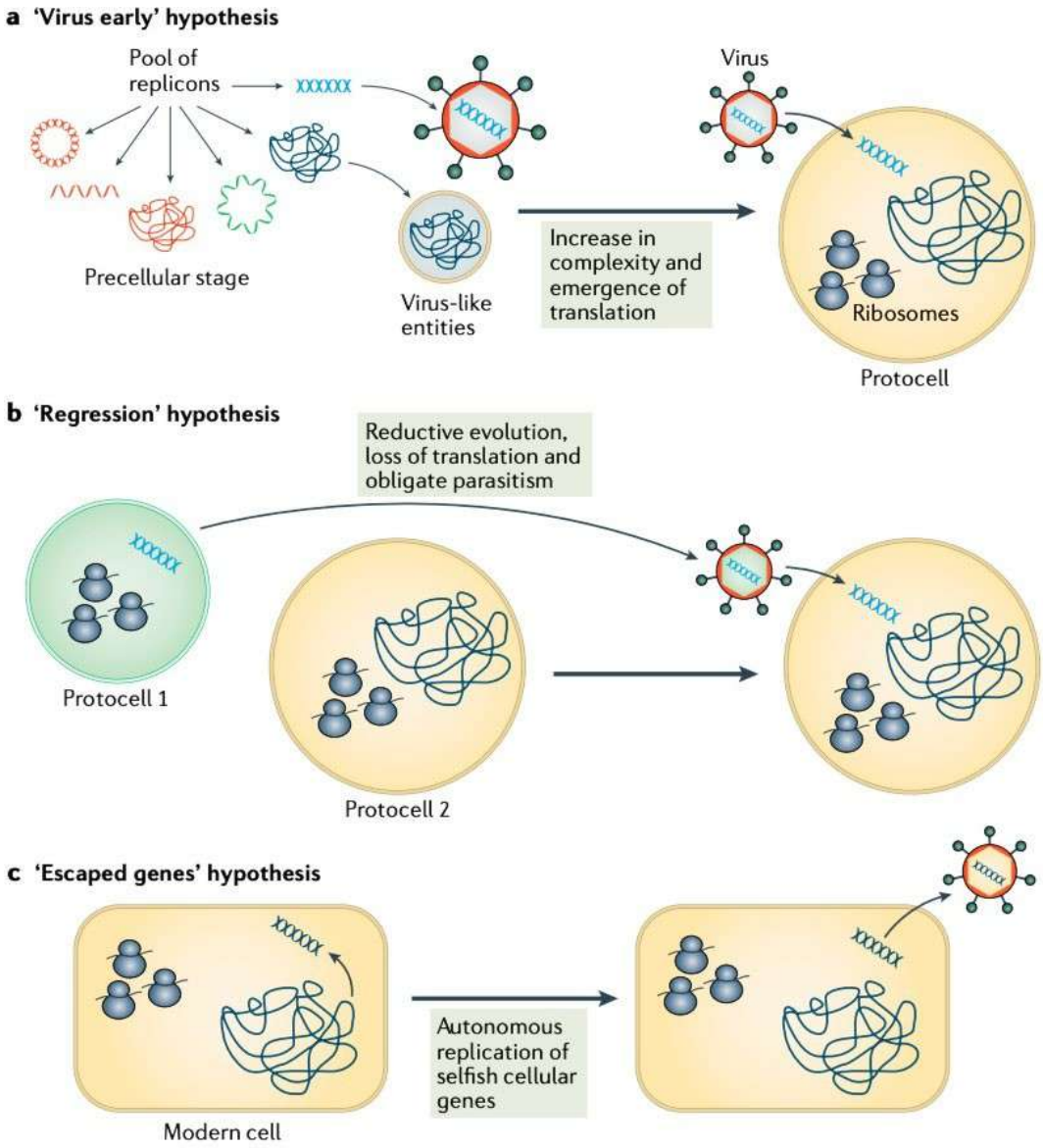
[23] Claverie JM.
Viruses take center stage in cellular evolution.
Genome Biol. 2006;7(6):110. doi:10.1186/gb-2006-7-6-110
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1779534/

[24] Dupré J, Guttinger S.
Viruses as living processes.
Stud Hist Philos Biol Biomed Sci. 2016 Oct;59:109-16. doi: 10.1016/j.shpsc.2016.02.010. Epub 2016 Mar 16. PMID: 26994935.
https://pubmed.ncbi.nlm.nih.gov/26994935/.

[25] Forterre P.

Theories of the origin of viruses and eukaryotic cells

Traditionally, three scenarios have been considered for the origin of viruses: descent from primordial pre-cellular genetic elements, reductive evolution from cellular ancestors, and gene escape from cellular hosts, all of which seek to explain the achievement of partial replicative autonomy and parasitic genetic elements.



https://www.nature.com/articles/s41579-019-0205-6

The three main scenarios for the origin of viruses.

a | The "early virus" hypothesis assumes that viruses evolved from the earliest replicative elements that preceded the earliest forms of cellular life.

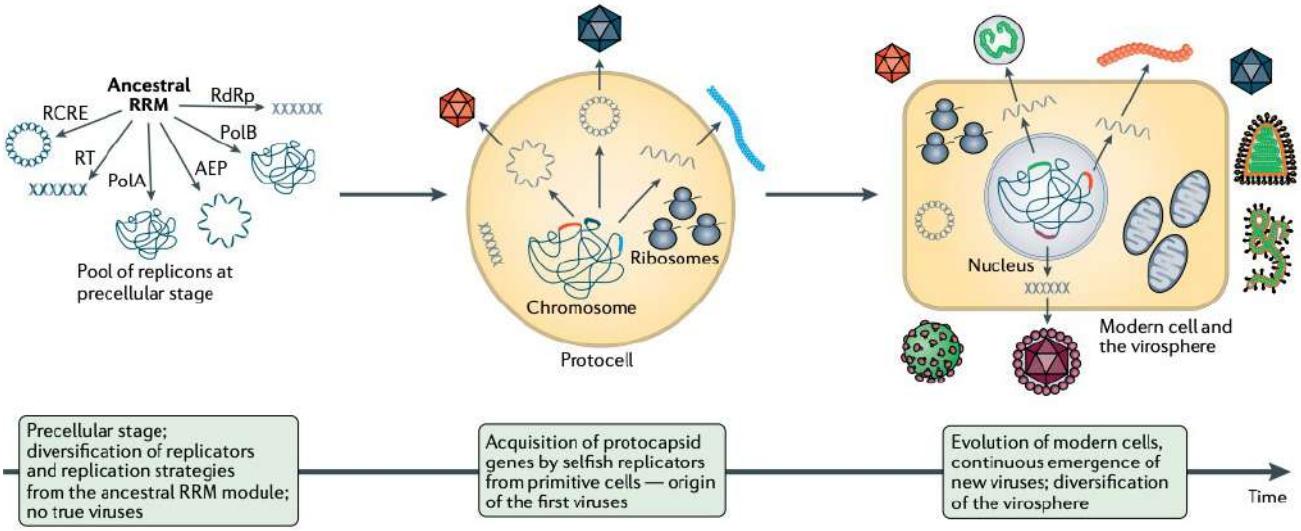
b | The "regression" hypothesis suggests that viruses emerged through the degeneration of cells that then assumed a parasitic lifestyle.

c | Finally, the "escaped genes" hypothesis proposes that cellular genes have acquired the capacity for "selfish" replication and spread

The two ages of the RNA world, and the transition to the DNA world: a story of viruses and cells.
Biochimie. 2005 Sep-Oct;87(9-10):793-803. doi: 10.1016/j.biochi.2005.03.015. Epub 2005 Apr 12. PMID: 16164990.
https://pubmed.ncbi.nlm.nih.gov/16164990/

These classical scenarios give different timelines for the origin of viruses and do not explain the origin of the two key functional modules that are responsible for viral genome replication and virion morphogenesis, respectively.

Recently, a new "chimeric" model of virus origin has been proposed by the group of Krupovic et al that involves a two-step process in which selfish replicators emerge before the appearance of the earliest forms of cellular life and then the capture of capsid protein genes from cellular organisms, enabling them to form virions. Continued evolution and adoption of cellular genes contributes to further diversification of the virosphere.[26]



https://www.nature.com/articles/s41579-019-0205-6
The chimerical scenario for the origin of viruses.
AEP, archaeal-eukaryotic primase; PolB, B-family DNA polymerase; RCRE, rolling circle replication endonuclease; RdRp, RNA-dependent RNA polymerase; RRM, RNA recognition motif; RT, reverse transcriptase.

Viruses that infect cells in the three domains of life, Archaea, Bacteria and Eukarya, share homologous characteristics, suggesting that viruses appeared very early in the evolution of life.
Most evolutionists agree that our present RNA/DNA/protein world originated from a simpler world in which RNA played both the role of catalyst and genetic material.

Recent findings from structural studies and comparative genomics now provide a clearer picture of this transition and suggest that evolution occurred in several steps, first from an RNA to an RNA/protein world (defining two ages of the RNA world) and finally to the current DNA-based world.

The DNA world itself probably originated in two stages, first the U-DNA world, following the appearance of ribonucleotide reductase, and later the T-DNA world, with the independent appearance of at least two thymidylate synthases.
Recently, several authors have suggested that evolution from the RNA world to the Last Universal Cellular Ancestor (LUCA) may have occurred before the appearance of cells. [27]

Peptide sharing analyses between five common human viruses (Borna disease virus, influenza A virus, measles virus, mumps virus, and rubella virus) and the human proteome

[26] Krupovic, M., Dolja, V.V. & Koonin, E.V.
Origin of viruses: primordial replicators recruiting capsids from hosts.
Nat Rev Microbiol 17, 449-458 (2019). https://doi.org/10.1038/s41579-019-0205-6
https://www.nature.com/articles/s41579-019-0205-6

[27] Forterre P.
The origin of viruses and their possible roles in major evolutionary transitions.
Virus Res. 2006 Apr;117(1):5-16. doi: 10.1016/j.virusres.2006.01.010. Epub 2006 Feb 14.
https://pubmed.ncbi.nlm.nih.gov/16476498/

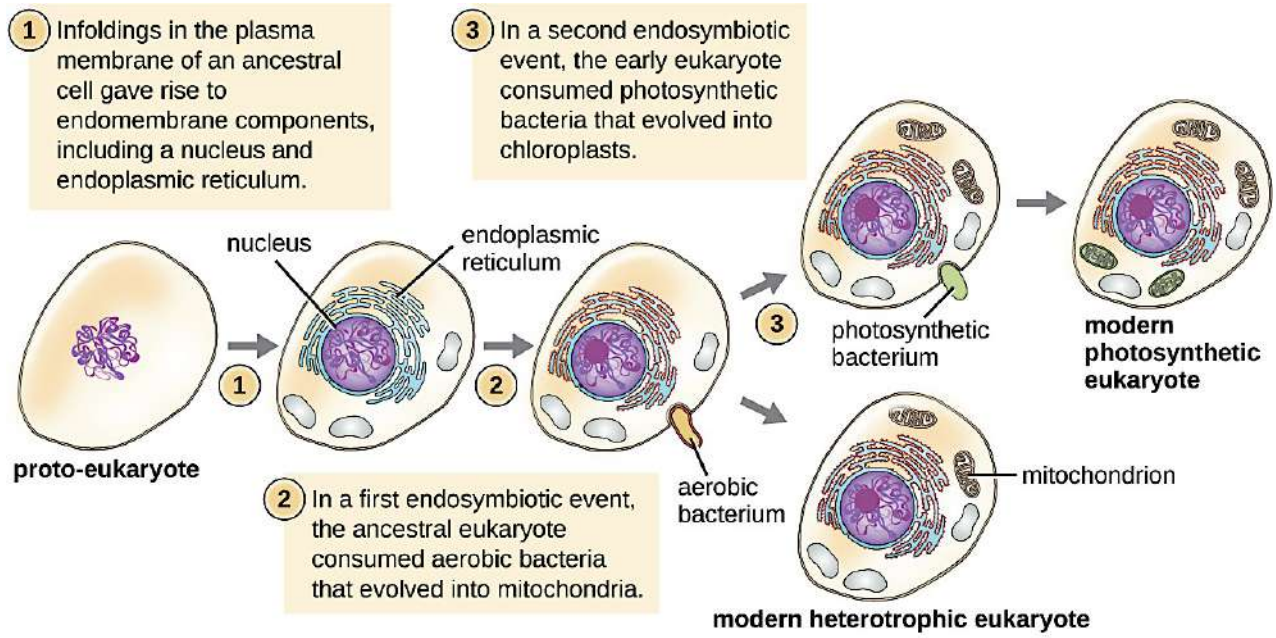
highlight a massive overlap between viral and human peptides that is mathematically unexpected. From an evolutionary perspective, the data underscore a close relationship between viruses and the origin of eukaryotic cells.

In fact, according to the v i r a l eukaryogenesis hypothesis [28] and in light of the endosymbiotic theory, the first eukaryotic cell (our lineage) originated as a consortium consisting of an archaeal ancestor of the eukaryotic cytoplasm, a bacterial ancestor of the mitochondria, and a viral ancestor of the nucleus. [29]

From a pathological point of view, as will be discussed later, the peptide sequence similarity between viruses and humans may provide a molecular platform for autoimmune cross-reactions during immune responses following viral infection/immunization.[30]

Below are figures related to the various theories on the origin of the eukaryotic cell and its nucleus. [31]

The endo-symbiotic theory



https://commons.wikimedia.org/wiki/File:OSC_Microbio_03_02_Endosymbio.jpg

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[28] Speijer D. Debating
Eukaryogenesis-Part 1: Does Eukaryogenesis Presuppose Symbiosis Before Uptake? Bioessays. 2020 Apr;42(4):e1900157. doi: 10.1002/bies.201900157. Epub 2020 Feb 20.
https://onlinelibrary.wiley.com/doi/full/10.1002/bies.201900157

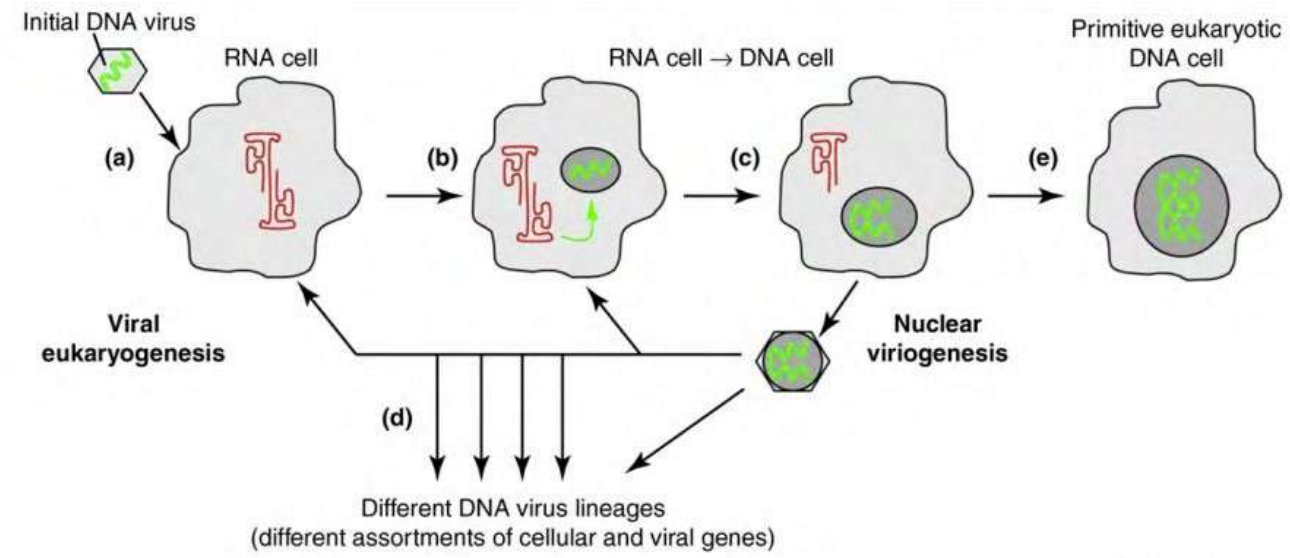
Dacks JB, Field MC, Buick R, Eme L, Gribaldo S, Roger AJ, Brochier-Armanet C, Devos DP.
The changing view of eukaryogenesis - fossils, cells, lineages and how they all come together.
J Cell Sci. 2016 Oct 15;129(20):3695-3703. doi: 10.1242/jcs.178566. Epub 2016 Sep 26.
https://jcs.biologists.org/content/129/20/3695.long

[29] Philip Bell (September 11th 2013).
Meiosis: Its Origin According to the Viral Eukaryogenesis Theory, Meiosis, Carol Bernstein and Harris Bernstein, IntechOpen, DOI: 10.5772/56876.
https://www.intechopen.com/books/meiosis/meiosis-its-origin-according-to-the-viral-eukaryogenesis-theory

[30] Kanduc D.
The comparative biochemistry of viruses and humans: an evolutionary path toward autoimmunity.
Biol Chem. 2019 Apr 24;400(5):629-638. doi: 10.1515/hsz-2018-0271.
https://pubmed.ncbi.nlm.nih.gov/30504522/

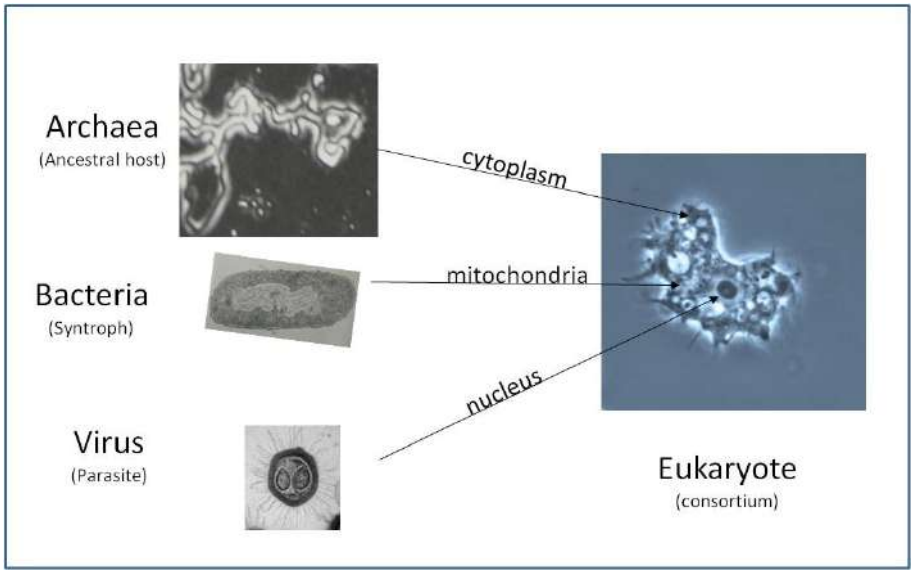
[31] Claverie JM.
Viruses take center stage in cellular evolution.
Genome Biol. 2006;7(6):110. doi:10.1186/gb-2006-7-6-110
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1779534/

Theory on the origin of the nucleus of the eukaryotic cell



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1779534/
An iterative model for the origin of the eukaryotic nucleus and the simultaneous emergence of several NCLDV families. (a) A primitive DNA virus (an ancestor of bacteriophage) becomes trapped within an RNA cell initiating a proto-nucleus. (b) Cell genes are recruited progressively into the proto-nucleus, driven by the selective advantages of DNA genomes. (c) This situation remains unstable for a while with some of the proto-nuclei reverting to a viral state (generalizing the use of the original capsid structure as a vehicle for the genome). (d) These viruses have infected other cells at various stages of their ongoing rapid evolution. (e) The emergence of nucleated cells with biochemically more stable DNA genomes may have coincided with the end of the pre-Darwinian era in which a collective mode of evolution (characterized by unstable protocellular organisms overwhelmed by too-frequent horizontal gene transfers) shifted to a cellular mode of evolution (with the dominance of the inherited gene from vertical descent, both in the cellular and viral worlds). This hypothetical scheme provides a mechanism for the emergence of various overlapping viral lineages, all using the capsid structure inherited from bacteriophage, which precedes the emergence of eukaryotes and exhibits various reassortments of viral and ancestral cellular genes

Theory of viral eukaryogenesis



https://www.intechopen.com/books/meiosis/meiosis-its-origin-according-to-the-viral-eukaryogenesis-theory
The eukaryotic cell evolves from a prokaryotic world community/consortium. In the theory of viral eukaryogenesis, the eukaryotic cell descends from a consortium of three originally independent prokaryotic world organisms. The eukaryotic cytoplasm descends from an archaeal cell that did not possess a cell wall and was subject to infection by a variety of agents including a range of bacteria and viruses. The eukaryotic mitochondrion is descended from an alpha-proteobacterium that was originally in a symplastic relationship with the archaeal host, and eventually invaded the host cytoplasm and established a permanent endosymbiotic presence in the host cell. The eukaryotic nucleus descended from an NCLDV virus that was originally in a parasitic relationship with the archaeal host and eventually established a permanent lysogenic presence within the host cytoplasm. The three consortium organisms eventually shared a common evolutionary trajectory and eventually evolved into the eukaryotic cell. In the process of permanently establishing the "eukaryotic cell," the viral core ancestor assumed the role of DNA replication and transcription through its capping/cap binding proteins that directed the preferential translation of viral transcripts into proteins. As a result, the virus became the genetic information processing center of the cell and gradually acquired genes from other organisms. The alpha proteobacterial ancestor of mitochondria transferred most of its genes to the virus/nucleus in the process of evolution in mitochondria and, in the case of hydrogenosomes, all of its genes. The archaeal host cytoplasm retained its role in the translation of mRNA transcripts and eventually transferred all its "eukaryotic" genes to the nucleus

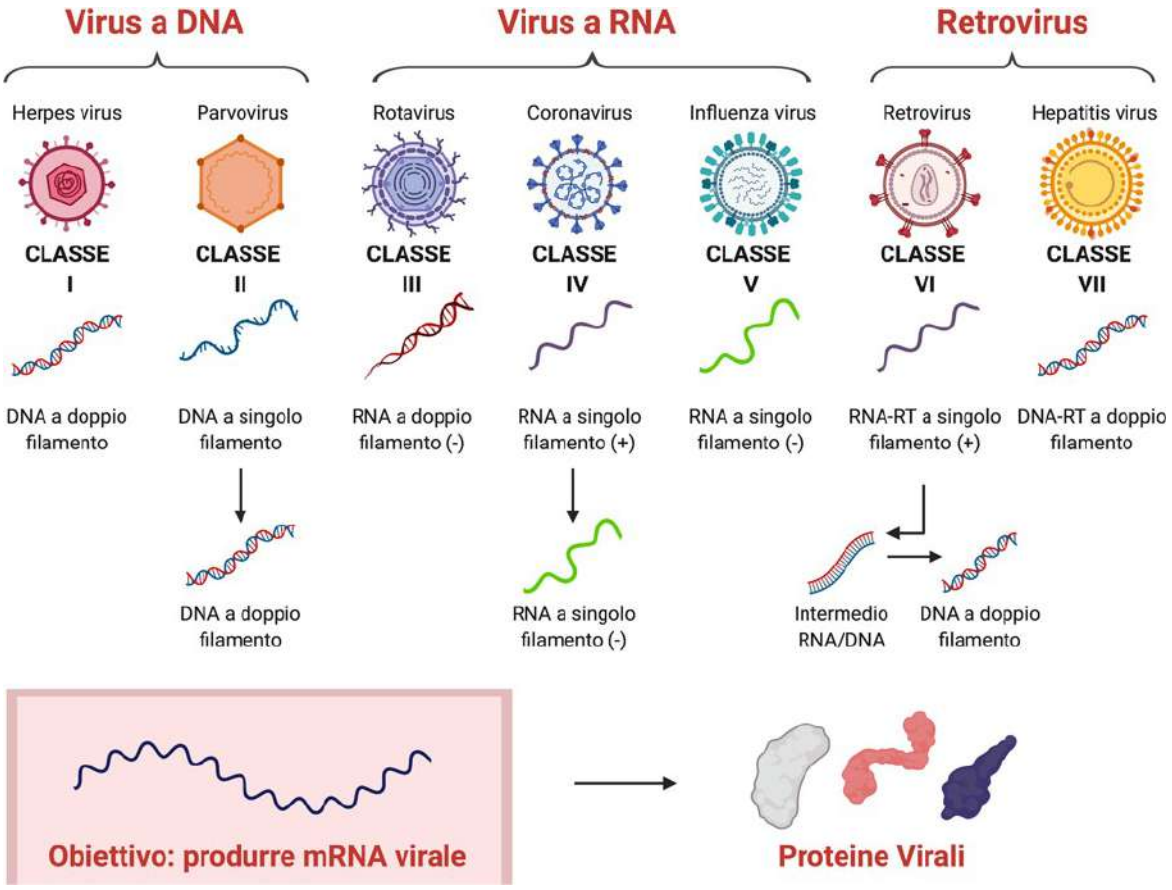
CLASSIFICATION OF VIRUSES

DNA viruses integrate their genome within that of the host cell and take advantage of normal cellular mechanisms for their own replication.

RNA viruses, on the other hand, use different mechanisms depending on the case:

- if you start with **a positive-sense RNA** this can already be used for translation into protein;
- if starting from **a negative-sense RNA,** the intervention of a viral dependent RNA-polymerase will be required for the synthesis of a translatable positive-sense RNA strand.
- **retroviruses** use a specific enzyme, reverse transcriptase, which enables them to synthesize DNA from RNA. [32]

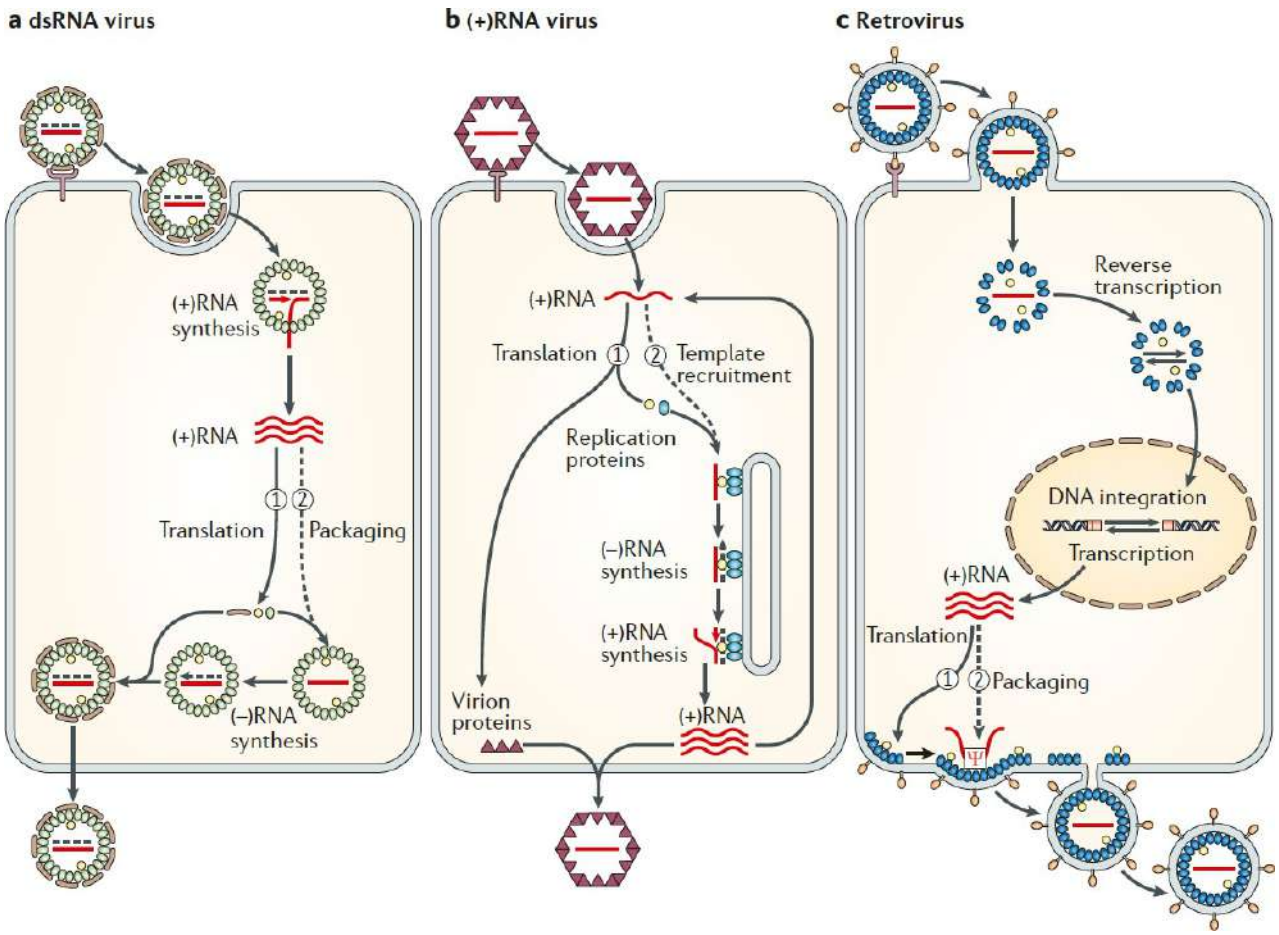
SARS-CoV-2 is an example of the viruses belonging to class IV: once infected the cell, its positive-sense RNA can be immediately translated into proteins and is used for the synthesis of complementary strands of negative-sense RNA, which, in turn, will be used for the production of new copies of positive-sense RNA to be incorporated into the virions being produced.



https://www.facebook.com/sciencenow.fb/ Classification of Baltimore viruses

[32] Ramasamy, Pathmanaban. (2015).
Reconstruction of Phylogenetic Relationships between Nucleic Acid Polymerases of Viruses with RNA Genome.
10.13140/RG.2.1.1380.6162.
https://www.researchgate.net/publication/288827106_Reconstruction_of_Phylogenetic_Relationships_between_Nucleic_Acid_Polymerases_of_Viruses_with_RNA_Genome

Ahlquist, P.
Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses.
Nat Rev Microbiol 4, 371-382 (2006). https://doi.org/10.1038/nrmicro1389
https://www.nature.com/articles/nrmicro1389



https://www.nature.com/articles/nrmicro1389
Distinct life cycles of dsRNA viruses, RNA (+) viruses, and retroviruses. All three classes of viruses replicate through positive-strand ((RNA) (+) RNA intermediates (red strands in the figure) that are templates for both translation and genome replication. For each class, a representative simplified life cycle is shown in the figure.
Double-stranded (ds) RNA viruses: As shown in part a of the figure, virus attachment and endocytosis provide a virionic core that contains viral genomic dsRNA and viral RNA polymerase (yellow) in the cytoplasm. The nucleus transcribes and extrudes (+) mRNAs that are first translated (1) and then packaged (2) by the resulting viral proteins into new virion nuclei. Nuclei mature by synthesizing negative-stranded (-) RNA (dotted strand) and adding external proteins. They exit by cell lysis or secretion.
RNA (+) viruses: As shown in part b of the figure, endocytosed virions release sense messenger genomic RNA into the cytoplasm for translation. Newly translated viral RNA replication proteins recruit this genomic RNA into a membrane-associated intracellular RNA replication complex. Small amounts of RNA(-) are produced and used as templates to greatly amplify viral RNA(+) that is encapsulated in newly progeny virions.
Retroviruses: As shown in part c of the figure, virion attachment and envelope fusion release a subviral complex containing viral genomic RNA (+) and reverse transcriptase (yellow). After cDNA synthesis by reverse transcription, the proviral cDNA is integrated into the host chromosome and transcribed to produce RNA (+) that is translated (1) and then packaged (2) into new virions that are released by budding.

General Virology
Classification of Viruses
The Nature of Viruses
Viruses

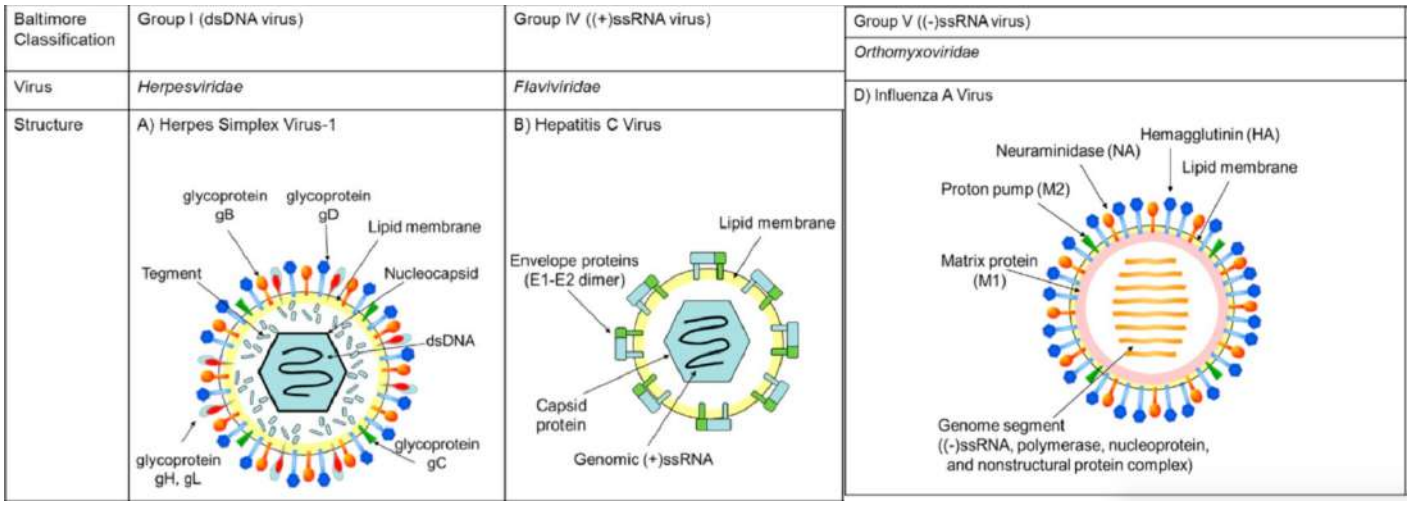
RNA: the molecule that shaped the phenomenon of life

Examples of genomes [33]

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) belong to the family Herpesviridae, have a complex structure, comprising an icosahedral capsid containing double-stranded DNA, located within the virion and surrounded by a heterogeneous membrane envelope studded with morphologically distinct spikes formed by 12 different species of glycoproteins. Transcription and replication of the viral genome, as well as the assembly of progeny capsids, occur within the nucleus. Viral mRNA is synthesized by RNA-polymerase II of the host cell with the participation of viral factors at all stages of infection. The capsid is transported from the nucleus to the cytoplasm, where virion maturation and outer shell formation occur. Release of the virion from the cell by exocytosis leads to the formation of the envelope.

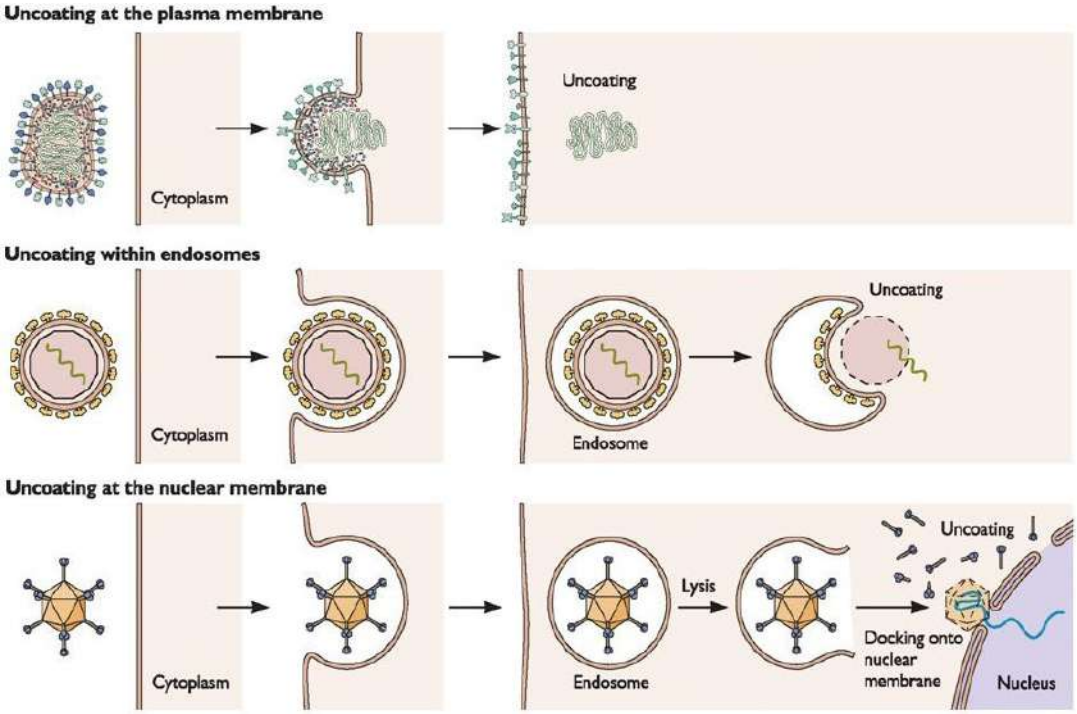
HCV (hepatitis C), ZIKV (Zika virus), WNV (West Nile irus) and DENV (Dengue virus) are all part of the Flaviviridae family and possess envelopes with icosahedral and spherical geometries. HCV, of the genus Hepacivirus, is a major cause of liver disease, while ZIKV, WNV and DENV, of the genus Flavivirus, cause a range of viral diseases transmitted mainly by arthropods. HCV and DENV are envelope viruses with a diameter of about 40-65 nm and have a 9.6 to 11 kb single-stranded, non-segmented, positive-sense RNA genome. Once inside cells, the genome is translated into proteins, then proteolytically processed by viral and cellular proteases to produce three structural and nonstructural (NS) proteins. The NS proteins recruit the viral genome into an RNA replication complex, and RNA replication occurs via viral RNA-dependent RNA polymerase. After the negative-stranded RNA is synthesized, it serves as a template for producing new positive-stranded viral genomes. The nascent genomes can then be translated, further replicated or packaged within new viral particles and released onto the cell surface.

Influenza A virus (IAV) is a member of the Orthomixoviridae family, an enveloped, roughly spherical virus with a diameter of about 50-120 nm and eight distinct negative-sense (or antisense) single-stranded RNA genome segments. Influenza virus has three membrane proteins: hemagglutinin (HA), proton pump (M2) and neuraminidase (NA). The inner membrane of the virion is supported by the matrix protein (M1), and the interior of the virion contains eight different genome segments. Each genome segment is a ribonucleoprotein complex (RNP) consisting of a negative-stranded RNA genome together with an RNA polymerase complex (PA, PB1, PB), nucleoprotein (NP) and nonstructural protein (NS).
IAV binds to host cell glycoproteins or glycolipids via the HA protein and enters cells through receptor-mediated endocytosis. At the low pH of the late endosome, HA induces fusion of viral and endosomal membranes. After replication and transcription of genomic RNAs in the nucleus by the polymerase complex, viral proteins enter the endoplasmic reticulum. Transport of the viral protein to the plasma membrane probably requires host factors.



[33] Kaihatsu K, Yamabe M, Ebara Y.
Antiviral Mechanism of Action of Epigallocatechin-3-O-gallate and Its Fatty Acid Esters.
Molecules. 2018;23(10):2475. Published 2018 Sep 27. doi:10.3390/molecules23102475
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6222519/

https://www.mdpi.com/molecules/molecules-23-02475/article_deploy/html/images/molecules-23-02475-g002.png



https://docplayer.it/1256495-Replicazione-e-genetica-virale.html
Three different strategies for undressing (uncoating) virions

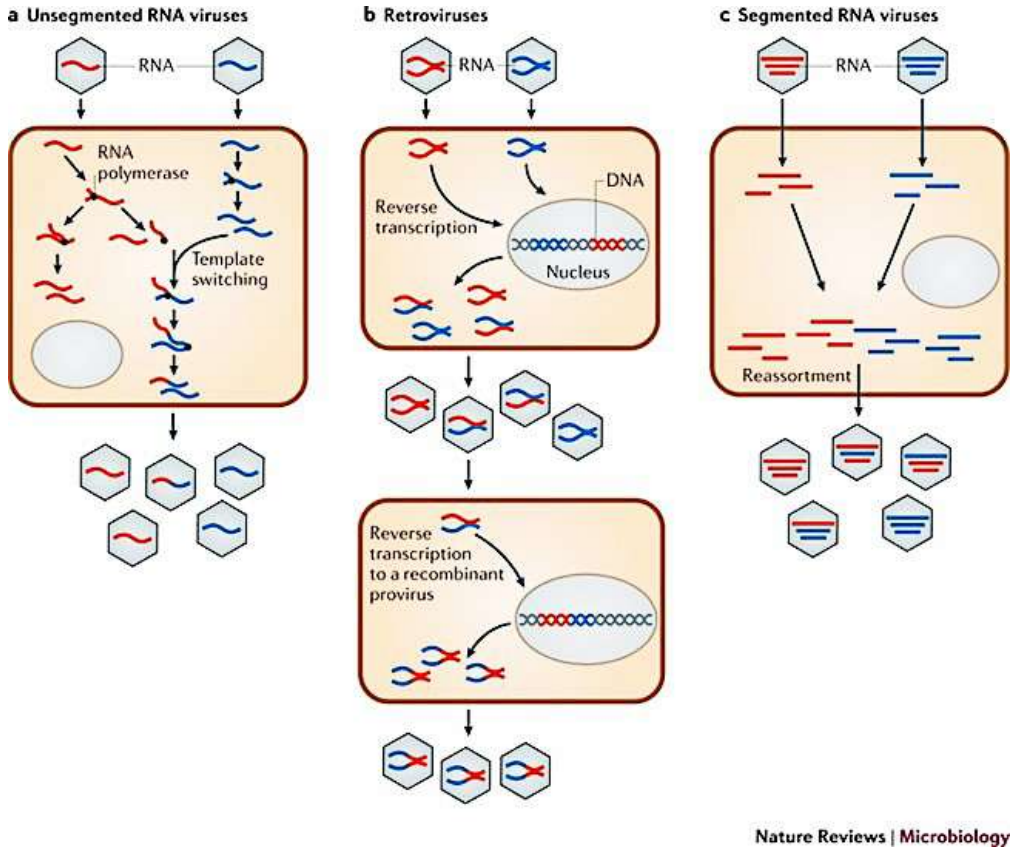
RECOMBINATION AND REASSORTMENT

The recombination process that occurs in RNA viruses corresponds to the formation of chimeric molecules from parental genomes of mixed origin. This process can occur within a single genomic segment (in which case it is often referred to as RNA **recombination**) or, for those viruses that possess segmented genomes, as an exchange of entire genomic segments between viruses. In this case the exchange is usually referred to as **reassortment**.

Although RNA recombination and reassortment are mechanistically very different, both require two or more viruses to infect the same host cell. [34]

[34] Simon-Loriere, E., Holmes, E.
Why do RNA viruses recombine?
Nat Rev Microbiol 9, 617-626 (2011). https://doi.org/10.1038/nrmicro2614
https://www.nature.com/articles/nrmicro2614

Modrow S., Falke D., Truyen U., Schätzl H. (2013)
Viruses with Single-Stranded, Segmented, Negative-Sense RNA Genomes.
Molecular Virology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-20718-1_16
https://link.springer.com/referenceworkentry/10.1007%2F978-3-642-20718-1_16



https://www.nature.com/articles/nrmicro2614
a)Co-infection of a cell by genetically distinct viral strains can lead to the generation of recombinant viruses. This process can occur both in unsegmented viruses (as shown here) and within a segment of a segmented virus. **b)**Co-infection of a cell by genetically distinct strains of a retrovirus can lead to the generation of "heterozygous" viral particles, after which a pattern-change event can lead to a recombinant provirus. **c)**Co-infection of a cell by genetically distinct strains of a segmented virus can generate different combinations of reassortment progeny.

Influenza viruses tend to reassort very frequently in vivo, [35] a phenomenon also exploited to select vaccine antigens used in attenuated virus influenza vaccines, as exemplified in the following figure. [36]

[35] Urbaniak K, Markowska-Daniel I.
In vivo reassortment of influenza viruses.
Acta Biochim Pol. 2014;61(3):427-31. Epub 2014 Sep 3.
http://www.actabp.pl/pdf/3_2014/427.pdf

https://nieman.harvard.edu/wp-content/uploads/pod-assets/microsites/NiemanGuideToCoveringPandemicFlu/TheScience/HowFluVirusesChange.aspx.html#reassortment

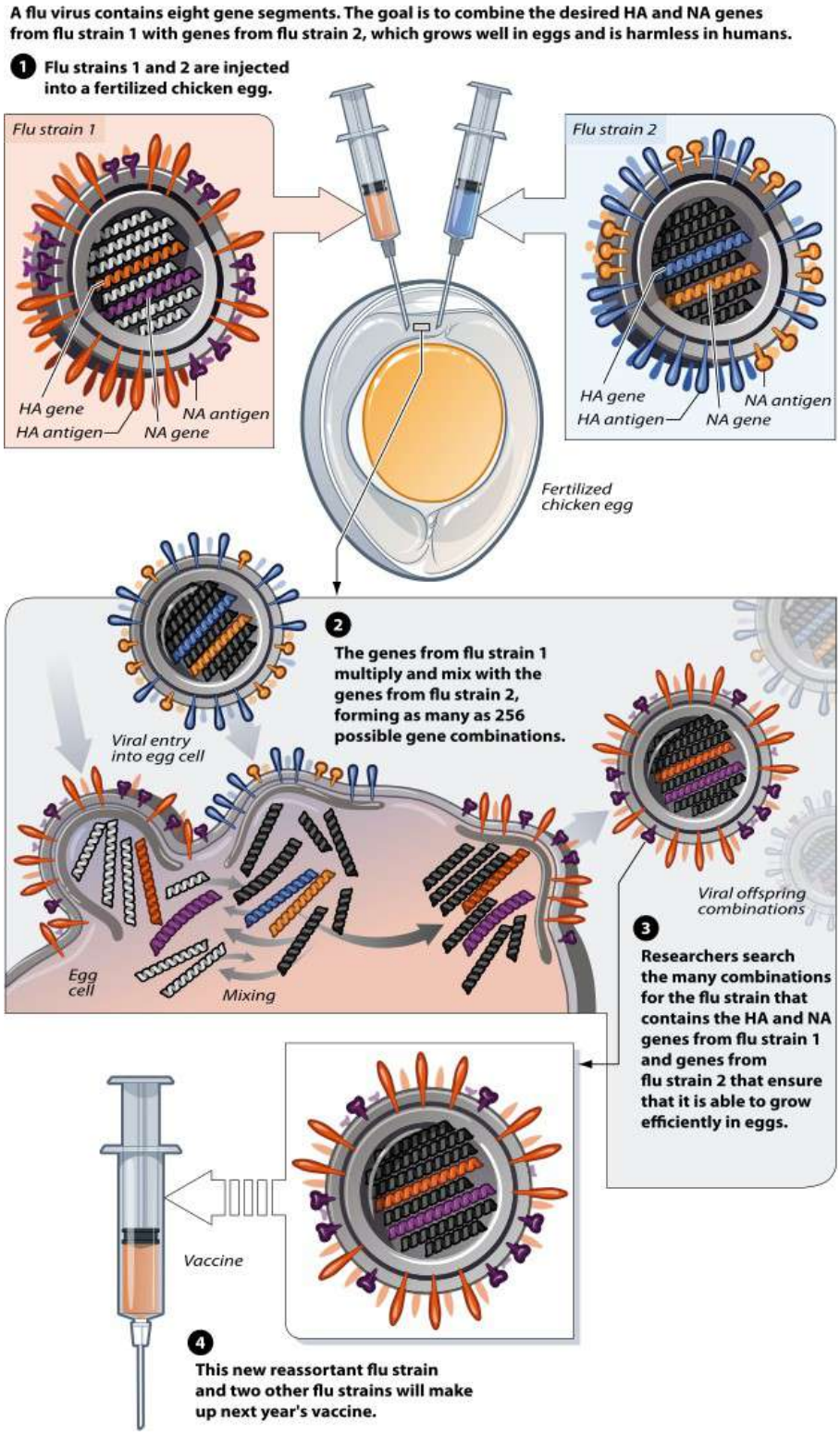
Squires, Richard. (2008).
An influenza virus molecular infection model and discrete event, stochastic simulation.
https://www.researchgate.net/publication/238732853_AN_INFLUENZA_VIRUS_MOLECULAR_INFECTION_MODEL_AND_DISCRETE_EVENT_STOCHASTIC_SIMULATION

[36] McDonald SM, Nelson MI, Turner PE, Patton JT.
Reassortment in segmented RNA viruses: mechanisms and outcomes.
Nat Rev Microbiol. 2016;14(7):448-460. doi:10.1038/nrmicro.2016.46
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5119462/

Blanco-Lobo P, Nogales A, Rodríguez L, Martínez-Sobrido L.
Novel Approaches for The Development of Live Attenuated Influenza Vaccines.
Viruses. 2019;11(2):190. Published 2019 Feb 22. doi:10.3390/v11020190
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6409754/.

Nogales A, Martínez-Sobrido L.
Reverse Genetics Approaches for the Development of Influenza Vaccines.
Int J Mol Sci. 2016;18(1):20. Published 2016 Dec 22. doi:10.3390/ijms18010020
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5297655/

Schultz-Cherry S, Jones JC.
Influenza vaccines: the good, the bad, and the eggs.
Adv Virus Res. 2010;77:63-84. doi: 10.1016/B978-0-12-385034-8.00003-X. PMID: 20951870.



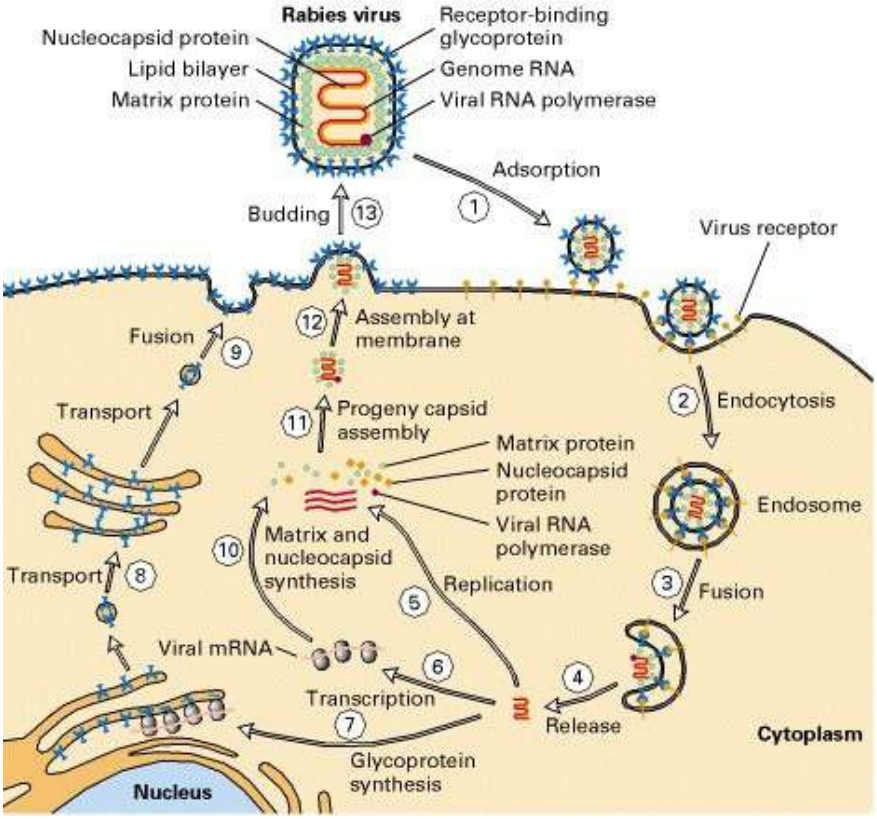
https://upload.wikimedia.org/wikipedia/commons/thumb/d/d7/Reassortment.svg/632px-Reassortment.svg.png

LYTIC CYCLE AND LYSOGENIC CYCLE

After the synthesis of new virions, from hundreds to thousands, has been completed, most bacterial cells and some infected plant and animal cells rupture, or lyse, releasing all virions simultaneously. In many viral infections of plants and animals, however, no discrete lytic event occurs; rather, the dead host cell releases virions as it gradually disintegrates.

https://pubmed.ncbi.nlm.nih.gov/20951870/

These events-adsorption*, penetration, replication and* release-describe the **lytic cycle** of viral replication. The result is the production of a new cycle of viral particles and cell death. Adsorption and release **of enveloped animal viruses** are somewhat more complicated processes, as virions "bud" from the host cell, thereby acquiring their outer phospholipid envelope, which contains mainly viral glycoproteins.[37]



https://www.ncbi.nlm.nih.gov/books/NBK21523/
The steps in the lytic replication cycle of an enveloped virus are illustrated for rabies virus, which has a single-stranded RNA (-) genome After a virion adsorbs to a specific host membrane protein (*step 1*), the cell engulfs it into an endosome (*step 2*). A protein in the endosome membrane pumps protons from the cytosol into the endosome, resulting in a decrease in endosomal pH that induces a conformational change in the viral glycoprotein, leading to fusion of the viral envelope with the endosomal lipid bilayer membrane and release of the nucleocapsid into the cytosol (*steps 3 and 4*). Viral RNA polymerase uses triphosphate ribonucleosides in the cytosol to replicate the viral RNA genome (*step 5*) and synthesize viral mRNAs (*step 6*). One of the viral mRNAs encodes for viral transmembrane glycoprotein (*blue*), which is inserted into the lumen of the endoplasmic reticulum (ER) while being synthesized on ER-bound ribosomes (*passage 7*). The carbohydrate is added to the large folded domain within the ER lumen and is modified when the membrane and associated glycoprotein pass through the Golgi apparatus (*passage 8*). Vesicles with the mature glycoprotein fuse with the plasma membrane, depositing the viral glycoprotein on the cell surface with the large folded domain outside the cell, the α transmembrane helix crossing the plasma membrane and the small cytoplasmic domain inside the cell (*passage 9*). Meanwhile, other viral mRNAs are translated on host cell ribosomes into nucleocapsid protein, matrix protein and viral RNA polymerase (*passage 10*). These proteins are assembled with replicated viral genomic RNA (*dark red*) into progeny nucleocapsids (*passage 11*), which then associate with the viral transmembrane glycoprotein in the plasma membrane (*passage 12*). As additional copies of the matrix protein on a single nucleocapsid associate with the cytoplasmic domain of additional copies of the viral transmembrane glycoprotein, the plasma membrane is folded around the nucleocapsid, forming a "bud" that is eventually released (*passage 13*).

The lytic cycle of E. coli **bacteriophage** T4, which has a double-helix DNA genome, begins with adsorption *(phase 1)*, during which viral coating proteins (at the tip of the tail in T4) interact with specific receptor proteins outside the host cell. The viral genome is then injected into the host.

Subsequently, host cell enzymes transcribe "early" viral genes into mRNA and then translate them into "early" viral proteins *(phase 2)*, which replicate viral DNA and induce the expression of "late" viral proteins by host cell enzymes *(phase 3)*. Late viral proteins include capsid and assembly proteins and enzymes that degrade host cell DNA, providing nucleotides for viral DNA synthesis. The progeny virions are assembled in the cell *(stage 4)* and released

[37] Lodish H, Berk A, Zipursky SL, et al.
Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 6.3,
Viruses: Structure, Function, and Uses.
https://www.ncbi.nlm.nih.gov/books/NBK21523/

(Stage 5) when the cell is lysed by viral proteins. The newly released viruses initiate another cycle of infection in other host cells.

In some cases, after a bacteriophage DNA molecule enters a bacterial cell, it is integrated into the chromosome of the host cell, where it remains quiescent and is replicated as part of the cell's DNA from one generation to the next. This association is called **lysogeny**, and the integrated phage DNA is referred to as prophage. Under certain conditions, prophage DNA is activated, leading to its excision from the host cell chromosome and entry into the lytic cycle. Bacterial viruses of this type are called **temperate phages**. [38]

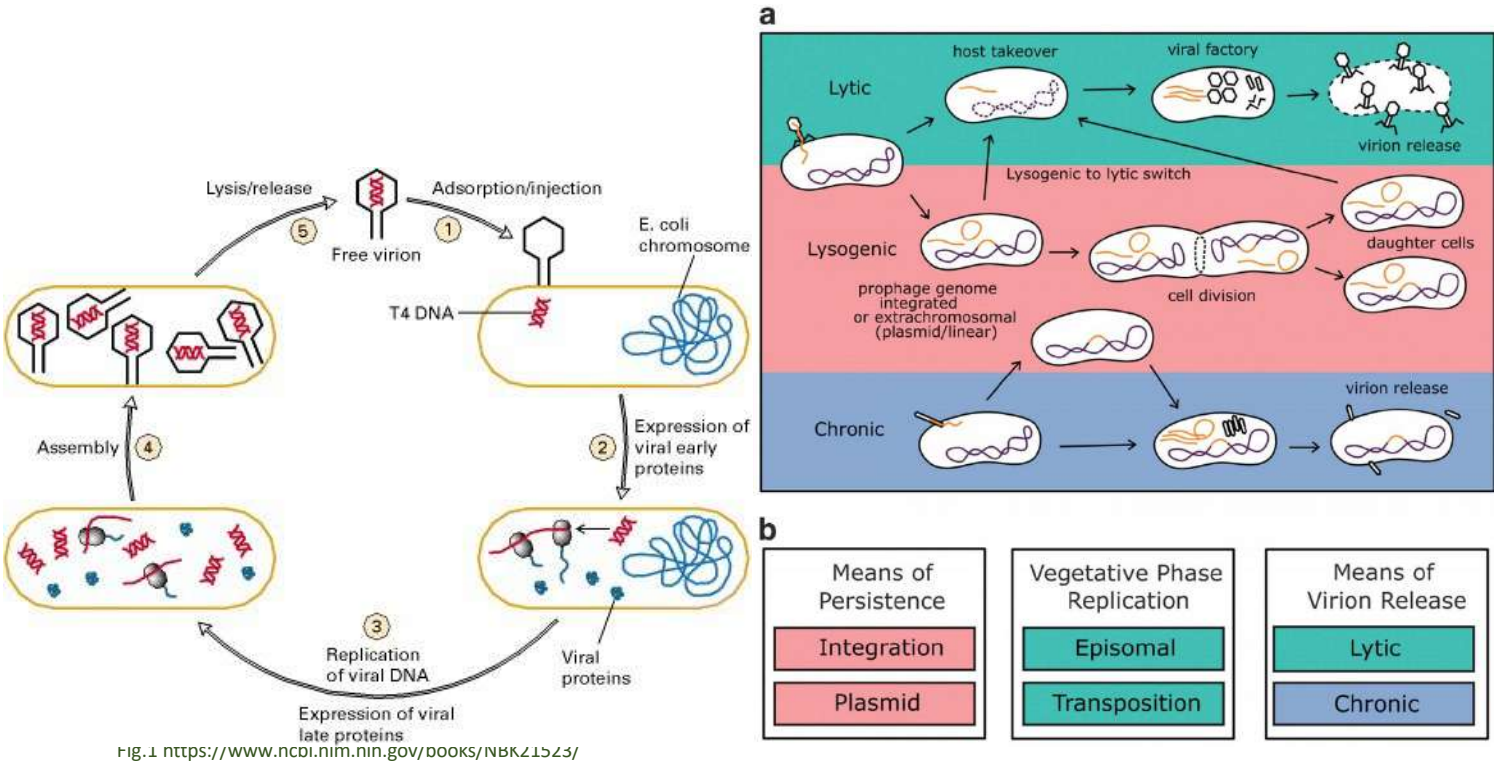


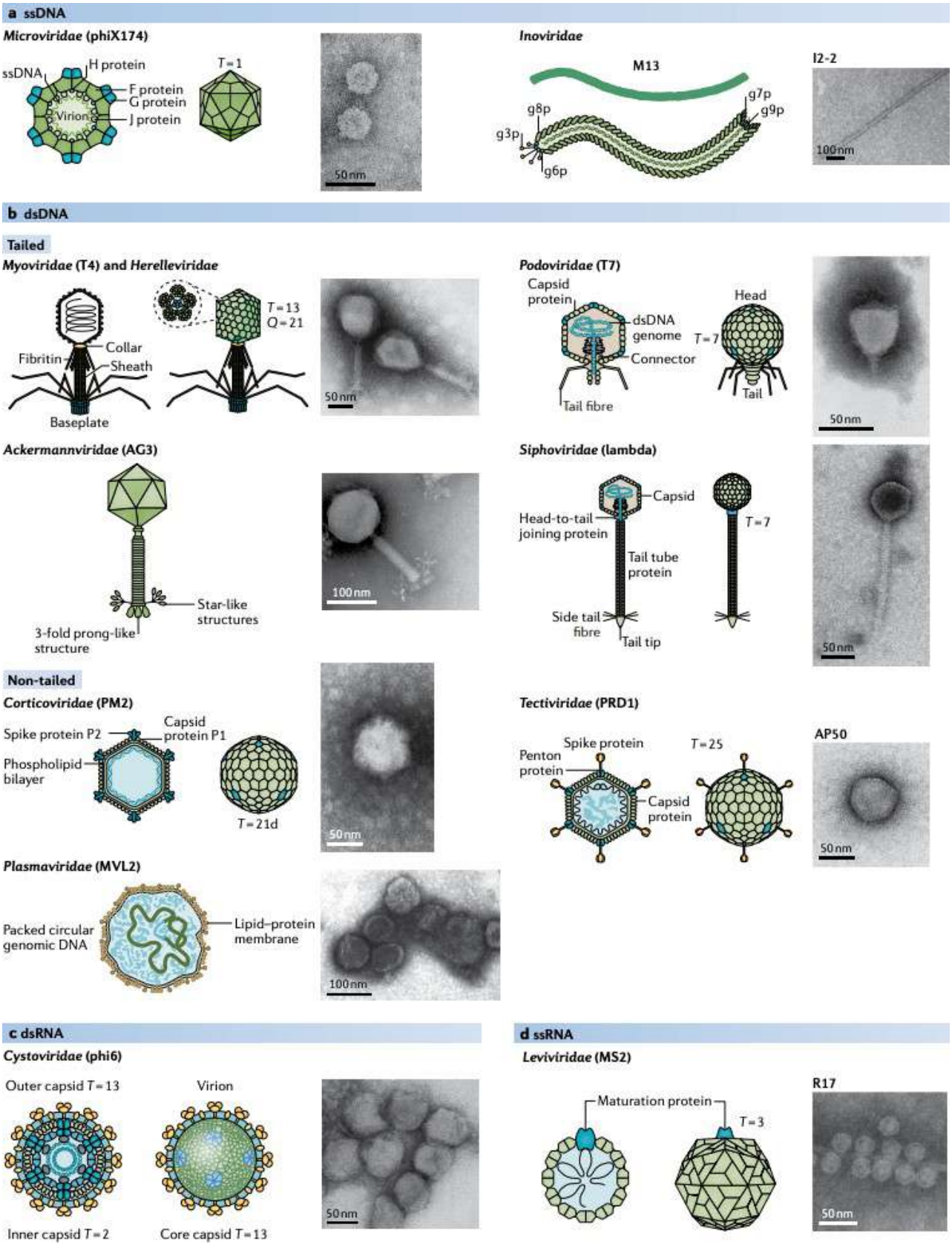
Fig.1 https://www.ncbi.nlm.nih.gov/books/NBK21523/
Bacteriophage λ undergoes lytic replication following infection with E. coli

Fig.2 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5520141/
Mode of infection of the temperate phage, from cell to community. (a) A temperate phage can infect a cell through virionic or lysogenic cycles, where it hijacks the metabolism of its host to produce new virion progeny or instead replicates its genome along with the host without producing new virions, respectively. The production of virion particles can occur either as a result of phage adsorption (productive cycle) or as a result of a switch from a lysogenic to a productive cycle (lytic or chronic temperate phage-dependent infection cycles). Although these are generalized dynamics of infection, the details may vary with specific phage-host types, ranging from efficient to inefficient infections, where the dynamics and outcome of infection may vary. (b) Summary of phage infection strategies by stage. Persistence describes the stage of the phage during the lysogenic cycle, replication describes the phage-genome state during production cycles, and release refers not only to the means by which progeny phage virions transit from intracellular to extracellular state, but also to the impact of production cycles on the infected phage-bacterium (i.e., lytic but not chronic causes phage-host phage phage physiological death).

The following are the various types of bacterial phages. [39]

[38] Howard-Varona C, Hargreaves KR, Abedon ST, Sullivan MB.
Lysogeny in nature: mechanisms, impact and ecology of temperate phages.
ISME J. 2017;11(7):1511-1520. doi:10.1038/ismej.2017.16
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5520141/

[39] Dion MB, Oechslin F, Moineau S.
Phage diversity, genomics and phylogeny.
Nat Rev Microbiol. 2020 Mar;18(3):125-138. doi: 10.1038/s41579-019-0311-5. Epub 2020 Feb 3.
https://pubmed.ncbi.nlm.nih.gov/32015529/

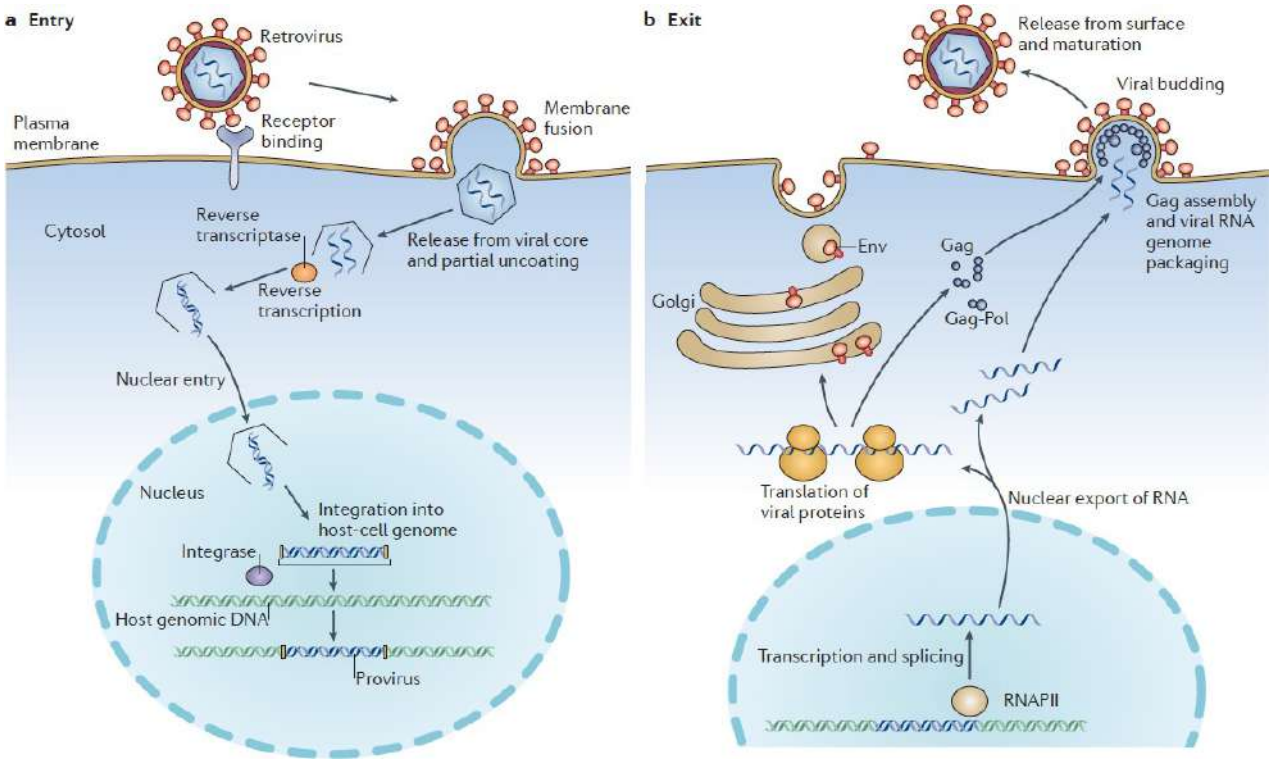


https://pubmed.ncbi.nlm.nih.gov/32015529/

Phage classification based on morphology and genome type. A schematic representation (SR) and a transmission electron micrograph (TEM) are shown for each morphology

The genomes of many animal viruses can also integrate into the genome of the host cell among which the most important are **retroviruses**.

The replication cycle of retroviruses is best known for the involvement of two unique enzymes encoded by the virus, reverse transcriptase and integrase, which enable the conversion of RNA to DNA, followed by the integration of viral DNA into the host genome, forming a DNA provirus.



https://www.nature.com/articles/nrmicro2783
Various events in the life cycle of retroviruses are illustrated**. a |** Viral entry into cells involves the following steps: binding to a specific receptor on the cell surface; membrane fusion either on the plasma membrane or from endosomes (not shown); viral core release and partial uncoating; reverse transcription; transit through the cytoplasm and nuclear entry and integration into cellular DNA to give a provirus. **b** | Viral output involves the following steps: transcription by RNA polymerase II (RNAPII); splicing and nuclear export of viral RNA; viral protein translation, Gag assembly and RNA packaging; budding across the cell membrane and release from the cell surface; and maturation of the virus.

After cell infection, viral RNA is retro-transcribed into double-stranded DNA and long terminal repeats (LTRs) are created. The viral DNA is subsequently integrated into the genomic DNA of the host cell to form a provirus that is subsequently transcribed by the host cell's RNA polymerase II (RNAPII), as already seen for herpes viruses.

The typical simple retrovirus has three genes: *gag, pol and env*. The *gag* gene encodes for the structural proteins that make up the viral core, the matrix (MA), capsid (CA) and nucleocapsid (NC), and these are initially synthesized as a single polyprotein. The *pol* gene encodes for the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). The initial translation is as a Gag-Pol fusion protein from the genome length RNA. The *env* gene encodes for surface (SU) and transmembrane (TM) proteins responsible for receptor binding and membrane fusion.

Because retroviruses usually do not lyse their target cell, integration allows for long-term association between cell and virus. If the infected cell is a germ cell, colonization of the germ line by the virus is possible.
Numerous studies indicate that such events have occurred several times throughout evolution.

Such inherited proviruses are called endogenous retroviruses (ERVs) and provide a "fossil record" of past retroviral infections dating back many millions of years. [40]

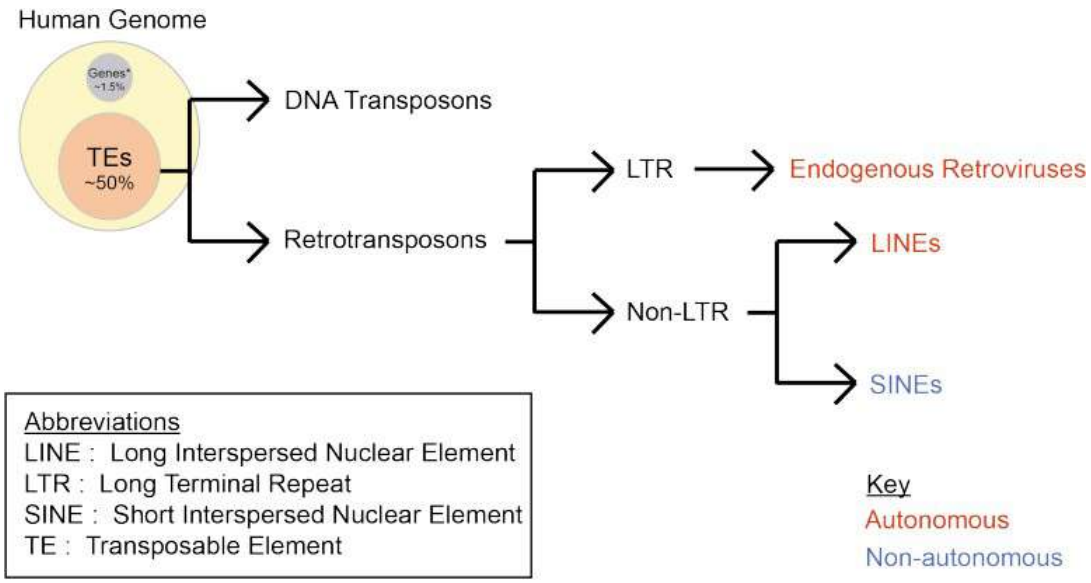
[40] Buzdin AA, Prassolov V, Garazha AV.
Friends-Enemies: Endogenous Retroviruses Are Major Transcriptional Regulators of Human DNA.
Front Chem. 2017;5:35. Published 2017 Jun 8. doi:10.3389/fchem.2017.00035
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5462908/

Stoye JP.
Studies of endogenous retroviruses reveal a continuing evolutionary saga.
Nat Rev Microbiol. 2012 May 8;10(6):395-406. doi: 10.1038/nrmicro2783.
https://pubmed.ncbi.nlm.nih.gov/22565131/

https://www.onb.it/2018/12/13/i-retrovirus-endogeni-nellevoluzione-umana/

Phylogenetic analysis revealed that the human genome contains at least 31 distinct groups of ERVs, with copy numbers ranging from one to many thousands.
Completed genome sequence analyses revealed that 4-10% of vertebrate DNA consists of retroviral residues.

This amount is several times higher than that corresponding to the sequences encoding the genes, but is substantially less than that derived from retro-transposons not containing LTRs (unlike HERVs that belong to LTR-retrotransposons), such as long interval nuclear elements (LINEs) and short interval nuclear elements (SINEs). [41]



https://discovery.ucl.ac.uk/id/eprint/10038410/1/Christopher%20Tie%20.pdf
Types of transposable elements (TEs) in the human genome.
Most transposon-derived repeats in the human genome are retrotransposons, which account for 50% of our genome. TEs can be classified according to the presence of LTRs and whether or not they can replicate autonomously. ERV and LINE are autonomous families while SINE are not autonomous

Together, these reverse transcription products make up nearly half of the vertebrate genome and thus pose a significant challenge for the development of whole-genome sequence assemblies using current short-read sequencing techniques.

It should also be noted that a small number of integration events involving RNA viruses other than retroviruses have been reported. These include bornaviruses and filoviruses. Such viruses normally do not undergo reverse transcription; it seems likely that such integrations occurred as a result of LINE element-mediated reverse transcription or after illegitimate recombination with an ERV.

[41] Tie, Christopher. (2017). Epigenetic control of endogenous retroviruses and their immune recognition in differentiated human cells. https://discovery.ucl.ac.uk/id/eprint/10038410/1/Christopher%20Tie%20.pdf

THE PRODUCTION OF VACCINES

CELL LINES USED FOR THE GROWTH OF VACCINE VIRUSES

Cell cultures [42] *are the in vitro* model par excellence, represented by a group of eukaryotic cells capable of growing and proliferating *in vitro* under particular conditions. Thanks to this simplified model, it is possible to perform studies on physiological and/or pathological processes, test substances such as drugs, herbal medicines, toxic substances or biological agents such as viruses or bacteria.
Cell cultures represent a simplified and easily reproducible substrate. It is a plastic system, and environmental conditions (temperature, oxygen, carbon dioxide, humidity) are easily controlled through the use of tools such as an incubator.
Like all *in vitro* methods, it has the advantage of reducing the use of animal guinea pigs for experimentation. However, it is worth noting that in pharmacological studies the *in vitro* system shows a dosing problem: the dose of a drug, for example, that shows beneficial effects in the in *vitro* system, may not give effects in an in *vivo* system, so after an initial in *vitro* screening it is still necessary to carry out *in vivo* tests.

While the "simplicity" of the system is an advantage, isolating the cells from the tissue of origin may result in loss of functions specific to the tissue or organ from which they were taken. Thus, while the most critical environmental variables (O_2, CO_2, humidity, etc.) are controllable, the microenvironment is not yet reproducible, so the system is not completely true to vivo and experimental results may be limited to the cell model. Therefore, these limitations remain to be taken into consideration when setting up any experiment.

Cell cultures: primary cultures and c o n t i n u o u s lines [43]

There are two main types of cell cultures: **primary** cell cultures and continuous growth **cell lines.**

Primary cultures are composed of cells that are derived from a tissue or organ explant. These cells are only able to duplicate for a limited number of passages, then they undergo senescence, and this type of culture is called "finite-lived."

Cell lines are composed of cells that can replicate for an unlimited number of passages.
The cell line is obtained from a stabilized primary culture that has undergone the **immortalization** process, either by spontaneous transformation (Vero cells, BHK-21, CHO, MDCK, Hi5) or chemical or viral agents (PER.C6). The cell line may also derive from tumor tissue that is by "nature" already immortalized, an example of which are HeLa cells.

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[42] https://www.biopills.net/colture-cellulari/

Cell Physiology and Cell Culture Laboratory
Role of Cell Culture Technology in new Vaccine Development
Vaccine manual - FAO

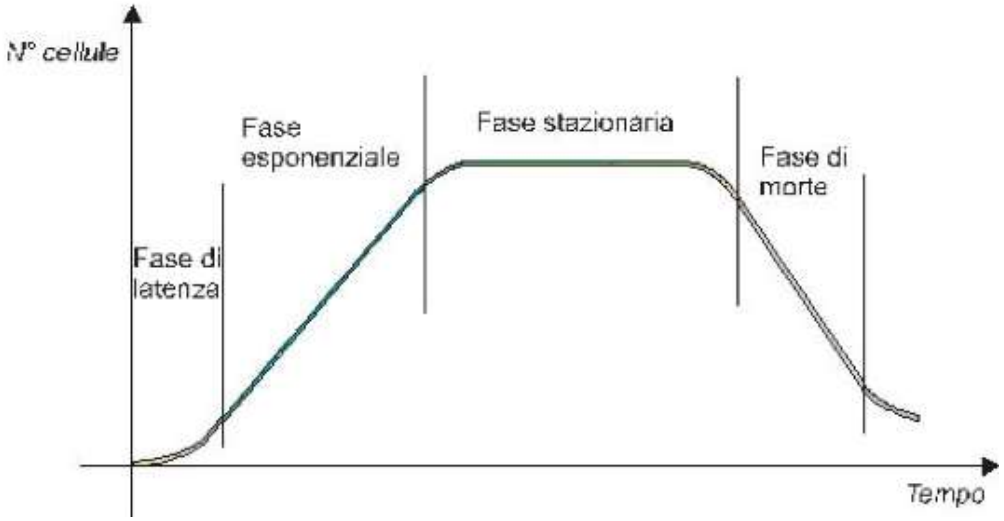
Zahoor, Muhammad Asif & Khurshid, Mohsin & Qureshi, Rabia & Naz, Aneeqa & Shahid, Muhammad
Cell culture-based viral vaccines: Current status and future prospects.
Future Virology.(2016). 11. 10.2217/fvl-2016-0006.
https://www.futuremedicine.com/doi/abs/10.2217/fvl-2016-0006

Genzel Y.
Designing cell lines for viral vaccine production: Where do we stand?
Biotechnol J. 2015 May;10(5):728-40. doi: 10.1002/biot.201400388. Epub 2015 Apr 22.
https://pubmed.ncbi.nlm.nih.gov/25903999/

[43] Jordan I, Sandig V.
Matrix and backstage: cellular substrates for viral vaccines.
Viruses. 2014;6(4):1672-1700. Published 2014 Apr 11. doi:10.3390/v6041672
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4014716/

Aubrit F, Perugi F, Léon A, Guéhenneux F, Champion-Arnaud P, Lahmar M, Schwamborn K.
Cell substrates for the production of viral vaccines.
Vaccine. 2015 Nov 4;33(44):5905-12. doi: 10.1016/j.vaccine.2015.06.110. Epub 2015 Jul 15.
https://www.semanticscholar.org/paper/Cell-substrates-for-the-production-of-viral-Aubrit-Perugi/45023a52e5f48615f0dfd3fa215943f81761122d

The growth and life **of** a cell culture is represented by the **growth curve**, which will be different depending on the type of culture.



https://www.biopills.net/colture-cellulari/
Growth curve of a primary cell culture

The typical growth curve of a primary culture can be divided into four stages:

• During the **first phase,** cells adapt to *in vitro* conditions. This is the most critical phase, is characterized by slow growth and is called the dormancy phase.

• During the **second phase**, the cells grow steadily; they have adapted to the *in vitro* conditions so they grow at a constant rate following a characteristic duplication time depending on the cell type. This phase is called exponential.

• During the **third phase** the process of senescence begins, there is an arrest of cell multiplication, and this phase is called stationary.

• The **fourth stage is** the death **stage, there is** a depletion of the cell culture-where the cell culture ends its life.

Stabilized cell lines show neither the stationary phase nor the death phase. In the case of these cultures, growth remains exponential because the cells have undergone the immortalization process. **Immortalization** can occur spontaneously, for example due to alterations in the cell cycle, or through the use of chemical (carcinogens), physical (UV irradiation) or biological (introduction of viral genes) agents.

Cell lines can also be derived from tumors, as in the case of HeLa cells: cancer cells proliferate uncontrollably, by various modifications they are able to evade the systems that regulate excessive cell proliferation, so immortalization is inherent.

Primary cultures are usually grown in the laboratory, while cell lines can be drawn from **Biobanks**.

The Biobank is a service that collects, stores and distributes biological material useful for research. It also stores along with the sample all the data and information associated with it.
Cell lines usually lose some features typical of the starting cell type (e.g., morphological or receptor characteristics), and often the immortalization process is the cause of these changes.

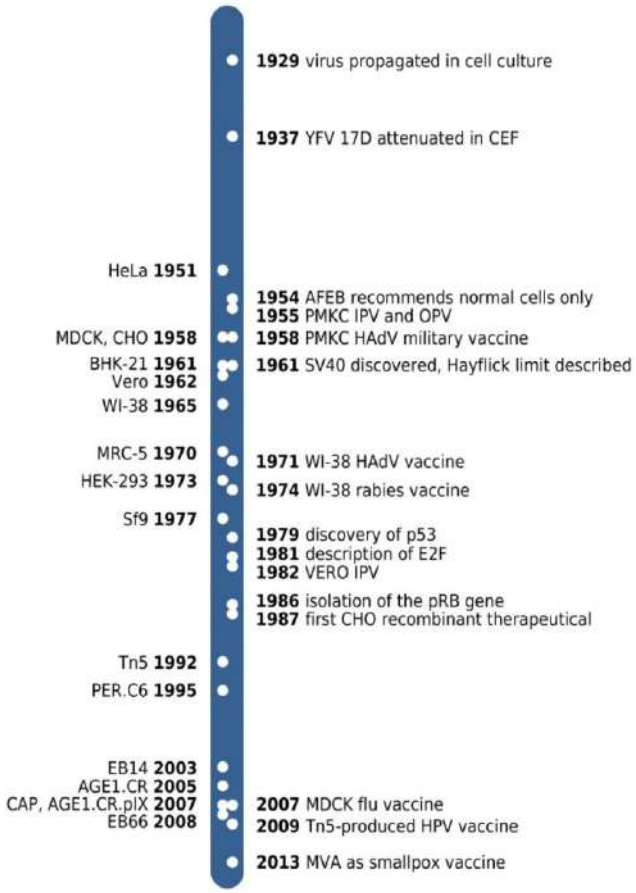
For this reason, primary cultures are the closest model to the *living*, but they are also more delicate because of the growth conditions they require.

There are also other types of cell cultures:
• **Clonal cell line**: derived from a single cell (clone) that produces a homogeneous population by mitosis

• **Diploid cell line (DCL)**: a cell line with a finite *in vitro* lifespan in which the chromosomes are paired (or euploid, i.e., with a normal number of chromosomes) and are structurally identical to those of the species from which they were derived. (e.g. WI-38, MRC-5)

• **Organ or histotypic cultures**: these are small portions of organ or tissue grown *in vitro*.
In this case, the relationship between the different cellular components is maintained, but these cultures are short-lived because the nutrients in the medium and oxygen cannot effectively reach the innermost portions of the organ or tissue. We can also include in this category histotypic cultures obtained by first isolating cells and then re-aggregating them using artificial **scaffolds** to recreate a tissue-like structure;

• **Co-culture**: two different cell types (e.g., endothelial and epithelial) are present in the same system so as to increase the complexity of the *in vitro* model.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4014716/
Time line of cell line derivations (left side of the time bar) and key developments (right side) referred to in the text. YFV 17D, yellow fever virus 17D strain; CEF, chicken embryo fibroblasts; AFEB, American Forces Epidemiological Board; PMKC, monkey primary kidney cell; IPV and OPV, inactivated and oral polio vaccine, respectively; HAdV, human adenovirus; MVA, modified Ankara vaccine virus.

The following are the types and characteristics of cell lines used for human vaccine production.

Most common cell substrates currently used for the production of human viral vaccines.

Cell line	Cell type and origin	Viral susceptibility (*not exhaustive list*)	Examples of vaccine candidate in development	Marketed vaccines for human use
Primary cells				
CEF	Chicken embryo fibroblast	Yellow fever; rabies; TBE; measles; mumps	MVA-based vaccines; HIV; Q fever	Rabies (Rabipur®); TBE (FSME-Immun®, Encepur®); measles (Attenuvax®); mumps (Mumpsvax®)
Diploid cell lines				
MRC5	Human embryonic lung	Varicella zoster virus; polioviruses; rabies; hepatitis A	Rabies	Varicella zoster virus (Varilrix®; Biopox®; ProQuad®); polioviruses (Poliovax®); rabies (Imovax®); hepatitis A (VAQTA®)
WI-38	Human embryonic lung	Rubella; adenoviruses	–	Rubella (Meruvax® II); adenoviruses (Adenovirus Type 4 and Type 7 Vaccine, Live, Oral®)
Continuous cell lines				
MDCK[a]	Canine kidney	Influenza virus type A and B; Human coxsackievirus B3, B4 and B5; reovirus type 2; AAV 4 and 5; vaccinia virus; VSV; Human poliovirus 2	Seasonal & pandemic flu; enterovirus	Seasonal flu (Optaflu®, Flucelvax®)
Vero	African green monkey kidney	Influenza virus type A and B; Measles virus; vaccinia virus; rubella virus; mumps virus; NDV; polioviruses; arbovirus (including dengue); rabies; RSV; parainfluenza viruses; reoviruses	Enterovirus; RSV; HFMD; influenza virus, rabies	Pandemic (Celvapan®) & seasonal flu (Preflucel®); smallpox (ACAM2000®); JEV (Ixiaro®,IMOJEV®); polioviruses (OPV®, IMOVAX Polio®, PolioRIX®, Adacel®); rabies (VERORAB®, Abhayrab®), rotaviruses (RotaRIX®, RotaTeq®)
PER.C6®	Human embryonic retina	Adenovirus; influenza virus; lentivirus; polioviruses; Ebola	Influenza virus; West Nile virus	–
AGE1.CR®	Duck retina	Smallpox; fowlpox; influenza virus; alphaviruses	MVA-based vaccines; influenza virus	–
EB66®	Duck embryos	Influenza virus; measles virus; mumps virus; HSV; poxviruses; NDV; Sindbis virus; Sendai virus; VEEV; yellow fever; VSV	Pandemic & seasonal flu; NDV; MVA recombinant vaccines	Pandemic flu (no commercial name yet)

Note: CEF, chicken embryo fibroblast; MRC, Medical Research Council; WI, Wistar Institute; MDCK, Madin Darby canine kidney; AAV, adeno-associated virus; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; RSV, respiratory syncytial virus; HSV, herpes simplex virus; MVA, modified vaccinia virus Ankara; JEV, Japanese encephalitis virus; HIV, human immunodeficiency virus; TBE, tick-borne encephalitis; HFMD, hand, foot and mouth disease; VEEV, Venezuelan equine encephalitis virus.

Cell substrates for the production of viral vaccines.
https://www.semanticscholar.org/paper/Cell-substrates-for-the-production-of-viral-Aubrit-Perugi/45023a52e5f48615f0dfd3fa215943f81761122d

Main characteristics of most common cell substrates currently used for the production of human viral vaccines–Biological characteristics.

Cell line	Process of derivation	Karyotype	Genetic stability	Tumorigenicity/ Oncogenicity	RT activity from retroviral origin	Adventitious agents
Primary cells						
CEF [87]	Derived from embryonated eggs	$2n-76$	–	NT/NT	Yes	Risk of endogenous retrovirus particles
Diploid cell lines						
MRC5 (ATCC CCL-171) [37]	Derived from a normal embryonic lung	46 (XY), polyploidy rate of 3.6%	Yes	No/NT	No	No
WI-38 (ATCC CCL-75) [37]	Derived from a normal embryonic lung	46 (XY)	Yes	No/NT	No	Micrococcus at P8 (cleared with neomycin)
Continuous cell lines						
MDCK [88–90]	Derived from canine kidney	Undisclosed (hyperdiploid)	Yes	Yes[a]/No	No	No
Vero [91]	Derived from African green monkey kidney	Hypodiploid ($2n-58$)	Yes	No/No[b]	No	No
PER.C6® [92]	Embryonic retinal cells transformed by the insertion of Ad5 E1 genes	46 (XX)	-	Yes[c]/No	No	No
AGE1.CR® [93]	Duck retinal cells immortalized with the Ad5 E1 genes	–	Yes	No/NT	No	No
EB66®	Derived from Duck embryonic stem cell	Diploid ($2n-78$)	Yes	Yes[d]/No	No	No

Note: RT, reverse transcriptase; NT, not tested; CEF, chicken embryo fibroblast; MRC, Medical Research Council; NT, not tested; WI, Wistar Institute; MDCK, Madin Darby canine kidney; ATCC, American Type Culture Collection; CCL, certified cell line; P, passage; Ad5, adenovirus 5.

[a] Both high [88,89] and low [90] tumorigenicity have been reported depending on the tested subclone.

[b] If passage level below p150.

[c] Weakly tumorigenic: only after injection of 10^7 cells.

[d] Weakly tumorigenic: only after injection of 10^7 cells (unpublished data).

For an in-depth look at the various cell lines, we suggest reading the article Matrix and Backstage: Cellular Substrates for Viral Vaccines

Critical issues in quality control

Since the first generation of vaccines produced using animal tissues, the main concerns of regulatory agencies (RAs), manufacturers, and public health authorities have been the possible presence of adventitious agents or cellular components (residual genetic material from cell culture and adventitious viruses), or the transformation of proteins in vaccine products (e.g., reversion of vaccine toxoids [44]).

In fact, several significant cases of contamination have been highlighted during the last century. [45], such as the discovery of SV40 in monkey kidney cells (RMK) used to produce polio vaccines in the 1960s [46], bacterial viruses identified in several live attenuated viral vaccines produced with bacteriophage-containing bovine sera in the early 1970s [47] and more recently, the detection of porcine circovirus sequences in rotavirus vaccines (Rotarix® and RotaTeq ®) [48].

The advancement of science and technology and the use of more powerful analytical methods, such as next-generation sequencing (HTS, NGS) [49] capillary electrophoresis and mass spectrometry applied to the study of

[44] Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed) Annex 4
https://www.who.int/biologicals/vaccines/Diphtheria_Recommendations_TRS_980_Annex_4.pdf?ua=1

[45] Petricciani J, Sheets R, Griffiths E, Knezevic I.
Adventitious agents in viral vaccines: lessons learned from 4 case studies.
Biologicals. 2014 Sep;42(5):223-36. doi: 10.1016/j.biologicals.2014.07.003. Epub 2014 Aug 16.
https://www.sciencedirect.com/science/article/pii/S1045105614000748?via%3Dihub

Klug B, Robertson JS, Condit RC, et al.
Adventitious agents and live viral vectored vaccines: Considerations for archiving samples of biological materials for retrospective analysis.
Vaccine. 2016;34(51):6617-6625. doi:10.1016/j.vaccine.2016.02.015
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5130882/

[46] Sweet Bh, Hilleman Mr. The
vacuolating virus, S.V. 40.
Proc Soc Exp Biol Med. 1960 Nov;105:420-7. doi: 10.3181/00379727-105-26128.
https://pubmed.ncbi.nlm.nih.gov/13774265/

Martini F, Corallini A, Balatti V, Sabbioni S, Pancaldi C, Tognon M.
Simian virus 40 in humans.
Infect Agent Cancer. 2007;2:13. Published 2007 Jul 9. doi:10.1186/1750-9378-2-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1941725/

Rotondo JC, Mazzoni E, Bononi I, Tognon M, Martini F.
Association Between Simian Virus 40 and Human Tumors.
Front Oncol. 2019;9:670. Published 2019 Jul 25. doi:10.3389/fonc.2019.00670
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6669359/

[47] Milch H, Fornosi F.
Bacteriophage contamination in live poliovirus vaccine.
J Biol Stand. 1975;3(3):307-10. doi: 10.1016/0092-1157(75)90034-7.
https://pubmed.ncbi.nlm.nih.gov/1158904/

Moody EE, Trousdale MD, Jorgensen JH, Shelokov A.
Bacteriophages and endotoxin in licensed live-virus vaccines.
J Infect Dis. 1975 May;131(5):588-91. doi: 10.1093/infdis/131.5.588.
https://pubmed.ncbi.nlm.nih.gov/805187/

[48] Victoria JG, Wang C, Jones MS, et al.
Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol. 2010;84(12):6033-6040. doi:10.1128/JVI.02690-09
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2876658/

[49] McClenahan SD, Krause PR.
Towards dynamic monitoring of cell cultures using high throughput sequencing.
Vaccine. 2019 Feb 8;37(7):1001-1005. doi: 10.1016/j.vaccine.2018.12.019. Epub 2019 Jan 11.
https://pubmed.ncbi.nlm.nih.gov/30642729/

Of complex matrices [50], capable of detecting undetectable or previously unknown contaminants has led RAs to implement new production and control procedures in the guidelines.

The basic principle of the guidelines is that vaccine quality, safety, potency, purity and efficacy are based on a comprehensive approach of risk assessment that affects the selection and characterization of raw materials, the control of the intermediate and final product but also the design and validation of the manufacturing process. [51] However, analyses of some commercial vaccines carried out with the

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Cheval J, Muth E, Gonzalez G, et al.
Adventitious Virus Detection in Cells by High-Throughput Sequencing of Newly Synthesized RNAs: Unambiguous Differentiation of Cell Infection from Carryover of Viral Nucleic Acids.
mSphere. 2019;4(3):e00298-19. Published 2019 Jun 5. doi:10.1128/mSphere.00298-19
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6553555/

Khan AS, Blümel J, Deforce D, et al.
Report of the second international conference on next generation sequencing for adventitious virus detection in biologics for humans and animals.
Biologicals. 2020;67:94-111. doi:10.1016/j.biologicals.2020.06.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7351673/

Cleveland MH, et al
Report of the 2019 NIST-FDA workshop on standards for next generation sequencing detection of viral adventitious agents in biologics and biomanufacturing.
Biologicals. 2020 Mar;64:76-82. doi: 10.1016/j.biologicals.2020.02.003. Epub 2020 Feb 22.
https://www.sciencedirect.com/science/article/abs/pii/S1045105620300245?via%3Dihub

Luciani F, Bull RA, Lloyd AR.
Next generation deep sequencing and vaccine design: today and tomorrow.
Trends Biotechnol. 2012;30(9):443-452. doi:10.1016/j.tibtech.2012.05.005
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7127335/

[50] Mao HH, Chao S.
Advances in Vaccines.
Adv Biochem Eng Biotechnol. 2020;171:155-188. doi:10.1007/10_2019_107
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7120466/

Advances and Challenges in Vaccine Development and Manufacture

Viviani L, et al.
Global harmonization of vaccine testing requirements: Making elimination of the ATT and TABST a concrete global achievement.
Biologicals. 2020 Jan;63:101-105. doi: 10.1016/j.biologicals.2019.10.007. Epub 2019 Nov 4.
https://www.sciencedirect.com/science/article/pii/S1045105619301137

[51] Lebron JA, Wolf JJ, Kaplanski CV, Ledwith BJ.
Ensuring the quality, potency and safety of vaccines during preclinical development.
Expert Rev Vaccines. 2005 Dec;4(6):855-66. doi: 10.1586/14760584.4.6.855.
https://pubmed.ncbi.nlm.nih.gov/16372881/

McClenahan SD, Krause PR.
Towards dynamic monitoring of cell cultures using high throughput sequencing.
Vaccine. 2019 Feb 8;37(7):1001-1005. doi: 10.1016/j.vaccine.2018.12.019. Epub 2019 Jan 11.
https://pubmed.ncbi.nlm.nih.gov/30642729/

Knezevic I, Stacey G, Petricciani J,
WHO Study Group on cell substrates. WHO Study Group on cell substrates for production of biologicals, Geneva, Switzerland, 11-12 June 2007.
Biologicals, 2008;36:203-11. https://www.who.int/biologicals/publications/meetings/areas/vaccines/cells/Cells.FINAL.MtgRep.IK.26_Sep_07.pdf?ua=1

Investigating Viruses in Cells Used to Make Vaccines; and Evaluating the Potential Threat Posed by Transmission of Viruses to Humans
https://www.fda.gov/vaccines-blood-biologics/biologics-research-projects/investigating-viruses-cells-used-make-vaccines-and-evaluating-potential-threat-posed-transmission

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks
https://www.who.int/biologicals/Cell_Substrates_clean_version_18_April.pdf?ua=1

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks Replacement of Annex 1 of WHO Technical Report Series, No. 878
http://158.232.12.119/biologicals/vaccines/TRS_978_Annex_3.pdf

Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines
https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-clinical-aspects-live-recombinant-viral-vectored-vaccines_en.pdf

support of the COVELVA association have pointed out numerous critical issues regarding the quality of these vaccines.

For a more in-depth discussion of contaminants in commercial vaccines, see the section **VACCINEGATE** (CORVELVA) or **CORVELVA ANALYSIS DOSSIER** (studiesalute.it)

Tumorigenicity and oncogenicity of contaminants [52]

ONCOGENICITY: The ability of an acellular agent-such as a chemical agent, virus, viral nucleic acid, viral gene, or subcellular elements-to induce tumor formation in the normal cells of an animal.

TUMORIGENICITY: the ability of an inoculated cell population in an animal model to produce a tumor by proliferation at the site of inoculation and/or at a distant site by metastasis.

Tumors presenting in a tumorigenicity assay contain cells derived from the inoculated cells, whereas tumors presenting in an oncogenicity assay are host-derived. The oncogenic activity of cell substrates could be due to cell substrate DNA (and perhaps other cellular components) or to an oncogenic viral agent present in the cells.

Tumorigenicity describes the ability of inoculated cells to proliferate in a recipient. There appear to be no molecular markers for this property.
Although chromosomal aberrations are the hallmark of many cancers, they have also been observed in the MRC-5 cell line, which is considered non-cancerous by regulatory agencies [53] and therefore is considered

ICH Topic Q 5 D Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products
https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-d-derivation-characterisation-cell-substrates-used-production-biotechnological/biological-products-step-5_en.pdf

ICH Topic Q 5 A (R1) Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-r1-viral-safety-evaluation-biotechnology-products-derived-cell-lines-human-animal-origin_en.pdf

ICH Topic Q 6 B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products
https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-6-b-test-procedures-acceptance-criteria-biotechnological/biological-products-step-5_en.pdf

Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications
https://www.fda.gov/media/78428/download

European Pharmacopoeia 5.0. 5.2.3. Cell substrates for the production of vaccines for human use. January 2005.

[52] Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications (FDA)

[53] Jacobs JP, Jones CM, Baille JP.
Characteristics of a human diploid cell designated MRC-5.
Nature. 1970 Jul 11;227(5254):168-70. doi: 10.1038/227168a0.
https://www.nature.com/articles/227168a0

Jacobs JP.
Updated results on the karyology of the WI-38, MRC-5 and MRC-9 cell strains.
Dev Biol Stand. 1976 Dec 13-15;37:155-6. PMID: 1031681.
https://pubmed.ncbi.nlm.nih.gov/1031681/

von Seefried A, Walton MJ, MacMorine HG.

That the stability of the karyotype of a cell line is not predictive [54] and that tumorigenicity should therefore be determined experimentally.
The use of immortalized, cancer-causing cell lines for viral vaccine production has been under discussion since the 1990s [55] . The main concerns associated with tumorigenicity of cell substrates are induction of tumor allografts, transfer of known or unknown viruses, and transfer of oncogenes or cellular components that could trigger tumors. Therefore, regulatory agencies have issued guidelines for the study of tumorigenic activity for all diploid (except MRC-5 and WI-38) and continuous cell lines, since cells could acquire tumorigenic activity as they are expanded in culture [56].

Cell lines are classified as carcinogenic when they possess the ability to form a tumor after injection of intact cells into genetically immunocompromised animals (e.g., nude, severe combined immunodeficiency (SCID) mice or immunocompromised animals obtained by inactivating T-lymphocyte functions with anti-thymocyte globulin, anti-thymocyte or anti-lymphocyte sera).
The tumorigenic phenotype of the cell substrate can be defined by evaluating the kinetics of tumor formation at a dose of 10^7 , 10^5 , 10^3 and 10 cells/animal, for at least 4 months of observation. Determining the dose that induces tumor in 50% of animals ($_{TPD50}$), the time required for tumor development, and the ability to form metastases are parameters that need to be evaluated for a given cell substrate. The current understanding is that a low $_{TPD50}$ (ability to form tumors at 10 -10^{14} cells/animal; such as HeLa cells) correlates with a higher risk of inducing neoplastic development. There is no general rule for cell substrate and/or finished product acceptability in this regard, but rather a discussion with ARs on a case-by-case basis is necessary.

The use of tumorigenic and tumor-derived cells for vaccine production requires an oncogenicity study, which must be performed using cellular DNA (≥100 g) and cell lysate (obtained from 10^7 cells) injected into newborn nude mice (e.g., <3 days of age), hamsters, and newborn rats. The animals are examined for nodule formation for a period of at least 4 months. At the end of the observation period, the animals are sacrificed and examined for evidence of tumor formation at the injection site as well as for evidence of metastatic lesions. [57]

The most important component potentially associated with transformation activity is cell line-derived DNA.

Residual DNA could pose a risk to the final product due to oncogenic and/or infectious potential. There are several potential mechanisms by which residual DNA could be oncogenic, including integration and expression of encoded oncogenes or insertional mutagenesis following DNA integration. Residual DNA could also be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extrachromosomal genomes are present.

Karyology: vaccine manufacturers' views.
Dev Biol Stand. 1976 Dec 13-15;37:169-75. PMID: 801470.
https://pubmed.ncbi.nlm.nih.gov/801470/

[54] Annex 3 Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks Replacement of Annex 1 of WHO Technical Report Series, No. 878
https://www.who.int/biologicals/vaccines/TRS_978_Annex_3.pdf

[55] Lewis AM Jr, Krause P, Peden K.
A defined-risks approach to the regulatory assessment of the use of neoplastic cells as substrates for viral vaccine manufacture.
Dev Biol (Basel). 2001;106:513-35.
https://pubmed.ncbi.nlm.nih.gov/11761266/

[56] Manohar M, Orrison B, Peden K, Lewis AM Jr.
Assessing the tumorigenic phenotype of VERO cells in adult and newborn nude mice.
Biologicals. 2008 Jan;36(1):65-72. doi: 10.1016/j.biologicals.2007.06.002. Epub 2007 Oct 22.
https://pubmed.ncbi.nlm.nih.gov/17933552/

[57] WHO Expert Committee on Biological Standardization. Sixty-first report. WHO 579 Technical Report Series, No. 978, 2013 Annex 3.
Recommendations for the eval- 580 uation of animal cell cultures as substrates for the manufacture of biological 581 medicinal products and for the characterization of cell banks.
https://www.who.int/biologicals/expert_committee/TRS_978_61st_report.pdf?ua=1

The risks of oncogenicity and infectivity of DNA from the cell substrate can be reduced by decreasing its biological activity. This can be achieved by decreasing the amount of residual DNA and reducing the size of the DNA (e.g., by treatment with DNAase or other methods) below the size of a functional gene (based on current evidence, about 200 base pairs). Chemical inactivation can reduce both the size and biological activity of DNA. [58]
ARs, while requiring validation of methods of inactivation/elimination of residual DNA and adventitious agents nevertheless support the hypothesis that ribonucleoprotein complexes and other entities commonly found in cell lysates cannot persist long enough to support malignant transformation, which is hypothesized to proceed in multiple stages [59].

A maximum allowable amount of cell line-derived DNA in oral and intranasal vaccines has not been defined, and final formulations should be discussed with authorities [60].
Levels are also not defined for vaccines produced on diploid or primary cell substrate.

However, a parenteral vaccine produced on a continuous cell line may contain no more than 10 ng of residual DNA per dose [61]. This value is the result of considerations that estimate the probability of an oncogene being successfully encoded and transferred with a given segment of co-purified DNA. Enzymatic fragmentation or β-propiolactone treatment can further reduce any risk associated with DNA contamination [62], but some processing steps cannot be performed without losing the potency of live vaccines.

Although the use of the fetal lung-derived diploid MRC-5 cell line is well established (it has been used for vaccine production since the 1960s), the karyotype study has stopped with the latest study "*Chromosomal characterization of MRC-5 cell banks utilizing G-banding technique*" [63] in which it is argued that the t(7; 12) translocation detected in this line does not pose any risk of tumorigenicity in mice and that therefore such cell lines are safe biological substrates and should not require any chromosomal analysis before being used as cell substrates for the production of live virus vaccines. This consideration is reiterated in the FDA guideline:
Guidance for Industry (FDA) Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications:

[58] Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications (FDA)

[59] Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer.
Nat Rev Cancer. 2002 May;2(5):331-41. doi: 10.1038/nrc795.
https://doi.org/10.1038/nrc795

[60] World Health Organization; Geneva, Switzerland: 2013. Recommendations for the Evaluation of Animal Cell Cultures as Substrates for the Manufacture of Biological Medicinal Products and for the Characterization of Cell Banks. (WHO Technical Report Series). TRS 978, Annex 3.

[61] Grachev V, Magrath D, Griffiths E.
WHO requirements for the use of animal cells as in vitro substrates for the production of biologicals (Requirements for biological susbstances no. 50).
Biologicals. 1998 Sep;26(3):175-93. doi: 10.1006/biol.1998.0153.
https://pubmed.ncbi.nlm.nih.gov/20226252/

[62] Yang H, Zhang L, Galinski M.
A probabilistic model for risk assessment of residual host cell DNA in biological products.
Vaccine. 2010 Apr 26;28(19):3308-11. doi: 10.1016/j.vaccine.2010.02.099. Epub 2010 Mar 10.

[63] Rosolowsky M, McKee R, Nichols W, Garfinkle B.
Chromosomal characterization of MRC-5 cell banks utilizing G-banding technique.
Dev Biol Stand. 1998;93:109-17. PMID:9737385
https://pubmed.ncbi.nlm.nih.gov/9737385/

Prockop DJ, Keating A.
Relearning the lessons of genomic stability of human cells during expansion in culture: implications for clinical research. Stem Cells. 2012 Jun;30(6):1051-2. doi: 10.1002/stem.1103.
https://stemcellsjournals.onlinelibrary.wiley.com/doi/full/10.1002/stem.1103

• a diploid cell strain should remain diploid at all times. If these characteristics are not stable, it must be demonstrated that the instability does not adversely affect the production or conformity of the product.

• For widely used human diploid cell strains, such as MRC-5 and WI-38 cells, measurement of residual DNA may not be necessary because we do not consider residual DNA from these cells
diploid humans a security issue

• residual DNA for nontumorigenic continuous cells, such as low-passage VERO cells, should be limited to less than 10 ng/dose for parenteral inoculation as recommended by WHO. (pg 37)

• It is recommended to monitor the genetic stability of the diploid cell strain during production (p. 11)

Priorix Tetra contains viral strains produced separately in chicken embryo cells (mumps and measles) and in human MRC-5 diploid cells (rubella and varicella). The cell lines used for Priorix Tetra include human diploid cell lines that cannot continuously divide. ***It*** *should be noted that, according to the European Pharmacopoeia****, MRC-5 diploid cell lines are not tumorigenic, as demonstrated by decades of use and control, and therefore no upper limit for MRC-5 cell DNA applies****. (EMA request reference ASK-43967 August 3, 2018)*

Insight: *discussion-NGS-EMA*

As of that date, therefore, no other published studies characterizing the genetic stability of diploid cell lines used for vaccine production are known.
During the quality investigation of Priorix Tetra vaccine, the whole genome of fetal DNA was sequenced, detected in substantial amounts (up to 300 times higher than the 10 ng/dose limit) in all samples analyzed. The results indicate the presence of a highly modified genome even in oncogenes and thus with tumorigenic potential.
As will be seen below, Dr. Deisher's in vitro studies confirm the transforming capacity of fetal DNA contained in pediatric attenuated virus vaccines.

WHOLE GENOME SEQUENCING OF FETAL DNA PRESENT VEL VACCINE PRIORIX TETRA

METHOD: sequencing by next generation sequencing (NGS) technology of the whole genome residual DNA derived from the MRC-5 cell line for the purpose of mapping single nucleotide mutations (SNPs), insertions and deletions of longer or shorter sequences occurring at certain positions in the genome, and variations in the copy number of portions of the genome/gens (CNVs, copy number variants).
The human reference genome (MRC-5 cell line) was found to be 99.76% covered by reads originating from the vaccine DNA, thus almost its entirety. **The fetal human DNA represented in this vaccine is therefore a complete individual genome i.e., genomic DNA from all chromosomes of a male individual corresponding to that of the MRC-5 line is present in the vaccine**

• DNA single-base variants (SNPs) are polymorphisms, i.e., variations in genetic material at a single nucleotide. In contrast, 'InDels' are small insertions and deletions less than 50pb in length and constitute another class of genomic variants in the human genome.

A total of about 3.6 million SNPs have been identified in the human vaccine genome (including 98.31% already reported in the public DBSNP database and **61,805 new** or original ones of this DNA).
The amount of SNPs is in line with what has been reported in the literature in a 'typical human genome'

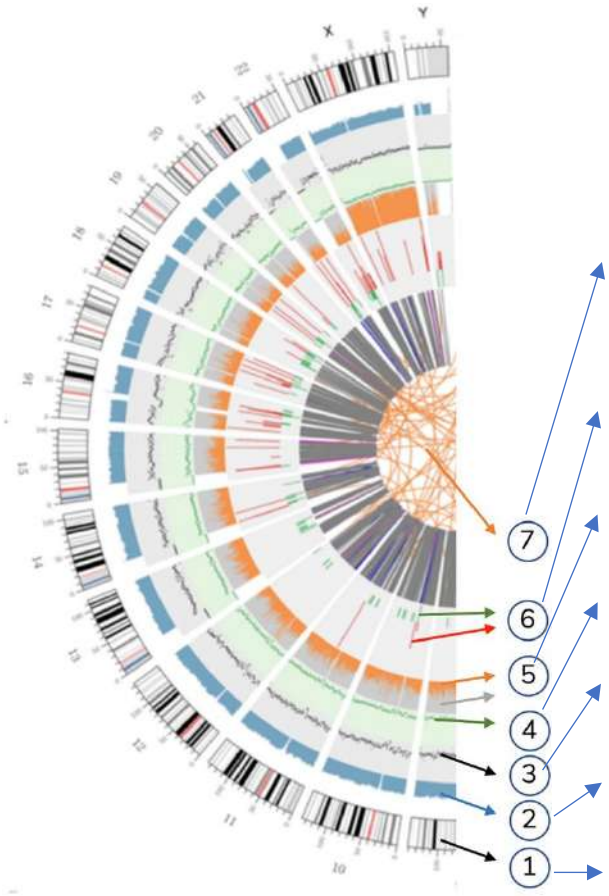
InDels are found to be in higher numbers than reported by '*The 1000 Genomes Project Consortium'* in 'A global reference for human genetic variation' (Nature, vol. 526, Oct. 10, 2015)' i.e. **800 thousand compared to 600 thousand**. 804 thousand InDels were identified (of which 89.42% were already reported in DBSNP and **85,106 were new**)

• copy number variants (CNVs) are genomic variants due to changes in the copy number of relatively large fragments (longer than 50 bp) between individual genomes. There are two types of CNVs: type

'gain' and 'loss' type. A total of **218 CNVSs** were detected in the human vaccine genome, **of which 82 were 'gain' type CNVSs** (covering a portion of the genome totaling about 6.9 million base pairs) **and 136 'loss' type CNVSs** (covering a portion of the genome of about 70 million bases).

- Analysis of variants in cancer genes

SNP, INDELS, CNV, SV variant analysis restricted to 560 genes involved in different forms of human cancer has highlighted the **presence of numerous 'original' variants,** i.e., not found in public databases and therefore not known in the literature.



MEANING OF CONCENTRIC CIRCLES

7) The most central ring represents the inference of SV (Structural Variants) in exonic and splicing regions. TRA (orange, translocations), INS (green, insertions), DEL (deletions, gray), DUP (duplications, pink) and INV (inversions, blue).

6) The sixth ring represents the inference of CNV (Variants in copy number). Red means gain of DNA pieces and green means loss.

5) The fifth ring represents the proportion of SNPs in homozygosity (orange) and heterozygosity (gray) in histogram style.

4) The fourth ring (green) represents snp density in "scatter plot" style.

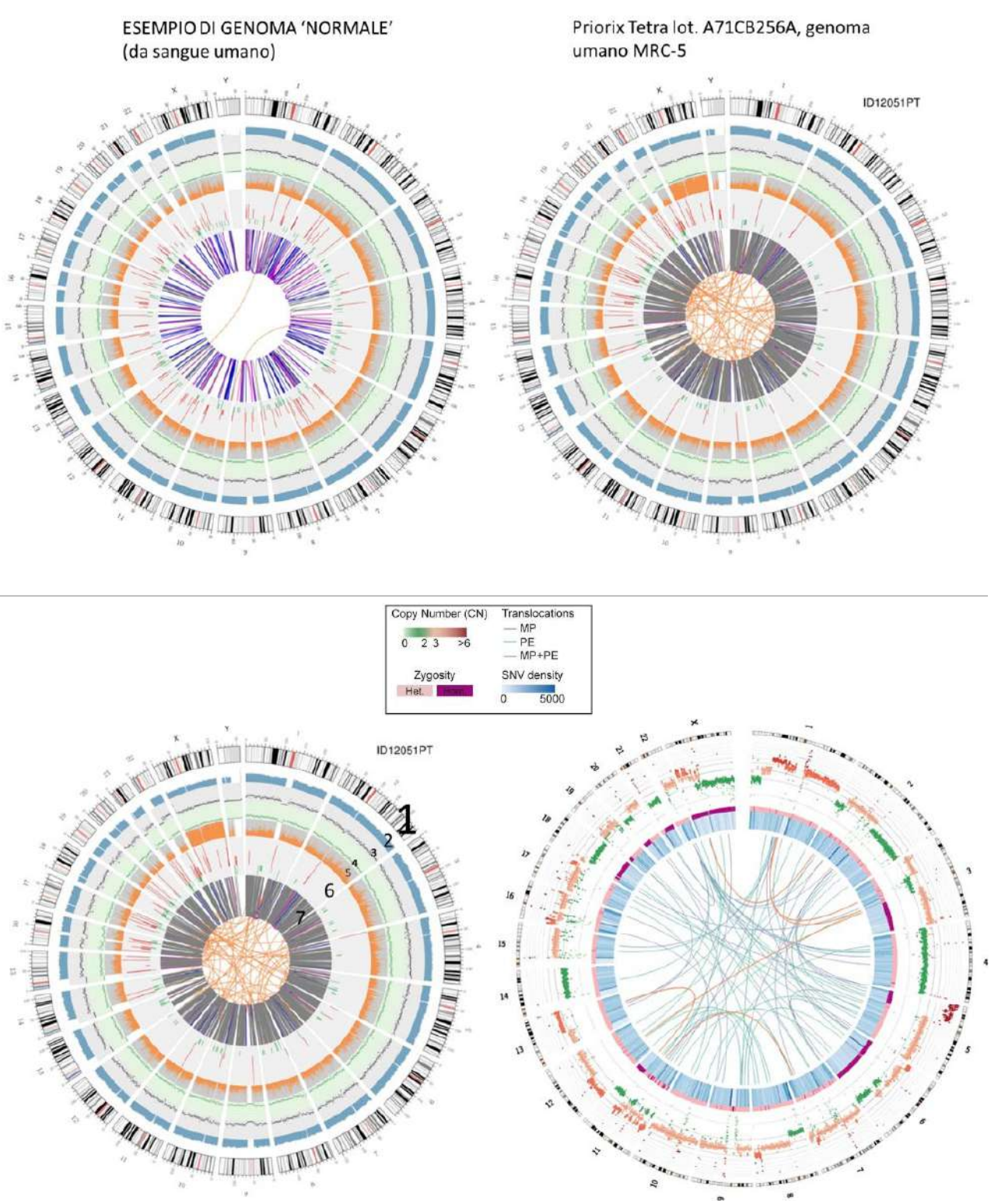
3) The third ring (black) represents the density of INDELs in "scatter plot" style.

2) The second ring (light blue) represents the coverage of histogram-style reads.

1) The outer circle (the first circle) is the chromosome number.

CIRCOS PLOT

A graphical representation of the vaccine genome called a "circos plot" (which is commonly used to represent a re-sequenced genome), is shown below, alongside another representing a re-sequenced genome from DNA extracted from blood of a healthy individual-"normal" genome



Rough comparison between **fetal DNA** (left) and DNA from **HeLa** cells (right), the immortalized cell line also used in polio vaccine production.

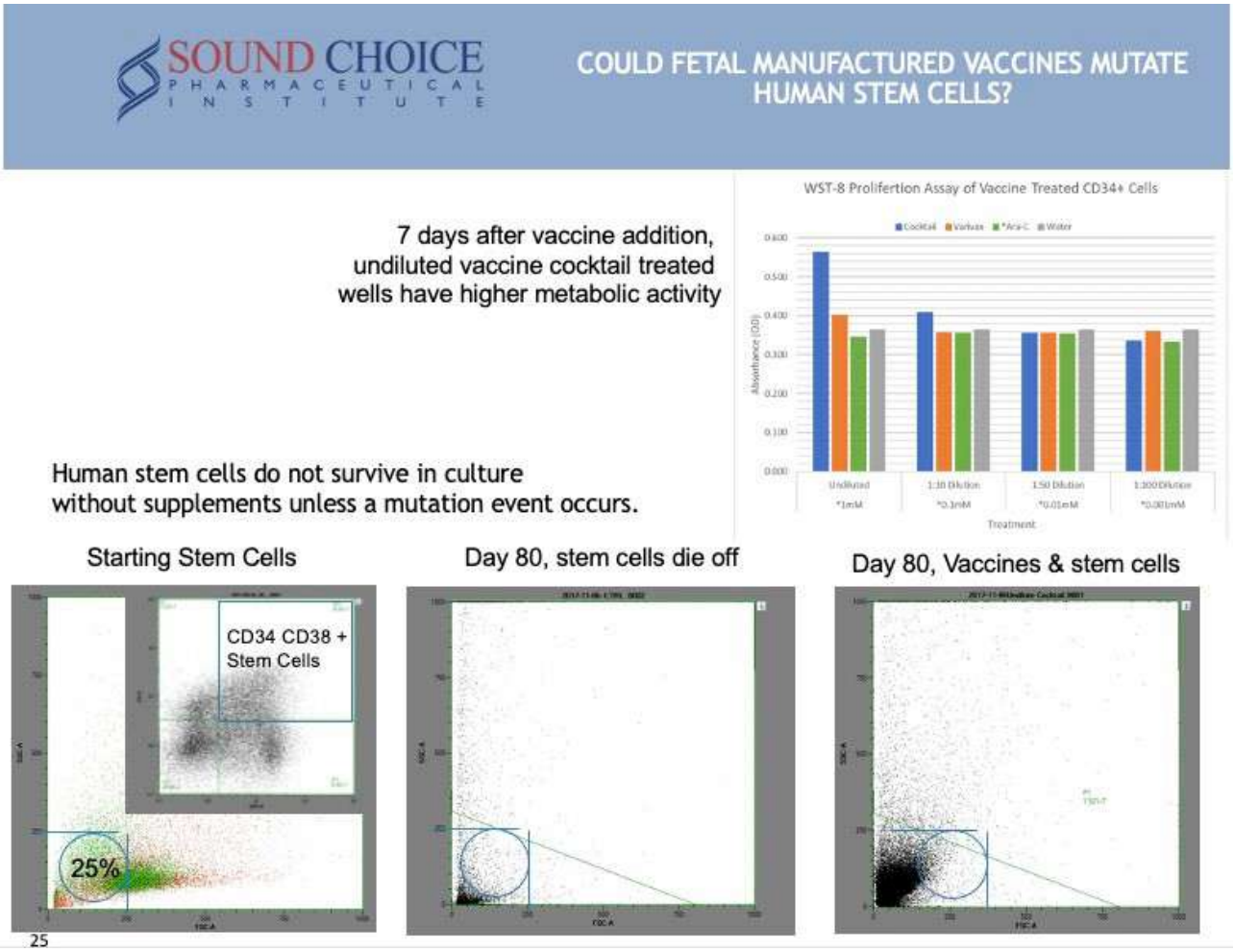
➢ translocations of HeLa cells represented in the circos plot by the nucleus lines refer to the entire genome (thus coding and noncoding part), whereas in the case of fetal vaccine cells they refer only to coding genes

Sequencing of the complete genome of MRC-5 contained in Priorix Tetra Comparison of human genome contained in Priorix Tetra and in the MRC-5 cell line

The studies of Dr. T. DEISHER [64] support the hypothesis that inoculation of human fetal DNA contamination from diploid cells, considered to date nontumorigenic by regulatory agencies, carries the risk of causing two established diseases:

• **insertional mutagenesis**: human fetal DNA, being hypomethylated, efficiently incorporates into the child's DNA causing mutations. Gene therapy using homologous recombination of small fragments has shown that amounts as small as 1.9 ng/mL of DNA fragments result in
in stem cell genome insertion in 100% of the injected mice. Levels of human fetal DNA fragments in children after vaccination with MMR, VARIVAX (varicella) or hepatitis A vaccines reach levels above 1.9 ng/mL.

• **autoimmune disease**: fetal human DNA stimulates the immune system's reaction to attack the vaccinee's body.



According to the WHO definition[65] , **adventitious agents** are microorganisms that contaminate cell culture or raw materials/greens, including bacteria, fungi, mollicutes (mycoplasmas or spiroplasmas),

[64] https://soundchoice.org/our-research/open-letter-to-legislators/ (letter to the rulers - April 8, 2019)
https://soundchoice.org/our-research/

[65] Annex 2 Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine
https://www.who.int/biologicals/vaccines/Annex2 Adventious_Agent_in_marketed_vaccine_eng.pdf?ua=1

Xu L, Lee SB, Fuchs C, Hyams KC, Brorson K, Swann P.
Role of risk assessments in viral safety: an FDA perspective.
PDA J Pharm Sci Technol. 2014 Jan-Feb;68(1):6-10. doi: 10.5731/pdajpst.2014.00959.
https://www.researchgate.net/publication/260120702_Role_of_Risk_Assessments_in_Viral_Safety_An_FDA_Perspective

Mycobacteria, rickettsia, protozoa, parasites, TSE-causing agents, and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

The source of the contaminant may be the cell line, the raw materials used in the culture medium to propagate the cells (such as human serum contaminated with hepatitis B virus used in the formulation of the 17D vaccine [66]), the environment, personnel, equipment ect. They are a major concern in processes obtained from p r i m a r y cell lines [67] and continuous, both invertebrate [68] and vertebrate [69].

In the FDA guideline *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks*, it is reported that "because a viral genome, once introduced, could amplify and produce many infectious particles, the risk of infectivity is likely to be greater than the oncogenic risk."

The polyoma virus genome is infectious in mice at about 50 pg, and a recent report showed that 1 pg of a proviral copy of a retrovirus is infectious in vitro. Because such low levels of DNA can be biologically active, the amounts of residual DNA (rcDNA) should be considered in safety assessments when tumorigenic cell substrates are used, especially for attenuated viral vaccines.

Recent studies conducted by the FDA support the use of new sequencing technologies (NGS) for screening vaccines to demonstrate the absence of contamination by viral agents.[70]

In the investigation of the quality of marketed vaccines, it emerged from interlaboratory confirmations of metagenomic analysis with NGS that the following adventitious and residual agents were present:

ICH Topic Q 5 A (R1) Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

[66] Frierson JG.
The yellow fever vaccine: a history.
Yale J Biol Med. 2010 Jun;83(2):77-85. PMID: 20589188; PMCID: PMC2892770
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2892770/

[67] Enserink M.
Influenza. Crisis underscores fragility of vaccine production system.
Science. 2004 Oct 15;306(5695):385. doi: 10.1126/science.306.5695.385.
https://science.sciencemag.org/content/306/5695/385/tab-pdf

[68] Li TC, Scotti PD, Miyamura T, Takeda N.
Latent infection of a new alphanodavirus in an insect cell line.
J Virol. 2007;81(20):10890-10896. doi:10.1128/JVI.00807-07
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2045576/

[69] Oehmig A, Büttner M, Weiland F, Werz W, Bergemann K, Pfaff E.
Identification of a calicivirus isolate of unknown origin.
J Gen Virol. 2003 Oct;84(Pt 10):2837-2845. doi: 10.1099/vir.0.19042-0.
https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.19042-0

[70] https://www.fda.gov/vaccines-blood-biologics/science-research-biologics/results-fda-study-support-development-high-throughput-sequencing-viral-safety-evaluation-biologic

Computational Pipeline Engine in FDA HIVE: Adventitious Agent Detection from NGS Data
https://www.fda.gov/media/141991/download

Cleveland MH,et al
Report of the 2019 NIST-FDA workshop on standards for next generation sequencing detection of viral adventitious agents in biologics and biomanufacturing.
Biologicals. 2020 Mar;64:76-82. doi: 10.1016/j.biologicals.2020.02.003. Epub 2020 Feb 22.
https://pubmed.ncbi.nlm.nih.gov/32094072/

Analisi vaccino Hexyon

Metagenomico

La presenza del materiale genetico contaminante è stata confermata con l'analisi interlaboratorio presso un service provider certificato europeo.

Il DNA presente è pari a 6,88 ng totali per dose (tale quantità è riferita al report divulgato. Il dato dell'analisi interlaboratorio è oggetto di peer review e quindi non divulgabile ma conferma l'ordine di grandezza), di cui lo 0,1% proveniente potenzialmente dalle cellule Vero (Cercopithecidae), cioè 688 pg/dose. Abbiamo identificato il Clostridium phage phiCT453A e SV40 assieme ad altri vettori per il clonaggio.

Presenza dei Poliovirus 1 e 2. In questo caso la risposta dell'EMA all'assenza del Poliovirus 3 è stata molto generica, l'assenza non è per loro una non conformità poiché danno per assodato la presenza dell'antigene D, capace di creare immunizzazione. Ovviamente abbiamo cercato questa proteina ma non siamo stati in grado di trovarla, sarebbe un ottimo approfondimento da sviluppare perché attualmente lasciamo su questo punto una risposta incerta.

DNA e RNA dei batteri utilizzati per la produzione degli antigeni di Corynebacterium diphtheriae (Diphtheria), Clostridium tetani (Tetanus), Bordetella pertussis (Pertussis) ed Haemophilus influenzae.

NOTA: il materiale genetico avventizio presente nel vaccino può essere legato all'alluminio adiuvante con possibile potenziamento degli effetti tossici (capacità infiammatoria, autoimmune e tumorale). Ribadiamo che, dai dati confermati interlaboratorio, rimane dubbia anche la sicurezza e l'efficacia di questo vaccino, risultando un prodotto del tutto non conforme per quanto riguarda la qualità.

Gardasil vaccine analysis
9

Metagenomico

La presenza del materiale genetico è stata confermata con l'analisi interlaboratorio presso un service provider certificato europeo e possiamo ripetere i precedenti dati, sono presenti:

- DNA umano e di topo (sotto i limiti di rilevabilità dello strumento)
- Virus avventizi:
  - Frammento L1 del virus HPV di DNA a doppia catena;
  - Fagi;
  - Molluscum contagiosum virus;
- Retrovirus:
  - Virus della leucemia murina;
  - Retrovirus endogeno umano K.
  - Saccharomyces

NOTA: il materiale genetico avventizio presente nel vaccino può essere legato all'alluminio adiuvante con possibile potenziamento degli effetti tossici (capacità infiammatoria, autoimmune e tumorale)

Analisi vaccino Priorix Tetra

Metagenomico

Quasi-specie virali: nel genoma della varicella vaccinale sono state individuate 245 varianti rispetto al genoma di riferimento utilizzato per l'analisi (genoma selvatico del ceppo Dumas). Di queste varianti, **154 sono varianti maggiori** mentre le restanti **91 sono varianti quasi-specie.** Dal confronto tra le varianti trovate nei due lotti non emerge alcuna differenza. Nel genoma vaccinale della parotite sono state individuate 40 varianti quasi-specie rispetto al genoma di riferimento utilizzato per l'analisi (genoma vaccinale Jeryl-Lynn), Dal confronto tra le varianti trovate nei due lotti emergono 4 differenze. L'EMA non è stata in grado di fornirci le sequenze dei virus vaccinali utilizzati dal produttore per questo vaccino, in quanto coperte da segreto industriale, motivo per cui non sappiamo quanto siano mutati i virus vaccinali rispetto a quanto dichiarato dal produttore
A causa delle coperture basse non è stato possibile rilevare varianti quasi-specie per i genomi di morbillo e rosolia.

Virus avventizi - Abbiamo confermato la presenza di questi virus avventizi:

- Human endogenous retrovirus K;
- Avian leukosis virus;
- HERV-H/env62.

La presenza del materiale genetico è stata confermata con l'analisi interlaboratorio presso un service provider certificato europeo
Le quantità sono riferite ai report divulgati. I dati dell'analisi interlaboratorio sono oggetto di peer review e quindi non divulgabili ma confermano l'ordine di grandezza
DNA - La quantità di DNA totale presente in questo vaccino oscilla da: 1.7 – 3.7 μg/dose ed è a tutti gli effetti il componente principale del vaccino. Il DNA è di tipo umano circa all'80% (74-88%) e di pollo (0-4%).

Il genoma umano è completo, cioè con geni e sequenze non codificanti, ad alto peso molecolare, di sesso maschile, qualificato come appartenente alla linea fetale MRC-5 cioè la linea cellulare continua derivata da tessuto polmonare di feto abortivo maschile degli anni '60. Il sequenziamento di questa linea cellulare ha dato prova di come fosse **altamente modificato dal punto di vista genetico e potenzialmente cancerogeno. Le analisi di sequenziamento del genoma intero del DNA fetale è stata effettuata presso un service provider (laboratorio) americano.**

RNA - Umano 68-87%. Pollo 0-0.2%

Virus attenuati - Sono stati confermati i seguenti virus attenuati lot. A71CB256A:

- **Varicella** (DNA) 11%;
- **Parotite** (RNA) 0.008%;
- **Morbillo** (RNA) 0.004%;
- **Rosolia** 0.00004%. (114 su 260 milioni di sequenze)

È stata confermata interlaboratorio una presenza irrilevante di rosolia nel vaccino (inferiore ai virus avventizi indicati qui sotto). Ciò mette in serio dubbio l'efficacia del vaccino.

Summary of data confirmation by interlaboratory analysis

COVID-19 VACCINE PLATFORMS

THE LIFE CYCLE OF SARS-COV-2 [71]

Similar to SARS-CoV and MERS-CoV. [72], the genome of SARS-CoV-2 is a positive-sense single-chain strand that encodes for nonstructural proteins (NSPs, such as 3-cymotrypsin-like protease, papain-like protease, helicase, and RNA-dependent RNA polymerase), structural proteins, and accessory proteins.

SARS-CoV-2 has four structural proteins: **spike protein** (S), **envelope protein** (E), **membrane protein** (M) and **nucleocapsid protein** (N).

Among these proteins, trimeric protein S is essential for virus-cell-receptor interactions during viral entry. The S protein comprises an S1 N-terminal subunit responsible for virus receptor binding and an S2 C-terminal subunit responsible for cell membrane-virus fusion.
S1 is further divided into an N-terminal domain (NTD) and a receptor binding domain (RBD). [73]

Khan AS, Blümel J, Deforce D, et al.
Report of the second international conference on next generation sequencing for adventitious virus detection in biologics for humans and animals.
Biologicals. 2020;67:94-111. doi:10.1016/j.biologicals.2020.06.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7351673/

Petricciani J, Sheets R, Griffiths E, Knezevic I.
Adventitious agents in viral vaccines: lessons learned from 4 case studies.
Biologicals. 2014 Sep;42(5):223-36. doi: 10.1016/j.biologicals.2014.07.003. Epub 2014 Aug 16.
https://www.sciencedirect.com/science/article/pii/S1045105614000748?via%3Dihub

[71] Liu X, Liu C, Liu G, Luo W, Xia N.
COVID-19: Progress in diagnostics, therapy and vaccination.
Theranostics 2020;10(17):7821-7835. doi:10.7150/thno.47987.
https://www.thno.org/v10p7821.htm

[72] Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S.
The spike protein of SARS-CoV--a target for vaccine and therapeutic development.
Nat Rev Microbiol. 2009 Mar;7(3):226-36. doi: 10.1038/nrmicro2090. Epub 2009 Feb 9.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2750777/

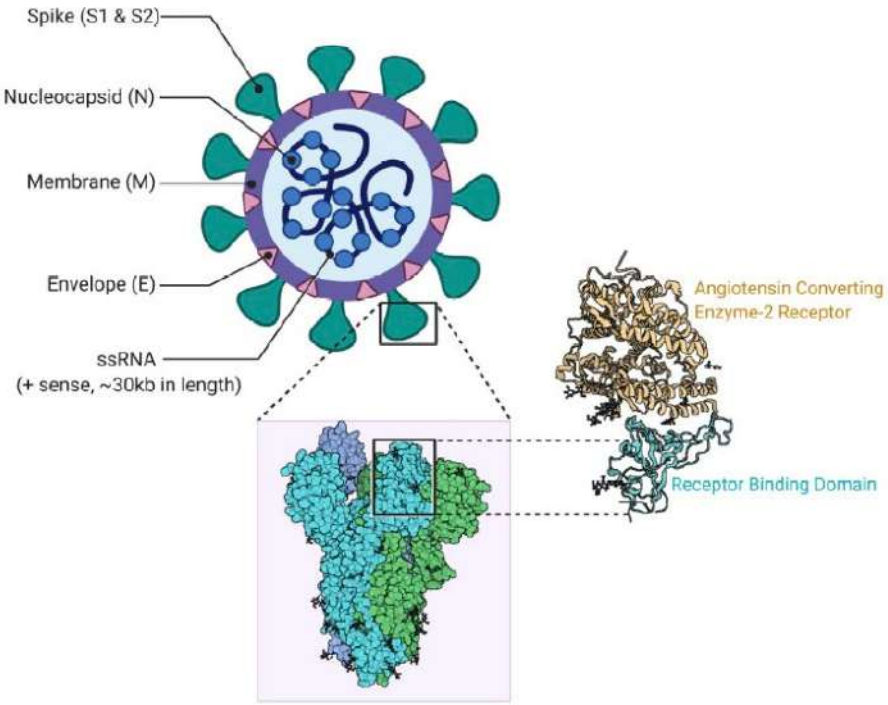
Du L, Yang Y, Zhou Y, Lu L, Li F, Jiang S.
MERS-CoV spike protein: a key target for antivirals.
Expert Opin Ther Targets. 2017;21(2):131-143. doi:10.1080/14728222.2017.1271415
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5457961/

[73] Lu R, Zhao X, Li J, et al.
Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding.
Lancet. 2020;395(10224):565-574. doi:10.1016/S0140-6736(20)30251-8
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7159086/

Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D.
Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein
[published correction appears in Cell. 2020 Dec 10;183(6):1735]. Cell. 2020;181(2):281-292.e6. doi:10.1016/j.cell.2020.02.058
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7102599/

Ge J, Zhang S, Zhang L, Wang X.
Structural basis of severe acute respiratory syndrome coronavirus 2 infection.
Curr Opin HIV AIDS. 2021 Jan;16(1):74-81. doi: 10.1097/COH.000000000658.
https://pubmed.ncbi.nlm.nih.gov/33186231/

Mahmood Z, Alrefai H, Hetta HF, A Kader H, Munawar N, Abdul Rahman S, Elshaer S, Batiha GE, Muhammad K.
Investigating Virological, Immunological, and Pathological Avenues to Identify Potential Targets for Developing COVID-19 Treatment and Prevention Strategies.
Vaccines (Basel). 2020 Aug 6;8(3):443. doi: 10.3390/vaccines8030443.
https://www.mdpi.com/2076-393X/8/3/443/htm



https://www.ncbi.nlm.nih.gov/books/NBK554776/
Genomic organization of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), including open reading frames (ORF1a and ORF1b), spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins. Three-dimensional protein structures of 3CL-protease, endoribonuclease and spike proteins related to human angiotensin-converting enzyme 2 (ACE2) receptor are illustrated. PDB: protein database

SARS-CoV-2 enters cells through the S protein, which binds to the human **angiotensin-converting enzyme 2** (ACE2) receptor and employs the cellular serine protease TMPRSS2 for activation of the S protein [74].

Such binding triggers a cascade of events leading to fusion between cell and viral membranes for virus entry into cells. The viral RNA genome is released into the cytoplasm after membrane fusion.
The polyproteins are subsequently synthesized to encode the viral replicase-transcriptase complex. The viral RNA is then synthesized by RNA-dependent RNA polymerase.
Structural protein synthesis is followed by the assembly and release of viral particles [75].

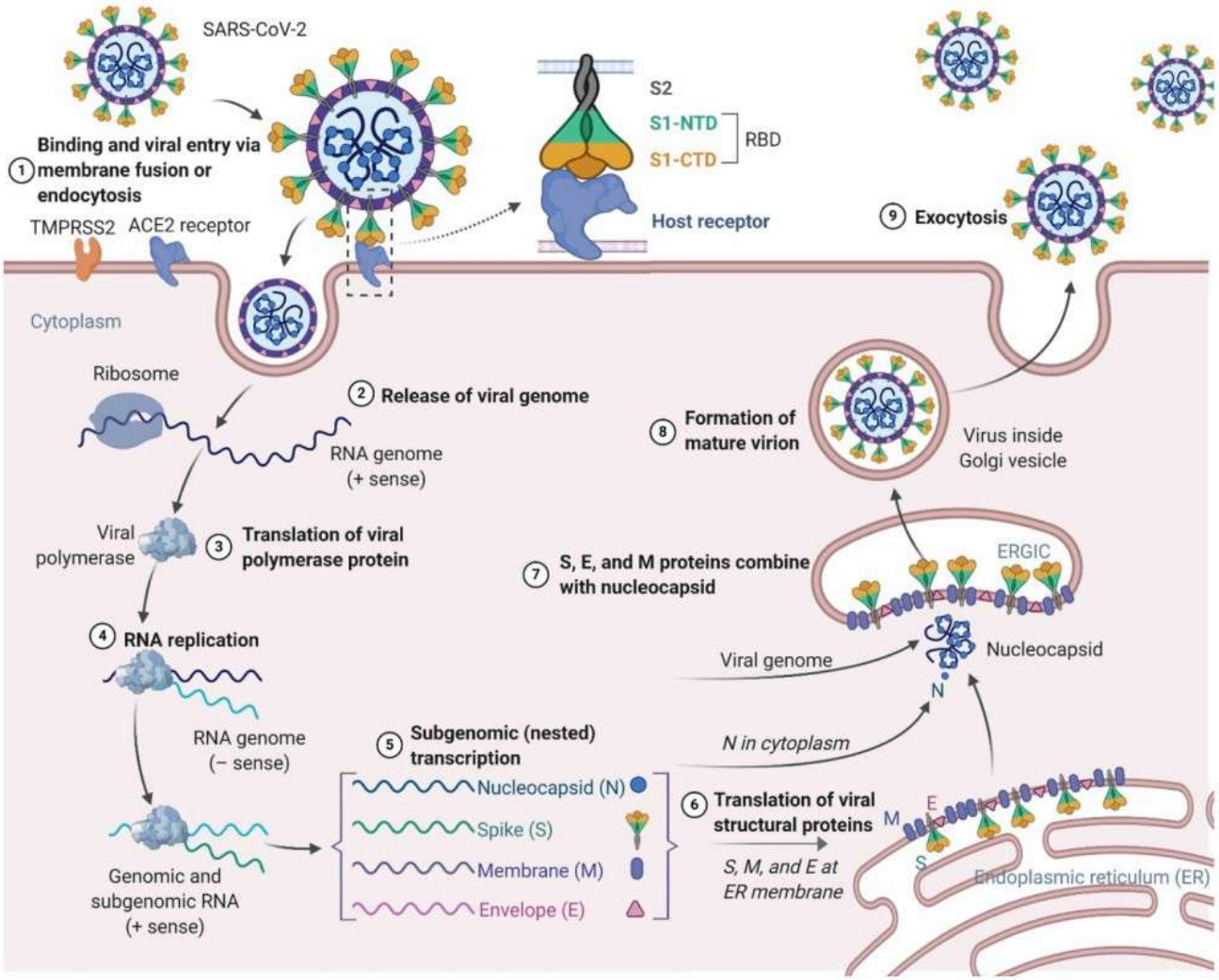
[74] Datta PK, Liu F, Fischer T, Rappaport J, Qin X.
SARS-CoV-2 pandemic and research gaps: Understanding SARS-CoV-2 interaction with the ACE2 receptor and implications for therapy.
Theranostics. 2020;10(16):7448-7464. Published 2020 Jun 12. doi:10.7150/thno.48076
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7330865/

Ou X et al
Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV.
Nat Commun. 2020 Mar 27;11(1):1620. doi: 10.1038/s41467-020-15562-9.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7100515/

Tai W, He L, Zhang X, Pu J, Voronin D, Jiang S, Zhou Y, Du L.
Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine.
Cell Mol Immunol. 2020 Jun;17(6):613-620. doi: 10.1038/s41423-020-0400-4. epub 2020 Mar 19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7091888/

Letko M, Marzi A, Munster V.
Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses.
Nat Microbiol. 2020;5(4):562-569. doi:10.1038/s41564-020-0688-y
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7095430/

[75] Chen Y, Liu Q, Guo D.
Emerging coronaviruses: Genome structure, replication, and pathogenesis
[published correction appears in J Med Virol. 2020 Oct;92(10):2249]. J Med Virol. 2020;92(4):418-423. doi:10.1002/jmv.25681
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7167049/



https://www.thno.org/v10p7821.pdf
Schematic presentation of the SARS-CoV-2 viral life cycle. SARS-CoV-2 enters host cells by first binding to angiotensin-converting enzyme 2 (ACE2) through the surface spike protein (S). After the virus enters the host cell, viral genomic RNA is released and translated into viral polymerase proteins. In this process, subgenomic (-) RNAs are synthesized and used as a mold to form subgenomic (+) messenger RNAs (mRNAs). Nucleocapsid (N) structural protein and viral RNA are replicated, transcribed, and synthesized in the cytoplasm, while other viral structural proteins, including protein S, membrane protein (M), and envelope protein (E), are transcribed and then translated into the endoplasmic reticulum (ER). The resulting structural proteins are further assembled into the nucleocapsid and viral envelope in the ER-Golgi intermediate compartment (ERGIC) to form a mature virion, followed by the release of the nascent virion from the host cell.

These stages of the viral life cycle provide potential targets for vaccines and therapies to prevent and treat SARS-CoV-2 infection. [76]

To elucidate the specific mechanisms of SARS-CoV-2 infection, the crystal structure of the spike of the RBD of SARS-CoV-2 bound to the cellular ACE2 receptor was recently determined with a resolution of 2.45 Å. In vitro binding experiments show that the RBD of SARS-CoV-2 has affinity for ACE2 in the nM order, indicating that the RBD is the key functional component of the S1 subunit responsible for the binding of ACE2 to SARS-CoV-2. [77]

[76] Uddin M, Mustafa F, Rizvi TA, et al.
SARS-CoV-2/COVID-19: Viral Genomics, Epidemiology, Vaccines, and Therapeutic Interventions.
Viruses. 2020;12(5):526. Published 2020 May 10. doi:10.3390/v12050526
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7290442/.

[77] Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, Zhang Q, Shi X, Wang Q, Zhang L, Wang X.
Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor.
Nature. 2020 May;581(7807):215-220. doi: 10.1038/s41586-020-2180-5. Epub 2020 Mar 30.
https://www.nature.com/articles/s41586-020-2180-5

TYPES OF VACCINE ANTIGENS

There are four main categories of vaccines used in clinical trials: whole virus (attenuated or inactivated, with or without adjuvant), protein subunit, viral vector, and nucleic acid (RNA and DNA). [78]

[78] https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines
Updated 12/29/2020

Fighting SARS-CoV-2: prevention and vaccines
July2020 Science Output
Antonia Mazzeo - Senior Consultant and Cecilia Parra - Project Manager
https://www.pqegroup.com/blog/2020/08/fight-against-sars-cov-2-prevention-and-vaccines/

Ura T, Yamashita A, Mizuki N, Okuda K, Shimada M.
New vaccine production platforms used in developing SARS-CoV-2 vaccine candidates.
Vaccine. 2021;39(2):197-201. doi:10.1016/j.vaccine.2020.11.054
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7685034/

National Academy of the Lynxes
COVID-19 Vaccines: Fall 2020 Report Covid-19 Commission Document
https://www.lincei.it/sites/default/files/documenti/Commissioni/ANL_Comm_Covid-19_Vaccini_30novembre2020.pdf

Funk CD, Laferrière C, Ardakani A.
A Snapshot of the Global Race for Vaccines Targeting SARS-CoV-2 and the COVID-19 Pandemic.
Front Pharmacol. 2020 Jun 19;11:937. doi: 10.3389/fphar.2020.00937.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7317023/
https://www.frontiersin.org/articles/10.3389/fphar.2020.00937/full

Vashishtha VM, Kumar P.
Development of SARS-CoV-2 vaccines: challenges, risks, and the way forward
[published online ahead of print, 2020 Dec 3]. Hum Vaccin Immunother. 2020;1-15. doi:10.1080/21645515.2020.1845524
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7754925/

Mathew S, Faheem M, Hassain NA, Benslimane FM, Thani AAA, Zaraket H, Yassine HM.
Platforms Exploited for SARS-CoV-2 Vaccine Development.
Vaccines (Basel). 2020 Dec 25;9(1):E11. doi: 10.3390/vaccines9010011. PMID: 33375677.
https://www.mdpi.com/2076-393X/9/1/11/htm.

Brisse M, Vrba SM, Kirk N, Liang Y, Ly H.
Emerging Concepts and Technologies in Vaccine Development.
Front Immunol. 2020;11:583077. Published 2020 Sep 30. doi:10.3389/fimmu.2020.583077
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7554600/

Dong, Y., Dai, T., Wei, Y. et al.
A systematic review of SARS-CoV-2 vaccine candidates.
Sig Transduct Target Ther 5, 237 (2020). https://doi.org/10.1038/s41392-020-00352-y
https://www.nature.com/articles/s41392-020-00352-y

Huang L, Rong Y, Pan Q, et al.
SARS-CoV-2 vaccine research and development: conventional vaccines and biomimetic nanotechnology strategies
[published online ahead of print, 2020 Sep 1]. Asian J Pharm Sci. 2020;10.1016/j.ajps.2020.08.001. doi:10.1016/j.ajps.2020.08.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7462629/

Yizhi Peng, Sheng Yin, Min Wang.
Extracellular vesicles of bacteria as potential targets for immune interventions.
Human Vaccines & Immunotherapeutics (2020) 0:0, pages 1-7.
https://www.tandfonline.com/doi/full/10.1080/07853890.2017.1407035?src=recsys

Xiaoxia Dai, Yongmin Xiong, Na Li and Can Jian (March 5th 2019).
Vaccine Types, Vaccines - the History and Future, Vijay Kumar,
IntechOpen, DOI: 10.5772/intechopen.84626. A
https://www.intechopen.com/books/vaccines-the-history-and-future/vaccine-types

Krammer, F.
SARS-CoV-2 vaccines in development.
Nature 586, 516-527 (2020). https://doi.org/10.1038/s41586-020-2798-3
https://www.nature.com/articles/s41586-020-2798-3

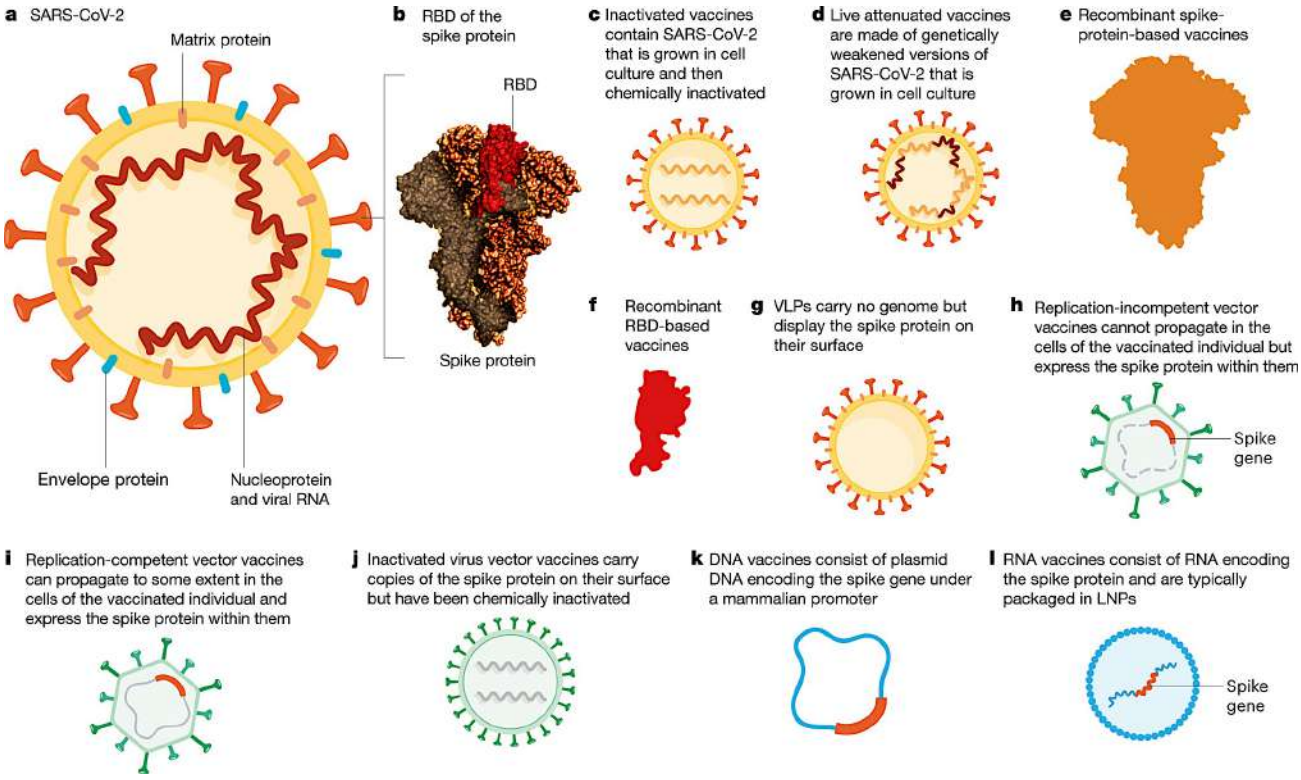
Liu Y, Wang K, Massoud TF, Paulmurugan R.
SARS-CoV-2 Vaccine Development: An Overview and Perspectives.
ACS Pharmacol Transl Sci. 2020 Sep 18;3(5):844-858. doi: 10.1021/acsptsci.0c00109.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7526333/

Dagotto G, Yu J, Barouch DH.

In general terms, vaccines require two components in order to act: antigens of the target pathogen that are provided or generated by the vaccine recipient and an infection signal (such as a pathogen-associated molecular pattern--PAMPS--or damage-associated molecular pattern--DAMPS) that alerts and activates the host immune system.

Live attenuated vaccines can naturally provide both of these components, while nonviral vaccine platforms can provide the antigens but often require adjuvant substances to create an artificial source of signals needed to alert the immune system.
Typically, these nonviral vaccine platforms require multiple vaccinations to induce protective immunity, whereas attenuated virus vaccines should be able to provide "one-shot" immunity.

Similar to nonviral platforms, vaccines with inactivated virus sometimes require the inclusion of an adjuvant and repeated administration to increase efficacy.



https://www.nature.com/articles/s41586-020-2798-3
a, A schematic of the structural proteins of the SARS-CoV-2 virion, including the lipid membrane, the genomic RNA covered by the nucleoprotein inside, the envelope and matrix proteins inside the membrane, and the spike protein on the surface of the virus. **b,** The structure of the spike protein; a monomer is highlighted in dark brown and the RBD is shown in red**. c - l**, Current SARS-CoV-2 vaccine candidates include inactivated virus vaccines **(c)**, live attenuated vaccines **(d)**, recombinant protein vaccines based on the spike protein **(e)**, RBD **(f)** or virus-like particles **(g)**, non-replicating vector vaccines **(h)**, replicating vector vaccines **(i)**, inactivated viral vector vaccines that have the spike protein on their surface **(j)**, DNA vaccines **(k)** and RNA vaccines **(l)**.

In addition to the careful selection of antigens and vaccine platform, the route of administration is a key consideration of vaccine strategies.

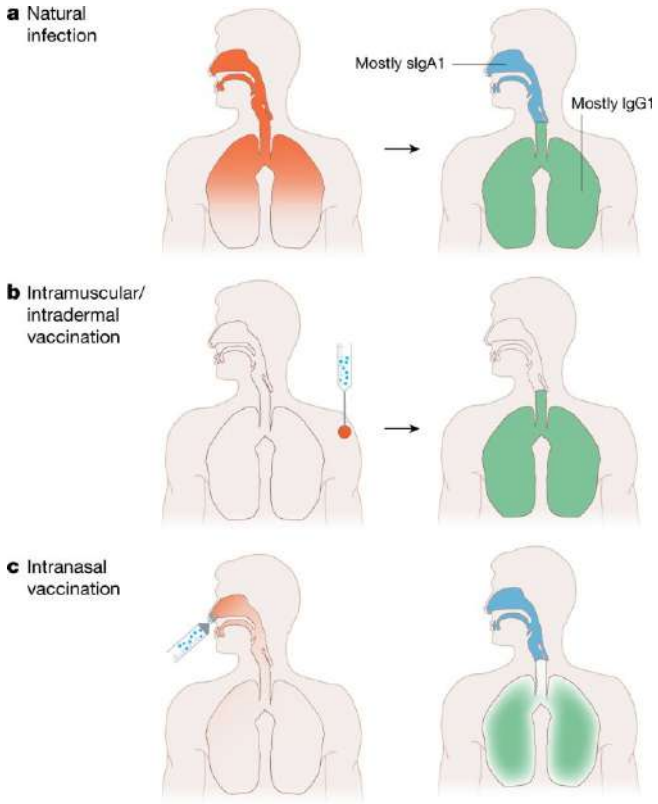
Approaches and Challenges in SARS-CoV-2 Vaccine Development.
Cell Host Microbe. 2020;28(3):364-370. doi:10.1016/j.chom.2020.08.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7416703/

Haque A, Pant AB.
Efforts at COVID-19 Vaccine Development: Challenges and Successes.
Vaccines (Basel). 2020;8(4):739. Published 2020 Dec 6. doi:10.3390/vaccines8040739
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7762169/

Karch CP, Burkhard P.
Vaccine technologies: From whole organisms to rationally designed protein assemblies.
Biochem Pharmacol. 2016 Nov 15;120:1-14. doi: 10.1016/j.bcp.2016.05.001. Epub 2016 May 6. PMID: 27157411;
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5079805/

This is especially important for mucosal pathogens such as SARS-CoV-2 and those pathogens against which optimal protection requires not only neutralizing antibodies but also innate and adaptive cellular immunity. [79] The best window of opportunity for control and elimination of SARS-CoV-2 is the asymptomatic or pre-symptomatic period of COVID-19 (2-12 days), which is likely to require that all elements of immune protection be present in the respiratory mucosa prior to viral entry [80].

The route of administration of vaccination plays a crucial role in determining this. The vaccine IgG antibodies induced by parenteral vaccination readily appear on the respiratory mucosa, however, this route of vaccination cannot effectively induce mucosal IgA antibodies or Tissue- resident memory cells (TRM) in the lungs. [81]



https://www.nature.com/articles/s41586-020-2798-3
The lower human respiratory tract is believed to be mostly protected by IgG (IgG1 is the most prevalent), the main type of antibody in serum, which is transported into the lung. The upper respiratory tract is thought to be mostly protected by secretory IgA1 (sIgA1). a**,** Natural respiratory virus infection induces both a systemic immune response, dominated by IgG1, and a mucosal immune response in the upper respiratory tract dominated by sIgA1. This process can lead to sterilizing immunity to many respiratory viruses. **b,** Intramuscular or intradermal vaccination leads in many cases to a strong induction of serum IgG but no induction of mucosal IgA. Although some IgG can also be found on mucosal surfaces of the upper respiratory tract, the lack of sIgA often leaves an individual vulnerable to upper respiratory tract infections. **c,** intranasal vaccination can effectively induce mucosal antibody responses, thus potentially providing sterilizing immunity in the upper respiratory tract. However, systemic immune responses are often lower after this type of vaccination. Currently, all candidate SARS-CoV-2 vaccines in clinical development are administered intramuscularly, and very few of the more than 180 vaccines in development are designed to induce mucosal immunity. Although mucosal immunity may not be required to protect against severe or even symptomatic disease, it may be necessary to achieve optimal protection from infection and subsequent transmission of SARS-CoV-2.

[79] Sariol A, Perlman S.
Lessons for COVID-19 Immunity from Other Coronavirus Infections.
Immunity. 2020;53(2):248-263. doi:10.1016/j.immuni.2020.07.005
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7359787/

[80] Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP.
The trinity of COVID-19: immunity, inflammation and intervention.
Nat Rev Immunol. 2020;20(6):363-374. doi:10.1038/s41577-020-0311-8
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7187672/

[81] Belyakov IM, Ahlers JD.
What role does the route of immunization play in the generation of protective immunity against mucosal pathogens?
J Immunol. 2009 Dec 1;183(11):6883-92. doi: 10.4049/jimmunol.0901466. PMID: 19923474.
https://www.jimmunol.org/content/183/11/6883.long

The following tables show the immunological properties of the main COVID-19 candidate vaccine platforms and non-antigenic vaccine components.

Vaccine platform	SARS-CoV-2 antigens	Neutralizing antibody response	T cell response			Pre-existing antivector immunity	Route of vaccination	Overall immunogenicity	Other attributes
			$CD4^+$ T_H cells	$CD8^+$ T cells	Lung T_{RM} cells				
Viral-vectored vaccines									
Ad5 (non-replicating)	S protein	Quality and durability affected by pre-existing antivector immunity	T_H1 cell	Potent response; negative effects from pre-existing antivector immunity	Induced by RM but not IM route	High, age-dependent, prevalence in blood; low prevalence in respiratory tract	Parenteral (IM) in clinical trials	Strong with single delivery but hindered by pre-existing antivector immunity	Ample human safety data; RM delivery helps bypass antivector immunity; can be delivered by inhaled aerosol
Ad26 (non-replicating)	S protein	Quality and durability affected by pre-existing antivector immunity	T_H1 cell	Moderate response; negative effects from pre-existing antivector immunity	Induced by RM but not IM route	Medium prevalence	Parenteral (IM) in planned clinical trials	Weak; requires repeated or heterologous boost vaccination	Established human safety from HIV and Ebola vaccine trials; RM delivery helps bypass antivector immunity
ChAd (non-replicating)	S protein	Unimpeded owing to lack of pre-existing antivector immunity	T_H1 cell	Potent response	Induced by RM but not IM route	Very low prevalence	Parenteral (IM) in clinical trials	Strong with single delivery	Well-established human safety data; amenable to RM delivery; can be used as a stand-alone vaccine or in prime–boost regimens
VSV (replicating)	S protein	Unimpeded owing to lack of pre-existing antivector immunity	T_H1 cell	Response not as strong as for Ad5 or ChAd when used as a stand-alone vaccine; strong T cell booster	Not induced by IM route	None	Parenteral (IM) in previous successful Ebola vaccine trials	Good with single delivery	Successfully licensed platform for Ebola; not known whether it protects against RM viral pathogens
Measles and influenza viruses (replicating)	S protein?	Quality and durability depend on whether there is pre-existing antivector immunity and vaccination route	T_H1 cell	Good response when delivered via RM route	Not induced by parenteral route	High prevalence owing to vaccination and natural infection	Parenteral or RM	Weak relative to adenovirus vectors	Not extensively tested in humans; potential recombination of live attenuated influenza vectors in the lung delivered via RM route

Vaccine platform	SARS-CoV-2 antigens	Neutralizing antibody response	T cell response			Pre-existing antivector immunity	Route of vaccination	Overall immunogenicity	Other attributes
			$CD4^+$ T_H cells	$CD8^+$ T cells	Lung T_{RM} cells				
Other vaccines									
mRNA-based vaccine	S protein or RBD encapsulated in lipid nanoparticle	Unimpeded owing to lack of pre-existing antivector immunity	T_H1 cell or T_H2 cell depending on adjuvant	Depends on choice of adjuvant and formulation	Not induced by parenteral route	None	Parenteral (IM) in clinical trials	Requires repeated delivery	Adjuvant required; unclear whether it is amenable to RM vaccination
DNA-based vaccine	S protein	Unimpeded owing to lack of pre-existing antivector immunity	T_H1 cell	Response not as strong as for some of the viral vectors	Not induced	None	Parenteral (IM) in clinical trials	Weaker than mRNA-based vaccine; requires repeated delivery	Adjuvant required; not amenable to RM vaccination
Live attenuated virus	Multiple viral antigens	Strong induction	T_H1 cell	Strong response	Induced by RM but not IM route	No cross-reactive antibodies; cross-reactive T cells from seasonal coronavirus infections	Parenteral (SC)	Requires only a single delivery	Extensive safety testing required for potential recombination with wild-type virus
Inactivated virus	Multiple viral antigens	Strong induction	T_H1 cell or T_H2 cell depending on adjuvant	Weak response	Not induced	None	Parenteral (IM)	Weak; requires repeated vaccination	Adjuvant required; alum often used, which enhances T_H2 cell responses possibly involved in ADE
Protein subunit vaccine	S protein or RBD	Strong induction	T_H1 cell or T_H2 cell depending on adjuvant	Weak response	Not induced	None	Parenteral (IM) in clinical trials	Weak; requires repeated vaccination	Adjuvant required; mostly unsuitable for RM vaccination
Virus-like particle	Multiple viral antigens	Strong induction	T_H1 cell or T_H2 cell depending on adjuvant	Weak response	Not induced	None	Parenteral (IM) or RM	Weak, but greater than for protein subunits; requires repeated vaccination	Well-established platform for several commercial human vaccines (hepatitis B and HPV vaccines); adjuvant required

https://www.nature.com/articles/s41577-020-00434-6

Immunological properties of the main COVID-19 candidate vaccine platforms.

Ad5, human serotype 5 adenovirus; Ad26, human serotype 26 adenovirus; ADE, antibody-dependent enhancement; ChAd, chimpanzee adenovirus; COVID-19, coronavirus disease 2019; HPV, human papillomavirus; IM, intramuscular; RBD, receptor-binding domain; RM, respiratory mucosal; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S protein, spike protein; SC, subcutaneous; TH cell, T helper cell; TRM cell, resident memory T cell; VSV, vesicular stomatitis virus.

Component	Clinical Vaccine Candidates Containing Adjuvant/Component (Antigen Type)	Description	Effect/Skew	Mechanism
Advax-SM	Vaxine Pty/Medytox (Recombinant Protein)	Delta-inulin (water-insoluble polysaccharide) microparticles mixed with CpG 1018	Adjuvant, Th1 skew (No skew without CpG) [67]	Unknown, antigen-presenting cell-dependent [68]
Alum	Sinovac (Inactivated Virus), Sinopharm (Inactivated Virus), Bharat Biotech (Inactivated Virus), Clover (With CpG 1018, Recombinant Protein), FBRI SRC VB VECTOR (Peptide Subunit), West China Hospital/-Sichuan University (Recombinant Protein)	Aluminum salts (aluminum hydroxide or aluminum phosphate)	Adjuvant, Th2 Skew	Multifaceted [69] [70]
AS03	Clover (Recombinant Protein), Medicago (VLP), Sanofi/GSK (Recombinant Protein)	Squalene and DL-α-tocopherol oil-in-water emulsion stabilized by polysorbate 80	Adjuvant, Th2 skew	Unknown, potentially innate immune recruitment and activation [71]
CpG 1018	Vaxine Pty/Medytox (Included in Advax-SM, Recombinant Protein), Medicago (VLP), Clover (With Alum, Recombinant Protein), Medigen/NIAID/Dynavax (Recombinant Protein)	Unmethylated oligodeoxynucleotide (ODN)	Adjuvant, Th1 skew	TLR9 stimulation [72]
Ionizable Lipid (various proprietary versions)	Moderna/NIAID (mRNA), Pfizer (mRNA and replicon RNA), Arcturus (replicon RNA), PLA Academy of Military Sciences/Walvax Biotech	Lipid molecules containing amino groups which become cationic at acidic pH.	Complexes anionic macromolecules (e.g. RNA) and promotes cytosolic delivery. Adjuvant, Th2 skew in absence of other adjuvants [73]	Unknown, potentially TLR2/TLR4 stimulation [74]
Matrix M	Novavax (Recombinant Protein)	Lipid nanoparticles containing cholesterol and immunostimulatory Quillaja triterpenoid saponins Matrix-A and Matrix-C in an 85:15 ratio [75]	Adjuvant, Balanced Th1/Th2 skew	Unknown, potentially innate immune recruitment and activation [76] [77]
MF59	Anhui Longcom (Recombinant Protein), Queensland/Seqirus/CSL (Recombinant Protein)	Squalene oil-in-water emulsion stabilized by polysorbate 80 and sorbitan trioleate	Adjuvant, Th2 skew	Unknown, potentially innate immune recruitment and activation [78] [79]
Polysorbate 80	Novavax (Recombinant protein)	Nonionic surfactant, a.k.a. Tween 80	Inhibits aggregation of emulsions and hydrophobic proteins	Stabilizes interfaces in emulsions, prevents protein adsorption to potentially denaturing interfaces, multimerizes transmembrane proteins [80,81].
RNA	Moderna/NIAID (mRNA), Pfizer/BioNTech (mRNA and replicon RNA), Curevac (mRNA), Arcturus (replicon RNA)	genetic material which encodes antigenic constructs and stimulates immune responses	Th1 skew	TLRs 3, 7, 8, 9, and 13 stimulation [82]

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7733686/pdf/main.pdf
Summary of non-antigenic components for SARS-CoV-2 vaccines.

Different vaccine platforms: advantages and disadvantages

Vaccine Platform	Advantages	Disadvantages
Viral vector-based	• Exhibit highly specific gene delivery into the host cell with rigorous immune response. • No infectious virus needs to be handled, shows significant preclinical and clinical data for many emerging viruses, including MERS-CoV.	• Host's immunity against the vector might negatively affect the effectiveness of the vaccine (depends on the vector chosen). • The integration of the viral genome into the host genome may cause cancer.
Live attenuated	• Develops long-lasting immunity • High potency and pre-existing infrastructure used for several licensed human vaccines. • Low-cost manufacturing.	• Possible regression to virulence strain. • Limited use in immunocompromised patients. • Making infectious clones for attenuated coronavirus vaccine seed may be time consuming because of its large genome size. • Extensive safety testing required.
Inactivated	• Stable and safe compared to live attenuated virus platform. • Pre-existing technology and infrastructure required for development are available. • Can be used in immunocompromised patients. • Has already been tested in humans for various diseases such as SARS-CoV-1 and adjuvants can be used to increase immunogenicity.	• Requires booster doses to maintain immunity. • Large amount of virus needs to be handled and antigen integrity needs to be confirmed. • Low production titer.
RNA	• Handling of infectious viral particle is not required. • Low-cost and ease of manufacturing. • Translation of mRNA occurs in the cytosol of the host cell thus reducing the risk of integration into the host genome.	• May have low immunogenicity due to instability. • Safety issue with reactogenicity have been reported for various RNA based vaccines. • Multiple doses may be required.
DNA	• Handling of infectious viral particle is not required. • Ease of manufacturing. • The synthetic DNA is temperature stable and cold-chain free	• The titer remains low, even though it elicits both cytotoxic and humoral immunity. • Potential integration to human genome causes abnormalities.
Protein subunit	• Can be used in immunocompromised patients. • Does not involve any live component of the viral particle	• Low immunogenicity. • Conjugation leads to batch-wise variation.

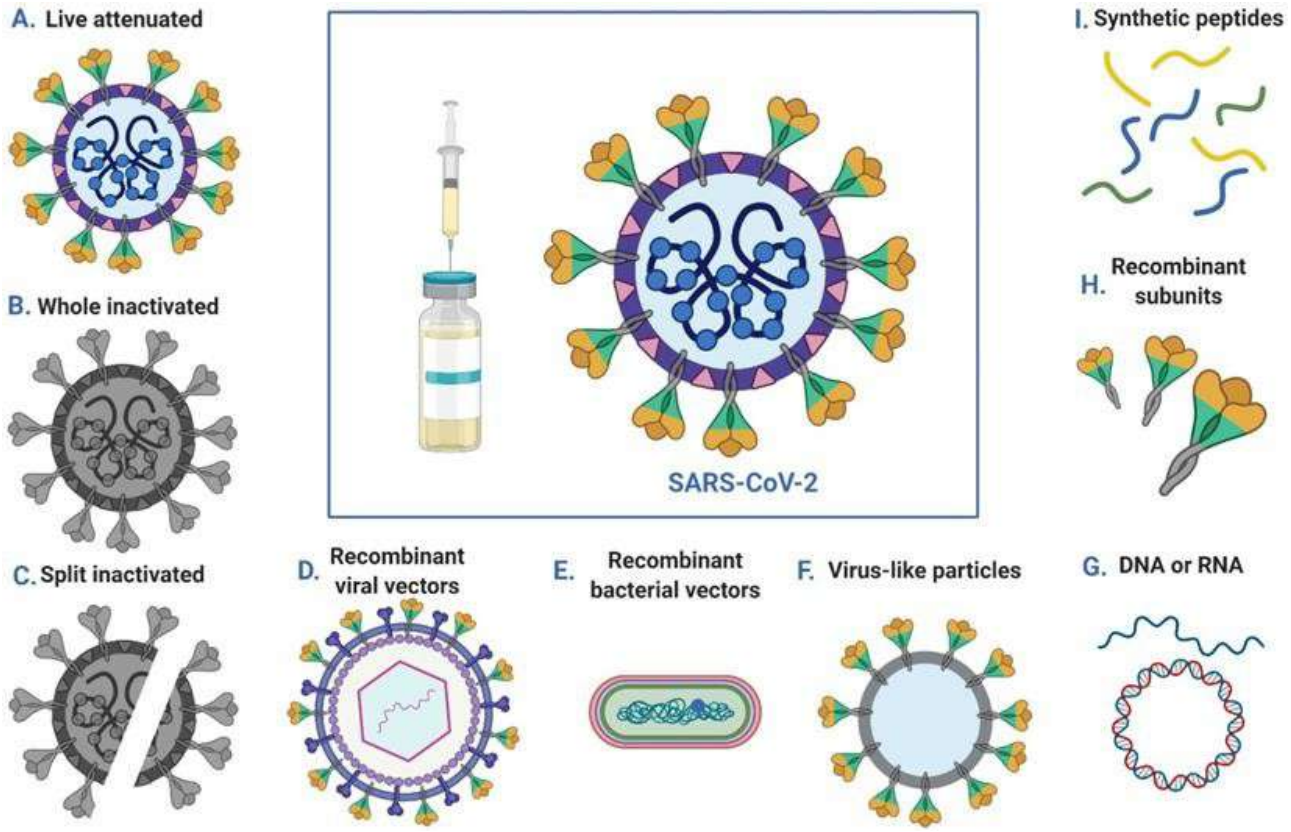
https://www.mdpi.com/2076-393X/9/1/11/htm
MERS-CoV, Middle East respiratory syndrome coronavirus, SARS-CoV, severe acute respiratory syndrome-corona virus.

VIDEO

There are four types of COVID-19 vaccines: here's how they

work Covid-19 vaccines: what they are and how they work

The following are the types of vaccines in clinical trials cited on the WHO website [82]



https://www.thno.org/v10p7821.pdf

[82] https://vac-lshtm.shinyapps.io/ncov_vaccine_landscape/
https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines
https://www.vfa.de/de/englische-inhalte/vaccines-to-protect-against-covid-19

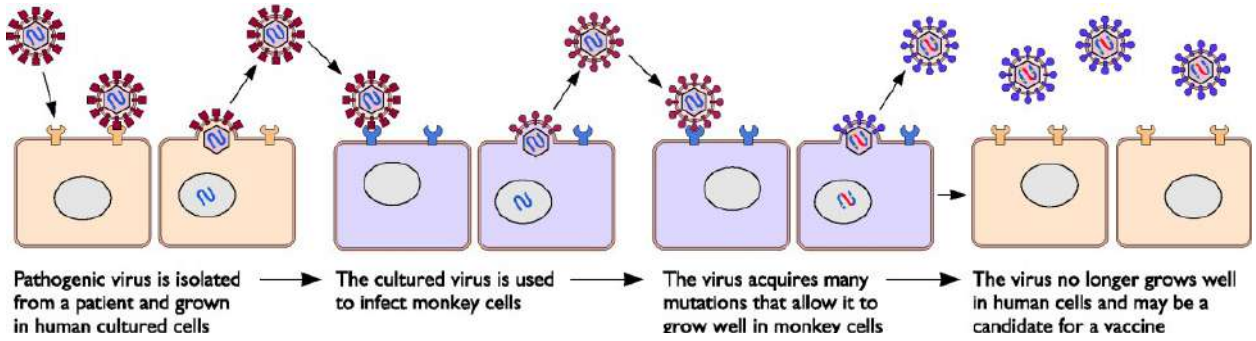
Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z.
Immunological considerations for COVID-19 vaccine strategies.
Nat Rev Immunol. 2020;20(10):615-632. doi:10.1038/s41577-020-00434-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7472682/

ATTENUATED VIRUS VACCINES

COVI-VAC: Serum Institute of India, (India)/Codagenix (New York)

Classical development of live attenuated vaccines requires that the organism be modified through multiple passages in culture under unique conditions in the laboratory until it loses pathogenicity so that it does not cause disease, but retains its ability to replicate for a short period in the vaccine recipient and stimulate a robust immune response both adaptive and innate.

These vaccines tend to be highly immunogenic and do not require an adjuvant. However, they are contraindicated in immunocompromised or pregnant people.



https://www.dbcf.unisi.it/sites/st13/files/allegatiparagrafo/28-04-2016/08_vaccini.pdf
Adaptation to a new cell substrate selects mutations that result in less ability to multiply and/or less ability to reach target organs in the original host

It is difficult to rapidly develop a live attenuated SARS-CoV-2 vaccine because of the time and knowledge required to ensure that it is properly attenuated and all virulence factors are removed. Long-term maintenance of substantial stocks of attenuated vaccines is also problematic. Because this approach relies on the use of a single viral strain, the vaccine may not cross-protect against other strains, particularly as the virus continues to spread worldwide and mutates with increasing selection pressure once immunity becomes more widespread. [83]

[83] Stern PL.
Key steps in vaccine development.
Ann Allergy Asthma Immunol. 2020 Jul;125(1):17-27. doi: 10.1016/j.anai.2020.01.025. Epub 2020 Feb 7.
https://www.annallergy.org/article/S1081-1206(20)30071-5/fulltext

Risk of evolutionary escape from neutralizing antibodies targeting SARS-CoV-2 spike protein
Debra Van Egeren, Alexander Novokhodko, et al medRxiv 2020.11.17.20233726; doi: https://doi.org/10.1101/2020.11.17.20233726
https://www.medrxiv.org/content/10.1101/2020.11.17.20233726v1.full

Kennedy DA, Read AF.
Monitor for COVID-19 vaccine resistance evolution during clinical trials.
PLoS Biol. 2020;18(11):e3001000. Published 2020 Nov 9. doi:10.1371/journal.pbio.3001000
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7676675/

Nyayanit DA, Yadav PD, Kharde R, Shete-Aich A.
Quasispecies analysis of the SARS-CoV-2 from representative clinical samples: A preliminary analysis.
Indian J Med Res. 2020 Jul & Aug;152(1 & 2):105-107. doi: 10.4103/ijmr.IJMR_2251_20. PMID: 32773417.
https://www.ijmr.org.in/temp/IndianJMedRes1521105-8570386_234823.pdf

Reverse genetics has been widely used both to understand the biology of SARS-Cov-2 [84] and for the rational design of the ideal attenuated vaccine virus, [85] for example by determining key virulence factors, as has been done for other coronaviruses [86].

IN-DEPTH STUDY: REVERSE GENETICS

Reverse genetics is a powerful technique for generating an infectious virus from the full-length cloned/synthesized DNA cDNA of a given virus. DNA manipulation by established molecular biology methods allows sequence modification prior to virus production, if desired. It allows unequivocal identification of molecular markers for a given virus, including genome features, virulence, host type, etc., of a given virus, and as such is a key component in the study of coronaviruses.

Classical genetics begins with the study of a mutant phenotype, proceeds to prove the existence of the relevant gene by analysis of progeny, and finally clones and sequences the gene to determine its DNA and protein sequence. In contrast, reverse genetics, a new approach made possible by recombinant DNA technology, works in the opposite direction. Reverse genetics starts with a protein or DNA for which there is no genetic information and then works backwards to create a mutant gene, ending up with a mutant phenotype.

GENETICS	REVERSE GENETICS
mutant phenotype	protein sequence
↓	↓
mutant allele	DNA sequence
↓	↓
DNA sequence	mutant allele
↓	↓
protein sequence	mutant phenotype

"Reverse genetics" describes the "gene to phenotype" approach, by which the functions of a gene of interest can be investigated by disrupting the physiological expression of this gene.
"Forward genetics" is a "phenotype to gene" approach seeks to find the genetic basis of a phenotype or trait without the need for prior knowledge.

[84] Thi Nhu Thao T, et al.
Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform.
Nature. 2020 Jun;582(7813):561-565. doi: 10.1038/s41586-020-2294-9. Epub 2020 May 4.
https://www.nature.com/articles/s41586-020-2294-9

Hou YJ, Okuda K, Edwards CE, et al.
SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract.
Cell. 2020;182(2):429-446.e14. doi:10.1016/j.cell.2020.05.042
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7250779/

[85] Todorov G, Uversky VN.
A Possible Path toward Rapid Development of Live-Attenuated SARS-CoV-2 Vaccines: Plunging into the Natural Pool.
Biomolecules. 2020;10(10):1438. Published 2020 Oct 14. doi:10.3390/biom10101438
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7602031/

Ma Z, Li Z, Dong L, Yang T, Xiao S.
Reverse genetic systems: Rational design of coronavirus live attenuated vaccines with immune sequelae.
Adv Virus Res. 2020;107:383-416. doi:10.1016/bs.aivir.2020.06.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7326460/

[86] Cockrell AS, Beall A, Yount B, Baric R.
Efficient Reverse Genetic Systems for Rapid Genetic Manipulation of Emergent and Preemergent Infectious Coronaviruses.
Methods Mol Biol. 2017;1602:59-81. doi:10.1007/978-1-4939-6964-7_5
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7120940/

Silva SJRD, Germano Mendes RP, Alves da Silva CT, Lorusso A, Kohl A, Pena L.
Insights into SARS-CoV-2, the Coronavirus Underlying COVID-19: Recent Genomic Data and the Development of Reverse Genetics Systems.
J Gen Virol. 2020;101(10):1021-1024. doi:10.1099/jgv.0.001458
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7660456/

Thi Nhu Thao, T., Labroussaa, F., Ebert, N. et al.
Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform.
Nature 582, 561-565 (2020). https://doi.org/10.1038/s41586-020-2294-9
https://www.nature.com/articles/s41586-020-2294-9

https://www.ncbi.nlm.nih.gov/books/NBK21843/

With the advent of modern DNA sequencing and PCR technologies, researchers were faced with a large number of gene sequences whose function was still unknown. Therefore, the need arose to alter the sequence and consequently the function of such genes in order to study their consequences at the phenotypic level.

Important tools for reverse genetics are in vitro mutagenesis and gene disruption, also known as gene knockout. One approach is to insert a selectable marker into the middle of a cloned gene and then use this construct to transform a wild-type recipient.

REVERSE GENETICS SYSTEMS (RGS) AND THE STUDY OF ANIMAL CORONAVIRUSES POTENTIALLY DANGEROUS T O HUMANS. [87]

RGSs have been widely used in the analysis of the occurrence and pathogenic potential of zoonotic CoVs. For example, civet-human chimeras of SARS-CoV have been used to demonstrate that the S gene of civet strains cannot efficiently mediate viral replication in human ACE2-expressing cells, suggesting that mutations in the S gene have been central to the onset in humans of SARS-CoV [88].
A significant obstacle to the study of zoonotic CoVs, including HKU3-CoV and HKU5-CoV, has been the difficulty in finding a viable culture system because of receptor incompatibility or possible problems with the overall viral genome.

Replacement of minimal portions of the S gene with *de novo* synthesized functioning HKU3 and HKU5 genomes was used to overcome receptor binding problems, **(Fig. A)**.
Once assembled, these chimeric viruses could efficiently infect and replicate in vitro and in vivo, demonstrating that receptor binding was a primary barrier for HKU3 and HKU5 infection of human cells [89].

A complementary strategy was used to test the ability of CoV S genes to mediate infection **(Fig. B)**. The S genes of the zoonotic CoVs SHC014-CoV and WIV1-CoV were inserted into a replication competent sequence in the mouse adapted for SARS-CoV MA15.
SHC014-MA15 and WIV1-MA15 chimeras have been shown to be able to replicate in vitro and in vivo, suggesting that these viruses could emerge in humans 90 . These initial studies have justified

[87] Johnson BA, Graham RL, Menachery VD.
Viral metagenomics, protein structure, and reverse genetics: Key strategies for investigating coronaviruses.
Virology. 2018 Apr;517:30-37. doi: 10.1016/j.virol.2017.12.009. Epub 2017 Dec 24.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5869085/

[88] Sheahan T, Rockx B, Donaldson E, Corti D, Baric R.
Pathways of cross-species transmission of synthetically reconstructed zoonotic severe acute respiratory syndrome coronavirus.
J Virol. 2008;82(17):8721-8732. doi:10.1128/JVI.00818-08
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2519660/

[89] Agnihothram S, Yount BL Jr, Donaldson EF, et al.
A mouse model for Betacoronavirus subgroup 2c using a bat coronavirus strain HKU5 variant.
mBio. 2014;5(2):e00047-e14. Published 2014 Mar 25. doi:10.1128/mBio.00047-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3977350/

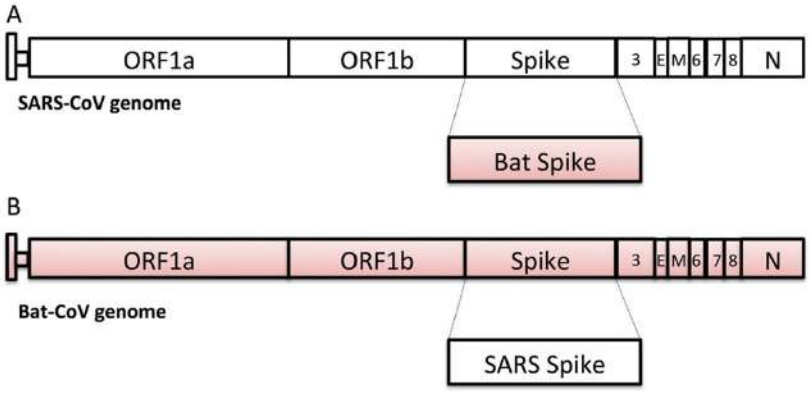
Becker MM, Graham RL, Donaldson EF, Rockx B, Sims AC, Sheahan T, Pickles RJ, Corti D, Johnston RE, Baric RS, Denison MR.
Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice.
Proc Natl Acad Sci U S A. 2008 Dec 16;105(50):19944-9. doi: 10.1073/pnas.0808116105. Epub 2008 Nov 26.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2588415/

[90] Menachery VD, et al.
A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence.
Nat Med. 2015 Dec;21(12):1508-13. doi: 10.1038/nm.3985. Epub 2015 Nov 9. Erratum in: Nat Med. 2016 Apr;22(4):446. Erratum in: Nat Med. 2020 Jul;26(7):1146.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4797993/

Menachery VD, Yount BL Jr, Sims AC, et al.
SARS-like WIV1-CoV poised for human emergence.

further examination and characterization of SHC014-CoV and full-length WIV1-CoV and indicated that specific mutations in the viral sequence are required f o r onset and pathogenesis.

Together, these two strategies exploit reverse genetics to create chimeric coronaviruses, circumventing the limitations of species-specific culture systems to analyze the potential for the occurrence of zoonotic CoVs in humans.



https://www.sciencedirect.com/science/article/pii/S0042682217304142
Dual approaches to exploit reverse genetics. Using coronavirus molecular clones, two strategies were employed to explore the emergence and pathogenic potential of sequences derived from zoonotic populations**. A)** Replacing wild-type spike proteins--This strategy explores the ability of spike proteins in the context of a viral sequence known to be capable of replication. These studies provide information on the potential of spike proteins to mediate infection of human cells and cause disease in vivo and help examine the broad efficacy of therapies directed against CoV spike proteins. **B**) Using replication-competent portions or whole spike proteins of CoV-this approach examines the ability of the viral sequence to mediate infection and pathogenesis. These studies provide information on the ability of the selected sequence to infect and cause disease when associated with receptor binding/entry. This approach can also evaluate the efficacy of therapies targeting portions of the CoV genome other than the spike. Both approaches have been used to examine bat viruses currently circulating in animal populations worldwide.

REVERSE GENETICS SYSTEMS USED FOR THE STUDY OF SARS-COV-2

In January 2020, the complete viral genome sequences of five patients in Wuhan during an early phase of the epidemic were published, and SARS-CoV-2 was found to be a new CoV, with sequence identity just less than 80 percent of that of SARS-CoV [91] .
The viruses most closely related to SARS-CoV-2 were coronaviruses isolated from bats, particularly RaTG13. Therefore, it was hypothesized that bats-a known reservoir of coronavirus-might serve as a reservoir for this new coronavirus.
Genomic sequences of numerous SARS-CoV-2 strains from around the world are now publicly available.[92] These data will allow detailed characterization of the sequence and protein functions to be obtained and comparative studies to be carried out.
Reverse genetics systems can be used to unequivocally identify key features, for example, molecular markers of virulence, host type, and transmissibility of SARS- CoV-2 can be compared with those of related viruses in order to shed light on the biology of this emerging pathogen.

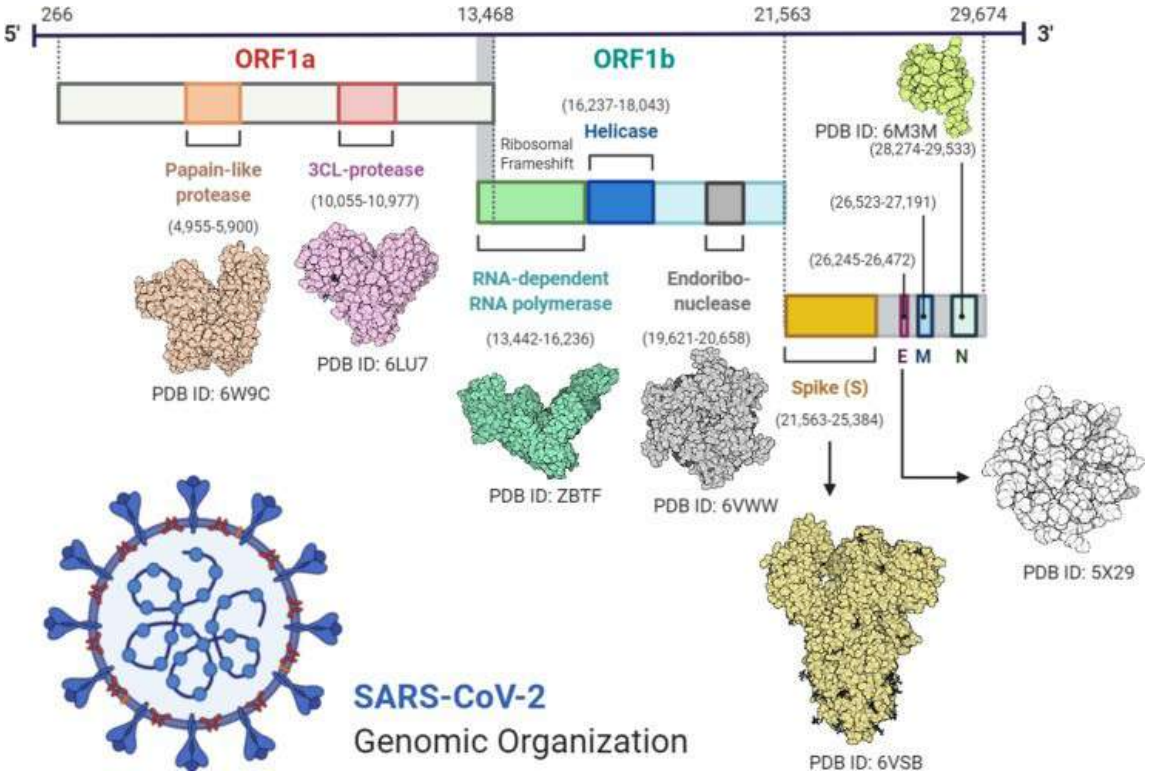
The organization of the SARS-Cov-2 genome and strategies for reverse genetics systems to generate recombinant viruses useful for studying the properties and evolution of the viral genome are summarized below.

Proc Natl Acad Sci U S A. 2016;113(11):3048-3053. doi:10.1073/pnas.1517719113
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4801244/

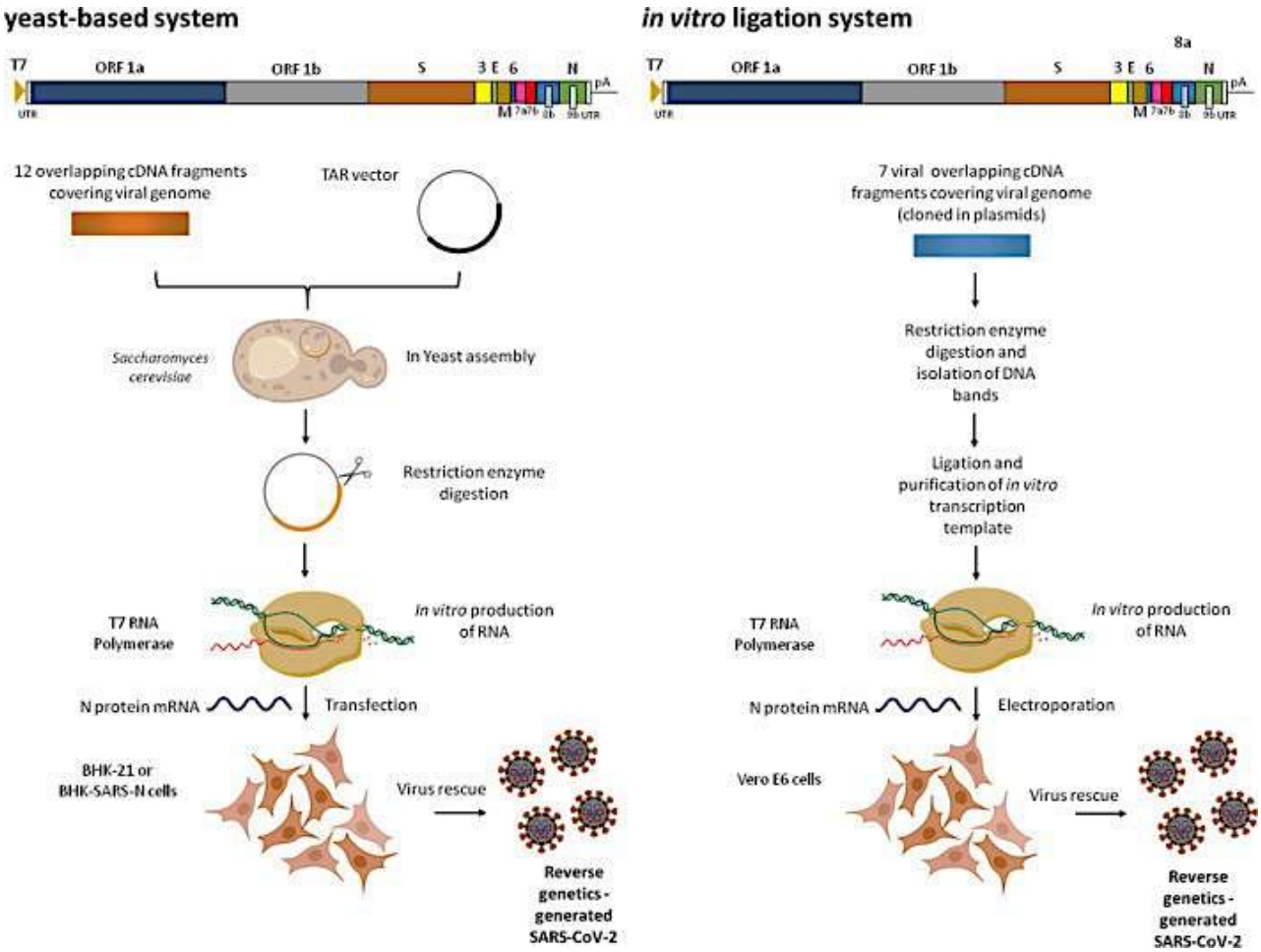
[91] https://www.ecdc.europa.eu/sites/default/files/documents/sequencing-of-SARS-CoV-2.pdf

[92] https://www.gisaid.org/
https://www.ncbi.nlm.nih.gov/sars-cov-2/

Thi Nhu Thao T,
Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform.
Nature. 2020 Jun;582(7813):561-565. doi: 10.1038/s41586-020-2294-9. Epub 2020 May 4.
https://www.nature.com/articles/s41586-020-2294-9



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7755200/ Diagram representing the genomic organization of SARS-CoV-2.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7660456/
Reverse genetics systems for SARS-CoV-2. **(a)** Description of the Saccharomyces cerevisiae-based assembly and retrieval system **(b)** Description of the in vitro ligation system. A schematic representation of the genome organization of SARS-CoV-2 is shown at the top of the panels. RNA polymerase promoter, T7; UTR, untranslated region; pA, poly(A) tail.

THE CONTROVERSY OVER THE ORIGIN OF SARS-COV-2

Although not the subject of this review, it is worth noting that the discussion of the origin of human SARS-Cov-2 outbreak has led to the publication of several studies, including some very recent ones on both the natural evolution of SARS-Cov-2 by recombination of CoVs strains from different animal species [93] and on the use of reverse genetics for the artificial selection of Covs chimeras capable of infecting humans. [94]

IN-DEPTH STUDY

Replication and viral genetics-Viral genetics

VIDEO
virus replicative mechanisms
What is recombinant DNA
Crispr-cas 9 the DNA scissors

BOOK Functional Genomics

METHODS FOR T H E ATTENUATION OF VACCINE VIRUSES

Coronaviruses have several genes that are not required for replication and can be deleted, leading to attenuation in vivo. Deletion of various nonstructural proteins and structural E protein, has been used as a strategy to design vaccine strains of several zoonotic and veterinary coronaviruses. Deletion of the E protein leads to attenuation and generation of an effective vaccine strain [95], but reversion of the attenuated phenotype has been reported[96] . **Deletion of virulence factors** is therefore the most appropriate attenuation mechanism.

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[93] Tagliamonte MS, Abid N, Borocci S, Sangiovanni E, Ostrov DA, Kosakovsky Pond SL, Salemi M, Chillemi G, Mavian C.
Multiple Recombination Events and Strong Purifying Selection at the Origin of SARS-CoV-2 Spike Glycoprotein Increased Correlated Dynamic Movements.
Int J Mol Sci. 2020 Dec 23;22(1):E80. doi: 10.3390/ijms22010080.
https://www.mdpi.com/1422-0067/22/1/80

[94] Secret R, Deigin Y.
The genetic structure of SARS-CoV-2 does not rule out a laboratory origin: SARS-COV-2 chimeric structure and furin cleavage site might be the result of genetic manipulation
[published online ahead of print, 2020 Nov 17]. Bioessays. 2020;e2000240. doi:10.1002/bies.202000240
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7744920/

[95] Enjuanes L, Zuñiga S, Castaño-Rodriguez C, Gutierrez-Alvarez J, Canton J, Sola I. Molecular Basis of Coronavirus Virulence and Vaccine Development.
Adv Virus Res. 2016;96:245-286. doi:10.1016/bs.aivir.2016.08.003
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7112271/

Netland J, DeDiego ML, Zhao J, Fett C, Álvarez E, Nieto-Torres JL, Enjuanes L, Perlman S.
Immunization with an attenuated severe acute respiratory syndrome coronavirus deleted in E protein protects against lethal respiratory disease.
Virology. 2010 Mar 30;399(1):120-128. doi: 10.1016/j.virol.2010.01.004. Epub 2010 Jan 27.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2830353/

Regla-Nava JA, Nieto-Torres JL, Jimenez-Guardeño JM, et al.
Severe acute respiratory syndrome coronaviruses with mutations in the E protein are attenuated and promising vaccine candidates.
J Virol. 2015;89(7):3870-3887. doi:10.1128/JVI.03566-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4403406/

Fett C, DeDiego ML, Regla-Nava JA, Enjuanes L, Perlman S.
Complete protection against severe acute respiratory syndrome coronavirus-mediated lethal respiratory disease in aged mice by immunization with a mouse-adapted virus lacking E protein.
J Virol. 2013;87(12):6551-6559. doi:10.1128/JVI.00087-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3676143/

[96] Jimenez-Guardeño JM, Regla-Nava JA, Nieto-Torres JL, et al.
Identification of the Mechanisms Causing Reversion to Virulence in an Attenuated SARS-CoV for the Design of a Genetically Stable Vaccine.
PLoS Pathog. 2015;11(10):e1005215. Published 2015 Oct 29. doi:10.1371/journal.ppat.1005215.

Another approach to viral attenuation is known as **codon deoptimization** [97], in which the nucleic acid sequence is modified so that suboptimal codons are used to encode the wild-type amino acid sequence, and translation of the viral protein during infection with the vaccine virus is greatly reduced.

However, generation of an attenuated strain of a pathogen for use as a vaccine requires demonstration of its inability to become pathogenic again by genetic reversion.[98]
This is particularly challenging in the case of coronaviruses because they are known to recombine in the wild [99] and an attenuated vaccine strain could, in theory, recombine with wild coronaviruses to recreate a pathogenic strain. [100]

So far, there are only three attenuated SARS-CoV-2 vaccines generated by codon deoptimization, 2 in preclinical development by Mehmet Ali Aydinlar University in Turkey, and Indian Immunologicals Ltd and

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4626112/

[97] Groenke N,
Mechanism of Virus Attenuation by Codon Pair Deoptimization.
Cell Rep. 2020 Apr 28;31(4):107586. doi: 10.1016/j.celrep.2020.107586. PMID: 32348767.
https://www.sciencedirect.com/science/article/pii/S2211124720305350

[98] Bull JJ.
Evolutionary reversion of live viral vaccines: Can genetic engineering subdue it?
Virus Evol. 2015 Jan;1(1):vev005. doi: 10.1093/ve/vev005. Epub 2015 Jan 1.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4811365/
Osterrieder N, Kunec D.
Attenuation of Viruses by Large-Scale Recoding of their Genomes: the Selection Is Always Biased.
Curr Clin Microbiol Rep. 2018;5(1):66-72. doi:10.1007/s40588-018-0080-3
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7100164/

Gonçalves-Carneiro D, Bieniasz PD.
Mechanisms of Attenuation by Genetic Recoding of Viruses.
mBio. 2021 Jan 5;12(1):e02238-20. doi: 10.1128/mBio.02238-20. PMID: 33402534.
https://mbio.asm.org/content/12/1/e02238-20.long

Hanley KA.
The double-edged sword: How evolution can make or break a live-attenuated virus vaccine.
Evolution (N Y). 2011;4(4):635-643. doi:10.1007/s12052-011-0365-y
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3314307/

[99] Tao Y, Shi M, Chommanard C, et al.
Surveillance of Bat Coronaviruses in Kenya Identifies Relatives of Human Coronaviruses NL63 and 229E and Their Recombination History.
J Virol. 2017;91(5):e01953-16. Published 2017 Feb 14. doi:10.1128/JVI.01953-16.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5309958/

Lau SK, Feng Y, Chen H, et al.
Severe Acute Respiratory Syndrome (SARS) Coronavirus ORF8 Protein Is Acquired from SARS-Related Coronavirus from Greater Horseshoe Bats through Recombination.
J Virol. 2015;89(20):10532-10547. doi:10.1128/JVI.01048-15
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4580176/

Luo CM, Wang N, Yang XL, et al.
Discovery of Novel Bat Coronaviruses in South China That Use the Same Receptor as Middle East Respiratory Syndrome Coronavirus.
J Virol. 2018;92(13):e00116-18. Published 2018 Jun 13. doi:10.1128/JVI.00116-18.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6002729/

[100] Gundlach BR, Lewis MG, Sopper S, et al.
Evidence for recombination of live, attenuated immunodeficiency virus vaccine with challenge virus to a more virulent strain. J Virol. 2000;74(8):3537-3542. doi:10.1128/jvi.74.8.3537-3542.2000
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC111861/

Kiseleva I, Dubrovina I, Bazhenova E, Fedorova E, Larionova N, Rudenko L.
Possible outcomes of reassortment in vivo between wild type and live attenuated influenza vaccine strains. Vaccine. 2012 Dec 7;30(51):7395-9. doi: 10.1016/j.vaccine.2012.09.076. Epub 2012 Oct 9.
https://pubmed.ncbi.nlm.nih.gov/23063833/

Griffith University and one in clinical phase by the Serum Institute of India/Codagenix. The latter is used as a nasal spray and is currently being tested in England. [101]

https://codagenix.com/technology/platform-overview/
Viral genomes are processed by the Codagenix computer algorithm to introduce hundreds of silent mutations into the genome to utilize codon pairs underrepresented in human cells (1). The resulting genome that is "de-optimized" for translation into the human host cell is synthesized from scratch (2), assembled into a whole genome, transfected into cells (3), and live, "de-optimized," attenuated viruses are recovered (4). This rapid cell culture-based process can generate animal study-ready master vaccine candidates in weeks versus months for traditional approaches to attenuate live viruses.

INSIGHT: GENETIC REVERSION [102]

Recombination describes a process by which nucleic acid sequences from two different parent viruses are exchanged so that the offspring contain sequences derived from both parents.
Both RNA and DNA viruses can undergo recombination when two related genomic variants of a virus co-infect a cell.

There are three different mechanisms of recombination in viral systems, dictated by the structures of viral genomes. For **DNA viruses**, recombination occurs by physical disruption and rejoining of parental DNA molecules across regions of sequence homology, similar or identical to the same process in bacteria or higher organisms.

For **RNA viruses** containing segmented genomes, gene exchange occurs primarily through **reassortment** of individual segments of the parental genome into progeny viruses, [103] however, intragenic recombination has also been reported for segmented orthomyxoviruses, reoviruses, and bunyaviruses [104].

[101] Safety and Immunogenicity of COVI-VAC, a Live Attenuated Vaccine Against COVID-19
https://clinicaltrials.gov/ct2/show/NCT04619628

https://codagenix.com/technology/platform-overview/

[102] Condit RC, et al Brighton Collaboration Viral Vector Vaccines Safety Working Group (V3SWG).
Unique safety issues associated with virus-vectored vaccines: Potential for and theoretical consequences of recombination with wild type virus strains.
Vaccine. 2016 Dec 12;34(51):6610-6616. doi: 10.1016/j.vaccine.2016.04.060. Epub 2016 Jun 23.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5204448/

[103] Muslin C, Mac Kain A, Bessaud M, Blondel B, Delpeyroux F.
Recombination in Enteroviruses, a Multi-Step Modular Evolutionary Process.
Viruses. 2019;11(9):859. Published 2019 Sep 14. doi:10.3390/v11090859
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6784155/.

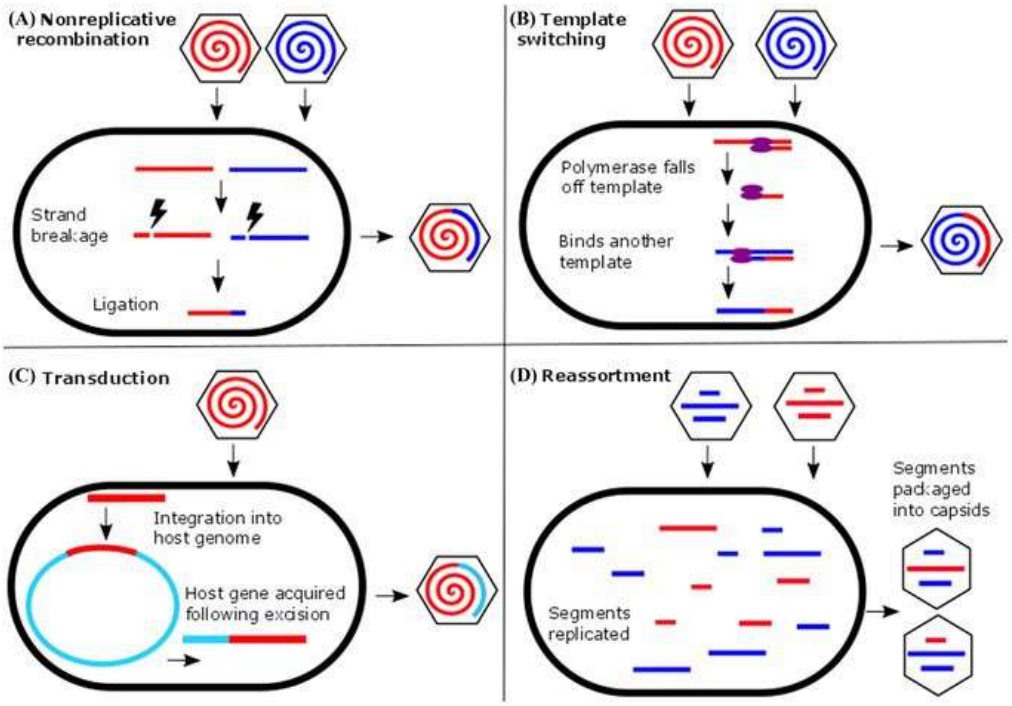
[104] McDonald SM, Nelson MI, Turner PE, Patton JT.
Reassortment in segmented RNA viruses: mechanisms and outcomes.
Nat Rev Microbiol. 2016;14(7):448-460. doi:10.1038/nrmicro.2016.46
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5119462/

Lowen AC.
It's in the mix: Reassortment of segmented viral genomes.
PLoS Pathog. 2018;14(9):e1007200. Published 2018 Sep 13. doi:10.1371/journal.ppat.1007200
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6136819/

Tao H, Steel J, Lowen AC. Intrahost dynamics of influenza virus reassortment. J Virol. 2014;88(13):7485-7492. doi:10.1128/JVI.00715-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4054463/

Recombination has been observed in several families of **single-stranded** (ssRNA) (-) and (+) **RNA viruses** both in the laboratory and in the wild; picornaviruses, coronaviruses, togaviruses and retroviruses, all with positive sense ssRNA genomes, show relatively efficient recombination. [105]

The frequency of recombination between negative-sense RNA viruses (excluding reassortment of segmented genomes) appears to be relatively low [106].



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7167663/
Main mechanisms of virus genetic recombination. **(A)** In nonreplicative recombination, nucleic acid strand breakage and repair allow recombination of genetic material from different sources into the same viral genome. Recombination can occur between homologous or nonhomologous sequences and between coinfecting viruses or between viruses and foreign nucleic acid strands. **(B)** In replicative recombination or mold switching, a polymerase molecule changes the mold during the process of replication of a nucleic acid strand. If the templates are derived from different sources, new genetic material can be introduced into the virus genome. **(C)** During the process of virus integration and excision from a host genome, viruses can acquire genetic material from the host. These genes may increase infectivity or aid in host suppression. **(D)** Reassortment occurs as a result of co-infection of a host cell by multiple segmented viruses. Replicated genome segments are packaged into procapsids independently of the parent of origin. In this way, segments from two or more parents can be packaged into the same procapsid, resulting in genetically different progeny from both parents.

Phan TG, Okitsu S, Maneekarn N, Ushijima H.
Evidence of intragenic recombination in G1 rotavirus VP7 genes.
J Virol. 2007;81(18):10188-10194. doi:10.1128/JVI.00337-07
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2045391/

[105] Bentley K, Evans DJ.
Mechanisms and consequences of positive-strand RNA virus recombination.
J Gen Virol. 2018 Oct;99(10):1345-1356. doi: 10.1099/jgv.0.001142. Epub 2018 Aug 29. PMID: 30156526.
https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.001142

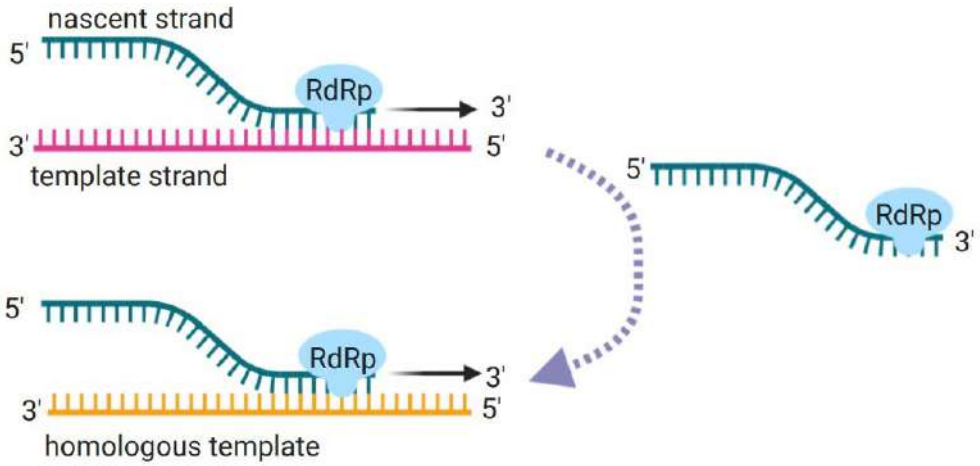
Zhu Z, Meng K, Meng G.
Genomic recombination events may reveal the evolution of coronavirus and the origin of SARS-CoV-2.
Sci Rep. 2020 Dec 10;10(1):21617. doi: 10.1038/s41598-020-78703-6. PMID: 33303849; PMCID: PMC7728743.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7728743/

Yi H.
2019 Novel Coronavirus Is Undergoing Active Recombination. C
lin Infect Dis. 2020 Jul 28;71(15):884-887. doi: 10.1093/cid/ciaa219.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7108124/pdf/ciaa219.pdf

Neches RY, McGee MD, Kyrpides NC.
Recombination should not be an afterthought.
Nat Rev Microbiol. 2020;18(11):606. doi:10.1038/s41579-020-00451-1
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7503439/

[106] Chare ER, Gould EA, Holmes EC.
Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses.
J Gen Virol. 2003 Oct;84(Pt 10):2691-2703. doi: 10.1099/vir.0.19277-0.
https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.19277-0

Recombination in RNA viruses, including coronaviruses [107] and SARS-Cov-2 [108], occurs during replication by "copy choice," (the host cell lacks the enzymes to recombine RNA and thus RNA-dependent RNA polymerase jumps from one die to another during RNA synthesis), resulting in the newly synthesized genome containing sequences from two different parental molecules.



https://www.biorxiv.org/content/10.1101/2020.09.01.278952v1.full.pdf
Copy choice recombination (copy choice) is the primary recombination mechanism hypothesized for RNA viruses. During negative strand synthesis, the replication complex and nascent strand dissociate from the template strand (template). From there, the replication complex can change template or reassociate with a homologous or replicated template strand. RdRp: RNA-dependent RNA polymerase

Although recombination clearly requires co-infection of a cell with two different viruses, the circumstances leading to such co-infection in vivo are not clearly understood. Co-infection could theoretically result from infection with a heterogeneous population of viruses, from simultaneous or overlapping serial infections with different viruses, or from infection of an individual harboring a persistent, latent, or reactivated infection with a different virus. [109]

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[107] Sawicki SG, Sawicki DL, Siddell SG.
A contemporary view of coronavirus transcription.
J Virol. 2007;81(1):20-29. doi:10.1128/JVI.01358-06
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1797243/

Pilipenko EV, Gmyl AP, Agol VI.
A model for rearrangements in RNA genomes.
Nucleic Acids Res. 1995;23(11):1870-1875. doi:10.1093/nar/23.11.1870
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC306956/pdf/nar00011-0040.pdf

Sola I, Almazán F, Zúñiga S, Enjuanes L.
Continuous and Discontinuous RNA Synthesis in Coronaviruses.
Annu Rev Virol. 2015;2(1):265-288. doi:10.1146/annurev-virology-100114-055218
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6025776/

Enjuanes L, Almazán F, Sola I, Zuñiga S.
Biochemical aspects of coronavirus replication and virus-host interaction.
Annu Rev Microbiol. 2006;60:211-30. doi: 10.1146/annurev.micro.60.080805.142157.
https://pubmed.ncbi.nlm.nih.gov/16712436/

[108] Brianna Chrisman et al
Structural Variants in SARS-CoV-2 Occur at Template-Switching Hotspots
bioRxiv 2020.09.01.278952; doi: https://doi.org/10.1101/2020.09.01.278952
https://www.biorxiv.org/content/10.1101/2020.09.01.278952v1.full.pdf

Gallaher WR.
A palindromic RNA sequence as a common breakpoint contributor to copy-choice recombination in SARS-COV-2.
Arch Virol. 2020;165(10):2341-2348. doi:10.1007/s00705-020-04750-z
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7394270/

Wang Q, Wu J, Wang H, et al.
Structural Basis for RNA Replication by the SARS-CoV-2 Polymerase.
Cell. 2020;182(2):417-428.e13. doi:10.1016/j.cell.2020.05.034
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7242921/

[109] Santacroce L, Charitos IA, Carretta DM, De Nitto E, Lovero R.

Several studies have explored from a bionformatics perspective the possible dynamics of recombination events that could have led to the selection of pandemic viruses (e.g., the 2018 "Spanish flu" virus that is believed to have originated by recombination between avian flu and human influenza A viruses [110]) and their occurrence in humans.

Moreover, recombination among viruses in the human population is a plausible phenomenon, as exemplified by recent studies describing recombinants of varicella zoster virus, hepatitis B virus, and enteroviruses.[111]

Vaccine viruses can be a reservoir for long-term recombination in nature, and recombination between attenuated vaccine strains and circulating wild viruses or even between two different live attenuated vaccine strains has been documented.

Specifically, there is evidence that the smallpox virus used as a vaccine during the smallpox eradication campaign in Brazil has created a durable reservoir in nature and is the cause of numerous cowpox-like infections in cattle and humans [112].
Similarly, bovine herpesvirus vaccine can form a latent reservoir in vaccinated animals that, through reactivation, can spread to other animals [113].
Numerous examples document the probable recombination between live attenuated vaccine viruses and wild viruses. Phylogenetic analysis revealed recombination between wild circulating strains of Newcastle disease virus (NDV), an avian paramyxovirus, and NDV attenuated vaccine strains [114].

The human coronaviruses (HCoVs) and the molecular mechanisms of SARS-CoV-2 infection.
J Mol Med (Berl). 2021;99(1):93-106. doi:10.1007/s00109-020-02012-8
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7710368/

Dennehy JJ.
Evolutionary ecology of virus emergence.
Ann N Y Acad Sci. 2017;1389(1):124-146. doi:10.1111/nyas.13304
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7167663/

Holmes EC, Drummond AJ.
The evolutionary genetics of viral emergence.
Curr Top Microbiol Immunol. 2007;315:51-66. doi: 10.1007/978-3-540-70962-6_3.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7120214/pdf/978-3-540-70962-6_Chapter_3.pdf

[110] He CQ, He M, He HB, Wang HM, Ding NZ.
The matrix segment of the "Spanish flu" virus originated from intragenic recombination between avian and human influenza A viruses.
Transbound Emerg Dis. 2019;66(5):2188-2195. doi:10.1111/tbed.13282
http://www.actabp.pl/pdf/3_2014/427.pdf

[111] Norberg P, Depledge DP, Kundu S, et al.
Recombination of Globally Circulating Varicella-Zoster Virus.
J Virol. 2015;89(14):7133-7146. doi:10.1128/JVI.00437-15
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4473579/

Castelhano N, Araujo NM, Arenas M.
Heterogeneous recombination among Hepatitis B virus genotypes.
Infect Genet Evol. 2017 Oct;54:486-490. doi: 10.1016/j.meegid.2017.08.015. Epub 2017 Aug 18. PMID: 28827173.
https://pubmed.ncbi.nlm.nih.gov/28827173/.

Kyriakopoulou Z, Pliaka V, Amoutzias GD, Markoulatos P.
Recombination among human non-polio enteroviruses: implications for epidemiology and evolution.
Virus Genes. 2015 Apr;50(2):177-88. doi: 10.1007/s11262-014-1152-y. Epub 2014 Dec 24. PMID: 25537948.
https://pubmed.ncbi.nlm.nih.gov/25537948/

[112] Moussatché N, Damaso CR, McFadden G.
When good vaccines go wild: Feral Orthopoxvirus in developing countries and beyond.
J Infect Dev Ctries. 2008 Jun 1;2(3):156-73. doi: 10.3855/jidc.258.
https://jidc.org/index.php/journal/article/view/19738346/146

[113] Dispas M, Schynts F, Lemaire M, Letellier C, Vanopdenbosch E, Thiry E, Kerkhofs P.
Isolation of a glycoprotein E-deleted bovine herpesvirus type 1 strain in the field.
Vet Rec. 2003 Aug 16;153(7):209-12. doi: 10.1136/vr.153.7.209. PMID: 12956298.
https://pubmed.ncbi.nlm.nih.gov/12956298/

[114] Chong YL, Padhi A, Hudson PJ, Poss M.
The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1.

Attenuated viruses contained in oral polio vaccine often recombine with indigenous strains of related human enteroviruses to produce circulating vaccine-derived polioviruses (cVDPV), which can cause paralytic disease [115].

Analysis of two independently isolated disease strains of bovine viral diarrhea virus (BVDV) pestivirus showed that these variants arose through both homologous and nonhomologous recombination between a persistent BVDV strain and a vaccine strain, resulting in the evolution of variants with increased pathogenicity compared with the parental strains [116].
In addition, recombination between independently derived attenuated avian herpesvirus vaccine strains can give rise to circulating pathogenic recombinant viruses [117].

Finally, and in a surprising way, there is evidence that retroviral disease reticuloendotheliosis was introduced into avian populations through contamination during the development of vaccines against birdpox (a poxvirus) and Marek's disease (a herpesvirus), and now circulates in the wild as a provirus integrated into some poultry genomes[118].

All of these examples attest to the potential for genetic interaction between vaccine viruses and viruses in the wild, which varies greatly depending on the viruses involved, and it is important that the unintended consequences of such an event be considered by both developers and regulators.

INTEGRATION OF NON-RETROVIRAL RNAE INTO GENOMIC DNA

RNA viruses (non-retroviral RNAs) have also been shown to be able to recombine with the host dsDNA genome of eukaryotic cells [119] and, more surprisingly, with the genomes of ssDNA viruses. [120]

PLoS Pathog. 2010;6(4):e1000872. Published 2010 Apr 22. doi:10.1371/journal.ppat.1000872
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2858710/

[115] Kew OM, Wright PF, Agol VI, et al.
Circulating vaccine-derived polioviruses: current state of knowledge.
Bull World Health Organ. 2004;82(1):16-23.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2585883/pdf/15106296.pdf

[116] Becher P, Orlich M, Thiel HJ.
RNA recombination between persisting pestivirus and a vaccine strain: generation of cytopathogenic virus and induction of lethal disease.
J Virol. 2001;75(14):6256-6264. doi:10.1128/JVI.75.14.6256-6264.2001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC114347/

[117] Lee SW, et al
Attenuated vaccines can recombine to form virulent field viruses.
Science. 2012 Jul 13;337(6091):188. doi: 10.1126/science.1217134.
https://pubmed.ncbi.nlm.nih.gov/22798607/

[118] Niewiadomska AM, Gifford RJ.
The extraordinary evolutionary history of the reticuloendotheliosis viruses.
PLoS Biol. 2013;11(8):e1001642. doi:10.1371/journal.pbio.1001642
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3754887/

[119] Stedman KM.
Deep Recombination: RNA and ssDNA Virus Genes in DNA Virus and Host Genomes.
Annu Rev Virol. 2015 Nov;2(1):203-17. doi: 10.1146/annurev-virology-100114-055127. Epub 2015 Sep 2.
https://pubmed.ncbi.nlm.nih.gov/26958913/

Pistello M, Antonelli G.
Integration of the viral genome into the host cell genome: a double-edged sword.
Clin Microbiol Infect. 2016 Apr;22(4):296-298. doi: 10.1016/j.cmi.2016.01.022. Epub 2016 Feb 3
https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(16)00082-3/fulltext

[120] Stedman K.
Mechanisms for RNA capture by ssDNA viruses: grand theft RNA.
J Mol Evol. 2013 Jun;76(6):359-64. doi: 10.1007/s00239-013-9569-9. Epub 2013 Jun 20.
https://pubmed.ncbi.nlm.nih.gov/23784142/

Diemer GS, Stedman KM.
A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses.
Biol Direct. 2012;7:13. Published 2012 Jun 11. doi:10.1186/1745-6150-7-13
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3372434/

RNA-DNA recombination gives ssDNA viruses access to a much wider variety of sequences than nucleotide substitution and DNA-DNA recombination alone, while deep integration of viral elements into genomic DNA appears to provide defense against future infection by the same or a similar virus.

This phenomenon was recently demonstrated in the pre-publication research by Liguo Zhang et al also for SARS-Cov-2 [121], with the finding of chimeric transcripts consistent with transcription of viral sequences integrated into the genome.

The authors found that endogenous human reverse transcriptase (LINE-1)* expression could be induced by SARS-CoV-2 infection, or by cytokine exposure in cultured cells, suggesting a molecular mechanism for retro-integration of SARS-CoV-2 in patients, in which infection and the subsequent cytokine storm characteristic of COVID-19 promote integration.

> * the human genome contains about 17% LINE-1. LINE-1 proteins have been shown to be nucleic acid chaperones with high binding affinity for RNA, so they can retro-integrate exogenous viral RNAs. [122]

This new feature of SARS-CoV-2 infection may explain why patients may continue to produce noninfectious viral RNA after recovery and continuous or recurrent positive molecular testing for long periods.

In fact, the retro-inserted SARS-CoV-2 sequences are most likely sub-genomic fragments, since the integration junctions are mostly enriched at the N sequence, ruling out infectious virus production, as is also the case with other viruses (e.g., bornavirus[123]). However, it cannot be ruled out at present that integration leads to chronic infection that can flare up following triggering stimuli.

From an evolutionary perspective, LINE-1-mediated viral RNA retro-integration could be an adaptive response by the host to provide sustained antigen expression, possibly for the purpose of enhancing protective immunity. [124]
On the other hand, back-integration of viral RNAs could be harmful and cause a more severe immune response in patients, such as a "cytokine storm" or autoimmune reactions, as is the case with other genome-integrated viruses. [125]

Stedman KM.
Deep Recombination: RNA and ssDNA Virus Genes in DNA Virus and Host Genomes.
Annu Rev Virol. 2015 Nov;2(1):203-17. doi: 10.1146/annurev-virology-100114-055127. Epub 2015 Sep 2.
https://pubmed.ncbi.nlm.nih.gov/26958913/

[121] Zhang L, Richards A, Khalil A, Wogram E, Ma H, Young RA, Jaenisch R.
SARS-CoV-2 RNA reverse-transcribed and integrated into the human genome.
bioRxiv [Preprint]. 2020 Dec 13:2020.12.12.422516. doi: 10.1101/2020.12.12.422516.
https://www.biorxiv.org/content/10.1101/2020.12.12.422516v1.full.pdf

[122] Richardson SR, Doucet AJ, Kopera HC, Moldovan JB, Garcia-Perez JL, Moran JV.
The Influence of LINE-1 and SINE Retrotransposons on Mammalian Genomes.
Microbiol Spectr. 2015;3(2):MDNA3-2014. doi:10.1128/microbiolspec.MDNA3-0061-2014
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4498412/

[123] Horie M, Honda T, Suzuki Y, et al.
Endogenous non-retroviral RNA virus elements in mammalian genomes.
Nature. 2010;463(7277):84-87. doi:10.1038/nature08695
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2818285/

[124] https://pubmed.ncbi.nlm.nih.gov/29028182/
Hurwitz JL, Jones BG, Charpentier E, Woodland DL.
Hypothesis: RNA and DNA Viral Sequence Integration into the Mammalian Host Genome Supports Long-Term B Cell and T Cell Adaptive Immunity.
Viral Immunol. 2017 Nov;30(9):628-632. doi: 10.1089/vim.2017.0099. Epub 2017 Oct 13.

[125] Balada E, Vilardell-Tarrés M, Ordi-Ros J.
Implication of human endogenous retroviruses in the development of autoimmune diseases.

These results may also be relevant to current clinical trials of antiviral therapies.
The reliance on PCR assays to assess the effect of treatments on viral replication and viral load may not reflect the effectiveness of treatment in suppressing viral replication, because the PCR assay can detect viral transcripts from viral sequences stably integrated into the genome rather than due to the infectious virus.

INTERACTION OF RNA VIRUSES WITH THE GUT MICROBIOTA

A fairly recent strand of research is investigating the ways in which bacteria in the microbiota and viruses in eukaryotic cells interact.
Interestingly, intestinal bacteria can promote the infection of several mammalian enteric RNA viruses.

Erickson et al [126] examined a panel of 41 bacterial strains as a platform to determine how different bacteria affect the infection of poliovirus, a model enteric virus.
Most bacterial strains, including those extracted from the cecal contents of mice, bound poliovirus, and each bacterium was able to bind multiple virions.
Some bacterial strains have increased viral co-infection of mammalian cells even at a low virus/host cell ratio.
Bacteria-mediated viral co-infection correlated with bacterial adherence to cells.
Importantly, the bacterial strains that induced viral co-infection facilitated genetic recombination between different viruses, thus removing deleterious mutations and restoring viral fitness (replicative capacity).

Therefore, bacterial-virus interactions can increase the fitness of viruses through viral recombination at initial sites of infection.[127]

The following figure depicts the specific mechanisms of bacterial enhancement of mammalian enteric virus infections:

- bacterial glycans can stabilize virions and increase virus binding to target cells;
- Bacterial interactions with enteric viruses can regulate antiviral immune responses in a pro-viral manner.
- Finally, bacterial interactions may facilitate viral co-infection of target cells and subsequent viral recombination.

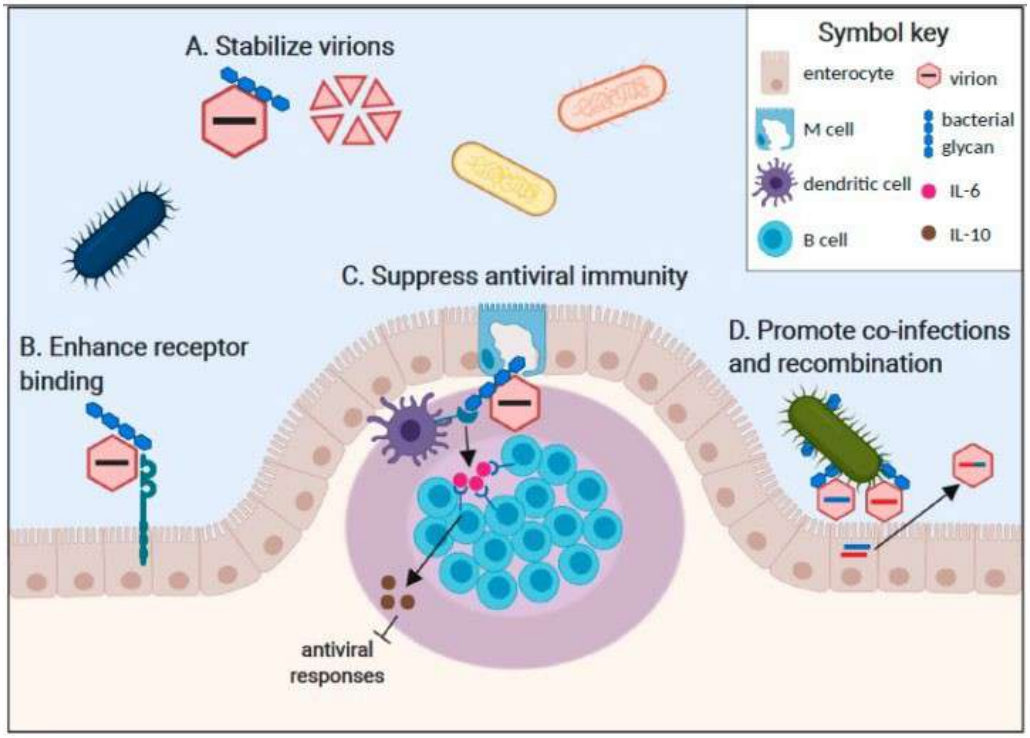
Int Rev Immunol. 2010 Aug;29(4):351-70. doi: 10.3109/08830185.2010.485333.
https://pubmed.ncbi.nlm.nih.gov/20635879/

Voisset C, Weiss RA, Griffiths DJ.
Human RNA "rumor" viruses: the search for novel human retroviruses in chronic disease.
Microbiol Mol Biol Rev. 2008 Mar;72(1):157-96, table of contents. doi: 10.1128/MMBR.00033-07.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2268285/

[126] Erickson AK, Jesudhasan PR, Mayer MJ, Narbad A, Winter SE, Pfeiffer JK.
Bacteria Facilitate Enteric Virus Co-infection of Mammalian Cells and Promote Genetic Recombination.
Cell Host Microbe. 2018;23(1):77-88.e5. doi:10.1016/j.chom.2017.11.007
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5764776/

[127] Roth AN, Grau KR, Karst SM.
Different Mechanisms Underlie Enhancement of Enteric Viruses by the Mammalian Intestinal Microbiota. Viruses. 2019;11(8):760. Published 2019 Aug 17. doi:10.3390/v11080760
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6722614/.

Neu U, Mainou BA.
Virus interactions with bacteria: Partners in the infectious dance.
PLoS Pathog. 2020;16(2):e1008234. Published 2020 Feb 11. doi:10.1371/journal.ppat.1008234
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7012391/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6722614/
Commensal bacteria increase enteric virus infections in several ways. **A**. Binding of the virion to bacterial glycans increases the stability of the particles in the face of environmental stresses and thus improves the efficiency of host-to-host transmission. Poliovirus, reovirus, and norovirus particles are stabilized by bacterial ligands. **B.** Direct interactions of glycan-bound viral particles with cellular entry receptors increase the stability of capsid-receptor interactions and promote the initiation of infection. A specific interaction between poliovirus, its receptor (PVR) and bacterial lipopolysaccharide (LPS) has been reported. **C.** Immune sensing of commensal bacterial components results in a tolerogenic gastrointestinal microenvironment that promotes enteric virus replication. For example, dendritic cell and macrophage detection of LPS-bound MMTV via TLR4 results in the release of IL-6, which stimulates B cells to express the anti-inflammatory cytokine IL-10. **D.** Multiviral clustering on bacterial surfaces increases the frequency of viral co-infection of a single cell, determining the potential for recombination that may lead to improved fitness of recombinant viral strains in the progeny.

A study on the interaction between SARS-Cov-2 and gut microbiota bacteria in Dr. Schmidtchen's laboratory [128] showed that the envelope glycoprotein of SARS-CoV-2 and SARS-CoV is able to bind to the lipopolysaccharide (LPS) of Escherichia coli and lipid A, the toxic portion of LPS and conserved in Gram-negative bacteria.

The authors observed that the Spike protein was able to bind to LPS with an affinity similar to CD14, the receptor used by immune cells to capture LPS and transfer it to toll-like receptors for inflammatory signaling, and that the combination of a low concentration of Spike and LPS, with minimal effect per se, was able to induce strong activation of the NF-KB promoter (dysregulated NF-κB activation is known to be a key pathogenic process in various inflammatory diseases, as it controls the activity of multiple proinflammatory cytokines).[129]

This finding supports the hypothesis of virus-microbiota interaction as a potential trigger mechanism for the cytokine storm observed in COVID19 patients, especially since the incidence of Gram-negative co-infection in severe cases is high.

Although the binding described by the authors of LPS to the SARS-CoV-2 S protein is novel, the interaction between S proteins and endotoxins is not necessarily new in nature.

[128] Petruk G, Puthia M, Petrlova J, Samsudin F, Strömdahl AC, Cerps S, Uller L, Kjellström S, Bond PJ, Schmidtchen A.
SARS-CoV-2 spike protein binds to bacterial lipopolysaccharide and boosts proinflammatory activity.
J Mol Cell Biol. 2020 Dec 9:mjaa067. doi: 10.1093/jmcb/mjaa067.
https://academic.oup.com/jmcb/advance-article/doi/10.1093/jmcb/mjaa067/6028992

[129] Dearest G, Ng LFP.
A promiscuous interaction of SARS-CoV-2 with bacterial products.
J Mol Cell Biol. 2020 Dec 16:mjaa068. doi: 10.1093/jmcb/mjaa068.
https://academic.oup.com/jmcb/advance-article/doi/10.1093/jmcb/mjaa068/6039176

In fact, interactions between viruses and bacteria for the induction of severe respiratory diseases have been described since the early 1930s.[130]

It is therefore possible that SARS-CoV-2 evolved to bind to LPS to induce a strong pro-inflammatory response in favor of viral replication or immune evasion.
However, there are many other evolutionary pressures that could explain the binding of LPS to the Spike protein. It could be hypothesized from examples in the literature that, similar to reoviruses, this interaction could affect the thermal stability of the Spike by allowing the viral particle to withstand a wider range of temperatures [131].

This interaction could also allow coronavirus to bind to bacteria by promoting multiple particle entry to a single epithelial cell in a manner similar to polioviruses.
Furthermore, it is also possible that this interaction explains the persistence of SARS-CoV-2 in the intestine, similar to the infectious mechanism of Noroviruses [132].

SARS-COV-2 BACTERIOPHAGE-LIKE BEHAVIOR

The research group of Dr. C. Brogna et have compiled a series of their own studies in the recent book "SARS- *CoV-2: THE COMPLETE TRUTH*" [133] observing with photographic documentation, that SARS- CoV-2 also behaves as a bacteriophage, that is, a virus capable of infecting the bacteria that make up the normal human intestinal (oro-rhino-alveolar-fecal) flora.

Photos of virus infection in bacterial cells can be found in Dr. Carlo Brogna's book

The study carried out on mixed bacterial cultures questions the validity of the approach used to define the life cycle of SARS-Cov-2, i.e., the exclusive use of culture in Vero cells treated with antibiotics, highlighting its limitation.

[130] Shope RE.
Swine influenza : i. experimental transmission and pathology.
J Exp Med. 1931;54(3):349-359. doi:10.1084/jem.54.3.349
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2131998/pdf/349.pdf

[131] Berger AK, Yi H, Kearns DB, Mainou BA.
Bacteria and bacterial envelope components enhance mammalian reovirus thermostability.
PLoS Pathog. 2017;13(12):e1006768. Published 2017 Dec 6. doi:10.1371/journal.ppat.1006768
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5734793/

[132] Berger AK, Mainou BA.
Interactions between Enteric Bacteria and Eukaryotic Viruses Impact the Outcome of Infection.
Viruses. 2018;10(1):19. Published 2018 Jan 3. doi:10.3390/v10010019
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5795432/.

Baldridge MT, Nice TJ, McCune BT, et al.
Commensal microbes and interferon-λ determine persistence of enteric murine norovirus infection.
Science. 2015;347(6219):266-269. doi:10.1126/science.1258025
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4409937/

[133] SARS-CoV-2: THE COMPLETE TRUTH by Carlo Brogna
BROGNA, CARLO. SARS-Cov-2: THE COMPLETE TRUTH: SARS-CoV-2: THE REAL TRUTH (Italian Edition) (p.2). Kindle edition.
https://www.amazon.it/SARS-Cov-2-COMPLETA-VERITA-SARS-CoV-2-TRUTH-ebook/dp/B08T234TMS

Petrillo, Mauro, Brogna, Carlo, Cristoni, Simone, Querci, Maddalena, Piazza, Ornella, & Van den Eede, Guy.
Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology (Version v1). Zenodo. (2020, October 14). http://doi.org/10.5281/zenodo.4088208
https://zenodo.org/record/4088208#.YA3t6y2h3jA

The author states that "*the exclusive study of the SARS-CoV-2 Coronavirus in a laboratory "True Cell," similar to the human eukaryotic cell but still not identical to it, is limiting and limited, and does not allow one to contemplate many other possible biological and biochemical interactions that the virus may establish with other cell types, such as those I observed between SARS-Cov-2 and bacteria of normal human intestinal flora.*

*So, Kock's (1843-1910) second postulate, which enunciates that "*it must be possible to isolate the microorganism from the diseased host and have it grow and reproduce in pure culture in the laboratory,*" could have been fine for a disease such as tuberculosis.*
*And also in light of this evidence this postulate should be supplemented as follows: "***Each pathogen should be observed as much in culture and in a mixed environment, together with other microorganisms, in order to analyze its interactions with other species, as as a pathogen isolated in pure culture.**"

The most significant results of the study conducted on fecal samples from patients infected with SARS-cov-2 are summarized from p. 36 of the book Home Pharmacological Therapy of the Symptomatic Patient, while the method is available in the pre-print article "*Detection of toxin-like peptides in plasma and urine samples from COVID-19 patients.*" [134]

It should be noted that among the proteins produced as a result of the bacterial-virus interaction, several forms of conotoxins* were found, and of these some were capable of occupying and blocking the nerve receptor sites to which acetylcholine binds to transmit up to the brain the olfactory information.
This represents a plausible mechanism of the loss of the sense of smell in those infected with SARS-Cov-2, namely that odor uptake occurs normally, but the acetylcholine-mediated transmission of information is blocked along the way, never reaching the temporal cerebral cortex, where the olfactory experience is realized at the conscious level. [135]

* Most conotoxins are gene-derived peptides that are synthesized at the ribosome level and further processed in the endoplasmic reticulum (ER) and Golgi apparatus of secretory cells of the venom gland of predatory sea snails. The five best-studied pharmacological classes of conotoxins are all ion channels expressed in the nervous and locomotor systems: α (acetylcholine receptor inhibitors, nAChR), ω (inhibitors of voltage-dependent calcium channels, VGCC), κ (inhibitors of voltage-dependent potassium channels, VGKC), μ (inhibitors of voltage-dependent sodium channels, VGSC) and δ (retarders of activation of voltage-dependent sodium channels, VGSC) [136]

The observation that the various forms of conotoxins found in plasma and urine differed in small amino acid sequences led the authors to test the hypothesis that they were produced by intestinal bacteria rather than epithelial cells. For the purpose, they inoculated the feces of healthy patients with SARS-CoV-2, obtaining confirmation of virus replication within the feces.
This was to mean that the virus found fertile ground to reproduce in feces and, more specifically, in bacteria in the feces of healthy patients.

[134] Brogna, Carlo, Petrillo, Mauro, Cristoni, Simone, Querci, Maddalena, Piazza, Ornella, & Van den Eede, Guy. Detection of toxin-like peptides in plasma and urine samples from COVID-19 patients (Version v1). Zenodo. (2020, October 27) http://doi.org/10.5281/zenodo.4139341 https://zenodo.org/record/4139341#.YA30ApNKjjA

[135] Brogna, C. The Covid-19 Virus Double Pathogenic Mechanism. A New Perspective. Preprints 2020, 2020040165 (doi: 10.20944/preprints202004.0165.v2). https://www.preprints.org/manuscript/202004.0165/v2

[136] Bjørn-Yoshimoto WE, Ramiro IBL, Yandell M, et al. Curses or Cures: A Review of the Numerous Benefits Versus the Biosecurity Concerns of Conotoxin Research. Biomedicines. 2020;8(8):235. Published 2020 Jul 22. doi:10.3390/biomedicines8080235 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7460000/

Because the loss of olfaction does not occur in all infected persons, it has been hypothesized that only in patients whose intestinal bacteria have produced relevant doses of conotoxins with the correct conformation and capable of occupying acetylcholine receptors, nerve transmission along the olfactory pathways is blocked and hyposmia is determined.

In addition to conotoxins, infection of bacteria by SARS-Cov-2 results in the production of other toxins including:

- **phospholipase A2**: phospholipases are important virulence factors in that they help bacterial pathogens invade host cells by destroying phospholipids of cell membranes [137]and their products could act as signaling molecules (thromboxanes and leukotrienes) [138].

For the mechanism of the eicosanoid storm in COVID-19 see the book "Home drug therapy of the symptomatic patient - anti-inflammatory section"

It has been reported that sPLA2 obtained from snake venom possesses potent virucidal (neutralizing) activity against dengue and yellow fever viruses due to its effect on the lipid bilayers of the viral envelope.[139]

- Prothrombin **activating protein:** [140] A protein that stimulates the events of the last stage of the coagulation cascade and induces an increase in coagulation, resulting in the micro-embolisms, found in the stages of severe fatal complication from COVID-19. [141]

For the mechanism of coagulopathy in COVID-19, see the book "Respiratory complications: immunopathology" - section Coagulopathy

Coagulation induction is a defense mechanism of innate immunity against the excessive spread of microorganisms that cause sepsis; however, it can be triggered by bacteria such as *S. Aureus* that use fibrin biofilms to protect themselves from attack by the immune system and antibiotics. [142]

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[137] Kumari Bandana, Kaur Jashandeep, Kaur Jagdeep.
Phospholipases in Bacterial Virulence and Pathogenesis.
Adv Biotech & Micro. 2018; 10(5): 555798. DOI: 10.19080/AIBM.2018.10.555798
https://juniperpublishers.com/aibm/pdf/AIBM.MS.ID.555798.pdf

[138] Hoxha M.
What about COVID-19 and arachidonic acid pathway?
Eur J Clin Pharmacol. 2020;76(11):1501-1504. doi:10.1007/s00228-020-02941-w
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7314570/

Robb CT, Goepp M, Rossi AG, Yao C.
Non-steroidal anti-inflammatory drugs, prostaglandins, and COVID-19.
Br J Pharmacol. 2020 Nov;177(21):4899-4920. doi: 10.1111/bph.15206. Epub 2020 Aug 27. PMID: 32700336; PMCID: PMC7405053.
https://pubmed.ncbi.nlm.nih.gov/32700336/

[139] Chen, M., Aoki-Utsubo, C., Kameoka, M. et al.
Broad-spectrum antiviral agents: secreted phospholipase A2 targets viral envelope lipid bilayers derived from the endoplasmic reticulum membrane.
Sci Rep 7, 15931 (2017). https://doi.org/10.1038/s41598-017-16130-w
https://www.nature.com/articles/s41598-017-16130-w

[140] Whelihan MF, Zachary V, Orpheus T, Mann KG.
Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation
[published correction appears in Blood. 2013 Aug 22;122(8):1532]. Blood. 2012;120(18):3837-3845. doi:10.1182/blood-2012-05-427856
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3488894/

[141] Castro RA, Frishman WH.
Thrombotic Complications of COVID-19 Infection:
A Review. Cardiol Rev. 2021 Jan/Feb;29(1):43-47. doi: 10.1097/CRD.000000000347.
https://pubmed.ncbi.nlm.nih.gov/32947478/

[142] Liesenborghs L, Verhamme P, Vanassche T.
Staphylococcus aureus, master manipulator of the human hemostatic system.
J Thromb Haemost. 2018 Mar;16(3):441-454. doi: 10.1111/jth.13928. Epub 2018 Jan 29.
https://onlinelibrary.wiley.com/doi/full/10.1111/jth.13928

- many other proteins, similar to phosphodiesterases, zinc-metal proteinases, serine proteases, bradykinins, etc.

The discovery of a role of gut bacteria in viral replication changes not only the therapeutic but also the epidemiological approach to the disease, since the presence of SARS-COV-2, active and still able to infect and replicate, is recorded in patients' stools.

This means that the contagiousness of a patient declared healthy through negativity to a nasopharyngeal swab is actually still going on and can occur by the fecal-oral route, that is, by direct contact with infected surfaces.

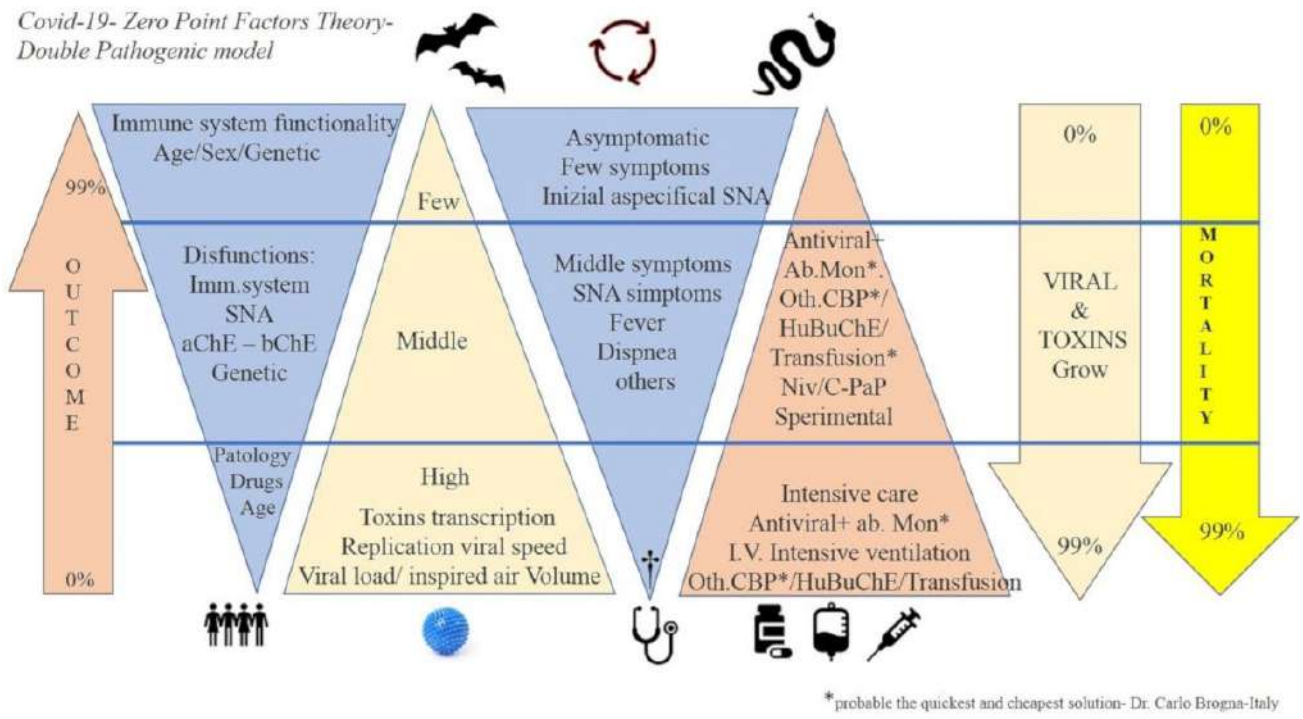
To conclude, a new **dual-pathogenicity model of SARS-Cov-2** is suggested below, in which the virus behaves as a "Trojan horse," which in passing through the intermediate host has probably acquired, randomly and naturally, simple or small gene sequences (micro-RNAs?) termed "**The** Zero Point Factors."

A circuit is generated during infection: the faster the viremia and replication, the more factors/toxins are translated, and the more viremia will increase due to the latter action on the immune system.
In addition, the immune system also has significant inter-individual variations in age, genetics, pathology, etc., and the longer it takes the immune system to produce antibodies against the virus, the more factors are released into the circulation and the more the immune system will be blocked by viremia and factors with a dual synergistic action.

In this model, the determining conditions are exposure time, initial and/or repeated viral load (number of viral particles/inhaled air volume). Zero-point factors could act at central and peripheral cholinergic synapses, nicotinic receptors, and coagulation factors by increasing the pathogenicity of the virus.

Individuals who might therefore be at increased risk are patients with ACE2 receptor overexpression (taking ACE inhibitor drugs); health care workers, due to increased exposure time; individuals with a depressed immune system (oncology, autoimmune diseases, treatment with immunosuppressants, etc.); patients with cardiac and respiratory diseases (either due to toxicity factors produced or viremia); patients with lower levels or impaired functioning of cholinesterase (differing in age, sex, race, and phenotype); neuronal diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), etc., or autoimmune patients such as Myasthenia Gravis who are treated with acetylcholinesterase inhibitors; farmers with seasonal exposure to organophosphates (pesticides).

The outcome of the disease would thus depend on the dual mechanism of action of the pathogens: on the one hand, factors related to viral infection and on the other hand, toxicity factors caused by the production of peptide toxins.



https://www.preprints.org/manuscript/202004.0165/v2
New probable dual-pathogenicity model (M.D.Carlo Brogna)

Finally, Dr. Brogna in his book indicates the most appropriate treatment approaches based on the results of his research:

- the disease should be treated first as a **poisoning syndrome**, and then by neutralizing the toxins, the cause of COVID-19 symptomatology with **antidotes**.

- In conjunction with antidotes, which inactivate the toxins already produced and circulating, it will be necessary to aim at **blocking the new production of toxins**. That is, it will be necessary to temporarily, and judiciously, "freeze" with bacteriostatic or bactericidal antibiotic drugs all those bacteria in the intestinal flora that, affected by SARS-COV-2, synthesize these proteins.

To this end, administered early, the **antibiotics** azithromycin, vancomycin and metronidazole have been shown (in vitro) to shut down both viral replication and toxin synthesis. Amoxicillin, on the other hand, while blocking viral replication, does not completely stop toxin synthesis. **Adjuvant probiotic therapy** should then be combined with antibiotic **therapy**.

Patients who took azithromycin or amoxicillin and probiotics (including Lactobacillus reuteri and Bacillus clausii) at "time zero," avoiding the administration of nonsteroidal anti-inflammatory drugs and mild cyclooxygenase 1 inhibitors (including acetaminophen), had a prompt recovery and mild initial symptoms.

- The positive clinical effects of antibiotic therapy (not usually indicated for the treatment of an infection of viral origin) further confirms the central role of gut flora bacteria in the genesis of clinical manifestations of COVID-19.

Similarly, the empirically found usefulness of **dexamethasone** in containing the symptomatology of COVID-19 is explained by its ability to inhibit Phospholipase A2, one of the toxin-like proteins found in infected patients and known to activate a cascade of biochemical events underlying the clinical picture of COVID-19.

- Especially in COVID-19-positive patients who are symptomatic and have already compromised general health status (patients with concomitant cardiovascular disease, for example), **the administration of drugs that could precipitate the underlying pathological condition** (cardiovascular disease, to follow with the above example) should be **avoided.**

The administration of, for example, Cycloxygenase-1 (COX-1) inhibitors, i.e., acetylsalicylic acid, ibuprofen, etc., is to be avoided because it potentiates the production of:

-Thromboxane A2, whose known vasoconstriction and platelet aggregation effects dramatically worsen the clinical picture of symptomatic COVID patients and those with existing cardiovascular disorders, promoting thrombotic and ischemic pictures.
-Leukotrienes, via hyperactivation of lipoxygenases, whose main effects are to enhance constriction of the bronchial territory, which in patients with concomitant respiratory tree diseases could undoubtedly prove fatal.

He reports other important thoughts at the conclusion of his discussion:

- toxins are more effective at low temperatures (winter season), and this may explain the seasonal pattern of infection.

- SARS-Cov-2, as a bacteriophage can pollute sewage, agribusiness, and seafood in particular.
- vaccination against SARS-Cov-2 will not protect vaccinees from the increased risk of developing cardiovascular, pulmonary, and degenerative diseases due to the production of bacterial toxins upon infection.

- the structure of SARS-Cov-2 will undergo many mutations due to the genetic changes induced by the bacteria to defend themselves from the bacteriophage attack, which will be different for each individual and replicative cycle.

- SARS-Cov-2, after infection, could be present in the bacteria with a structure similar to an artificial bacterial chromosome or plasmid, that is, it could be integrated by the replicative processes of the bacteria themselves into circular DNAs and be enveloped in a coronavirus surface protein envelope.
  This hypothesis is suggested by the fact that in COVID-19 patients there is neutrophilia, which is typical of bacterial infections (see book "Respiratory complications: immunopathology" - section Neutrophils), by its lower measurements (50-200 nm) than those described in the literature, and by a much more modest corona than that found in cultures of the virus in Vero cells.

  This working hypothesis deserves further investigation because it would explain the persistence of virus in feces for long periods. [143] However, although to date there is no evidence that bacterial ssRNA viruses are retrotranscribed and integrated into DNA as prophages or present extrachromosomally, as is the case with DNA phages, [144] retroviral elements (retrons) are known to be present in bacterial genomes with the production of reverse transcriptase and the formation of DNA-RNA hybrids.[145]

[143] Zhang Y, Chen C, Song Y, et al.
Excretion of SARS-CoV-2 through faecal specimens.
Emerg Microbes Infect. 2020;9(1):2501-2508. doi:10.1080/22221751.2020.1844551
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7717617/

[144] Witzany G. (eds) Biocommunication of Phages. Springer, Cham. https://doi.org/10.1007/978-3-030-45885-0

[145] Bacterial retrons function in anti-phage defense
Adi Millman, Aude Bernheim, Avigail Stokar-Avihail, Taya Fedorenko, Maya Voichek, Azita Leavitt, Rotem Sorek
bioRxiv 2020.06.21.156273; doi: https://doi.org/10.1101/2020.06.21.156273
https://www.biorxiv.org/content/10.1101/2020.06.21.156273v1.full.pdf

Lampson BC, Inouye M, Inouye S.
Retrons, msDNA, and the bacterial genome.
Cytogenet Genome Res. 2005;110(1-4):491-9. doi: 10.1159/000084982.
https://pubmed.ncbi.nlm.nih.gov/16093702/

INACTIVATED VIRUS VACCINES

- CoronaVac (PiCoVacc) / *Sinovac Research and Development Co., Ltd*
- Sinopharm + Wuhan Institute of Biological Products
- BBIBP-CorV / *Sinopharm + Beijing Institute of Biological Products*
- Institute of Medical Biology + Chinese Academy of Medical Sciences
- QazCovid-in / *Research Institute for Biological Safety Problems, Rep of Kazakhstan*
- BBV152 / *Bharat Biotech International Limited*
- Shenzhen Kangtai Biological Products Co., Ltd.
- VLA2001 / *Valneva, National Institute for Health Research, United Kingdom*

An inactivated vaccine consists of whole viral particles whose replicative capacity is blocked, but which retain the ability to induce an immune response.

CRITICALITY OF INACTIVATED VACCINES

In general, all inactivated viral vaccines follow a similar production cycle in which the pathogen is first grown on a substrate to produce large amounts of antigen. [146]
Historically, vaccine manufacturers have used primary cells, tissues, fertilized eggs, and even whole organisms as substrates for propagating viruses [147].

Today, industries are increasingly moving toward growing viruses on continuous cell lines*.
This brings some advantages such as reduced production costs, improved vaccine safety, and relatively simple increases in production capacity [148].

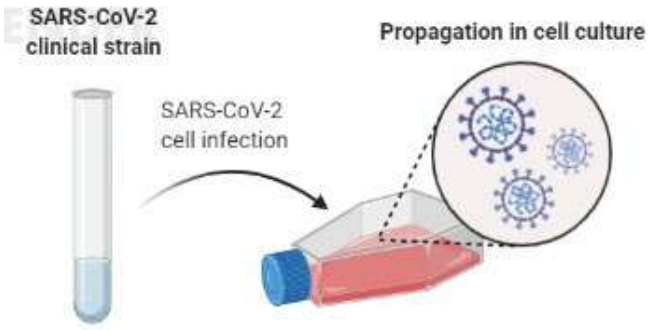
Once the virus has propagated, it is often purified and concentrated before inactivation. Inactivation can be performed using chemical or physical methods, or a combination of the two.
A wide range of well-established and novel inactivation agents or methods have been described to effectively inactivate viruses for vaccine purposes. [149]

[146] Sanders B, Koldijk M, Schuitemaker H.
Inactivated Viral Vaccines.
Vaccine Analysis: Strategies, Principles, and Control. 2014;45-80. Published 2014 Nov 28. doi:10.1007/978-3-662-45024-6_2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7189890/

[147] Hess RD, Weber F, Watson K, Schmitt S.
Regulatory, biosafety and safety challenges for novel cells as substrates for human vaccines.
Vaccine. 2012 Apr 5;30(17):2715-27. doi: 10.1016/j.vaccine.2012.02.015. Epub 2012 Feb 17.
https://pubmed.ncbi.nlm.nih.gov/22342707/

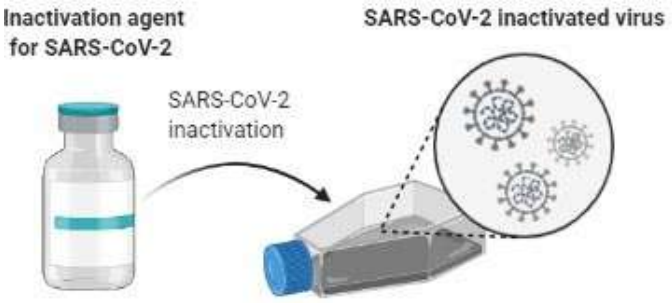
[148] Barrett PN, Mundt W, Kistner O, Howard MK.
True cell platform in vaccine production: moving toward cell culture-based viral vaccines.
Expert Rev Vaccines. 2009 May;8(5):607-18. doi: 10.1586/erv.09.19.
https://pubmed.ncbi.nlm.nih.gov/19397417/

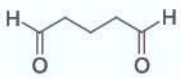
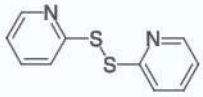
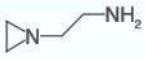
[149] Qiuhui Li, Huiya Ma, Yuanyuan Zhang, Kun Feng, Pengfei Yang, Jianjun Li, Hongli Zhu, Chao Chen, Kunping Yan.
HPLC method for Residual 2-Bromoethylamine Hydrobromide in Hemoglobin-based Oxygen Carriers Derived with 4-Methoxybenzenesulfonyl Chloride.
Journal of Liquid Chromatography & Related Technologies (2018) 41:13-14, pages 819-824.
https://www.tandfonline.com/doi/full/10.1586/erv.12.38



https://www.pqegroup.com/wp-content/uploads/2020/08/ITA-PQE-Group-infodemic-project-Fighting-SARS-CoV-2-prevention-and-vaccines.pdf

Inactivated viral vaccines are created by propagating viruses in cell cultures (as in Vero cells) followed by inactivation using a chemical reagent (such as beta-propiolactone). After vaccination, this allows the body to generate a diverse immune response against numerous viral antigens without having the threat of actually being infected because of the virus.



Method	Type	Mechanism
Formaldehyde 	Alkylating agent	Monohydroxymethylation of adenine Crosslinking of RNA to capsid proteins, causing a block of genome reading
	Crosslinker	Crosslinking of proteins by formation of inter- and intra-molecular methylene bridges between hydroxymethylated amines
Glutaraldehyde 	Crosslinker	Crosslinking of proteins by the same mechanism as formaldehyde (described above)
2,2′-dithiodipyridine 	Crosslinker	Crosslinking of proteins by oxidation of S-H groups causing formation of S-S bridges, which results in a covalent modification and functional inactivation of S-H-containing internal viral proteins
β-propiolactone 	Alkylating agent Crosslinker	Alkylation of RNA and DNA as a consensus mechanism Crosslinking of proteins
Binary ethylene imine 	Alkylating agent	Alkylation of RNA and DNA at low concentrations. Most likely genome reading is blocked by alkylation of guanine or adenine by binary ethylene imine Alkylation of proteins (nucleocapsid) at high concentrations
pH	Denaturation agent RNA degradation	Denaturation of viral functionally active proteins The close proximity of the hydroxyl group to the phosphor center of each internucleotide linkage facilitates transesterification under strongly acidic or strongly basic conditions, with a breakage of the phosphodiester bond as a consequence
Temperature	Denaturation agent RNA degradation	A high temperature denatures viral functionally active proteins Virus inactivation at 'low' temperature (below 41°C) is considered to be caused by degradation of the nucleic acid
Gamma irradiation	Radiation	Viruses are inactivated primarily by direct damage, via disruption of the genome Formation of free radicals that damage proteins
Ultraviolet light	Radiation	Induction of dimer formation between adjacent uracils in RNA. Dimer formation leads to pressure and breakage of the sugar backbone causing a block of genome reading Works more slowly, ultraviolet light also causes structural modifications of the capsid proteins resulting in the formation of large and small photoproducts

https://www.tandfonline.com/doi/full/10.1586/erv.12.38
Overview of inactivation methods for vaccine development with killed virus

Some examples of agents tested are ascorbic acid [150], ethyleneimine derivatives [151], psorlenes [152], hydrogen peroxide [153], gamma irradiation [154], UV treatment [155], heat [156] and many others [157].
However, only formaldehyde and β-propiolactone (GLP) have been widely used for inactivation of licensed human viral vaccines for decades. [158]

https://www.tandfonline.com/doi/full/10.1586/erv.12.38
Mechanism of reaction of formaldehyde with DNA/RNA or amino acids.
Reaction with (A) DNA or RNA (adenine) and, similarly, (B) amino acids (e.g., lysine) of proteins with a primary amine group: monohydroxy methylation and methylene bridge formation.
A: Adenine; Lys: Lysine.

https://www.tandfonline.com/doi/full/10.1586/erv.12.38
Reaction mechanism of glutaraldehyde with protein amino acids (e.g., with the primary amino group of lysine). Lys: Lysine.

https://www.tandfonline.com/doi/full/10.1586/erv.12.38
Reaction mechanism of β-propiolactone with DNA or RNA (guanine).

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[150] Madhusudana SN, Shamsundar R, Seetharaman S.
In vitro inactivation of the rabies virus by ascorbic acid.

Int J Infect Dis. 2004 Jan;8(1):21-5. doi: 10.1016/j.ijid.2003.09.002. PMID: 14690777.
https://www.ijidonline.com/article/S1201-9712(03)00004-3/fulltext

[151] Wide OP, Nebel AE.
Rabies virus inactivation by binary ethylenimine: new method for inactivated vaccine production.

J Clin Microbiol. 1980;11(2):120-122. doi:10.1128/JCM.11.2.120-122.1980
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC273335/pdf/jcm00175-0026.pdf

[152] Maves RC, Oré RM, Porter KR, Kochel TJ.
Immunogenicity and protective efficacy of a psoralen-inactivated dengue-1 virus vaccine candidate in Aotus nancymaae monkeys.
Vaccine. 2011 Mar 24;29(15):2691-6. doi: 10.1016/j.vaccine.2011.01.077. Epub 2011 Feb 17.
https://pubmed.ncbi.nlm.nih.gov/21303709/21303709.

[153] Amanna IJ, Raué HP, Slifka MK.
Development of a new hydrogen peroxide-based vaccine platform.
Nat Med. 2012;18(6):974-979. doi:10.1038/nm.2763
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3506259/

[154] Martin SS, Bakken RR, Lind CM, et al.
Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB.
Vaccine. 2010;28(4):1031-1040. doi:10.1016/j.vaccine.2009.10.126
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2815090/

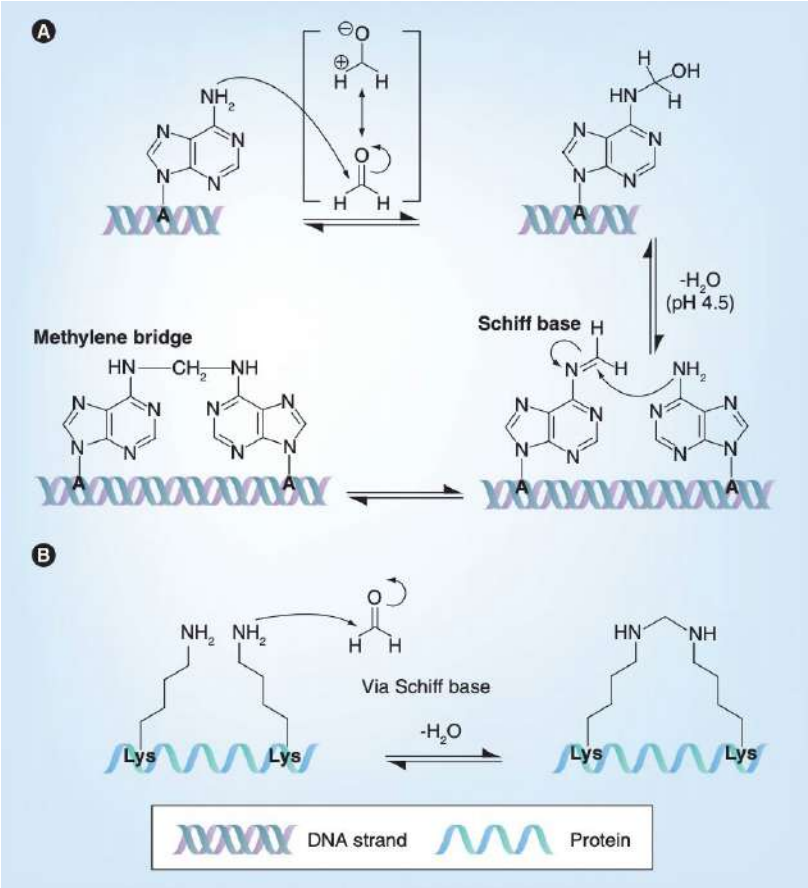
Alsharifi M, Müllbacher A.
The gamma-irradiated influenza vaccine and the prospect of producing safe vaccines in general.
Immunol Cell Biol. 2010 Feb;88(2):103-4. doi: 10.1038/icb.2009.81. Epub 2009 Oct 27.
https://pubmed.ncbi.nlm.nih.gov/19859081/

[155] Budowsky EI, Bresler SE, Friedman EA, Zheleznova NV.
Principles of selective inactivation of viral genome. I. UV-induced inactivation of influenza virus.
Arch Virol. 1981;68(3-4):239-47. doi: 10.1007/BF01314577.
https://pubmed.ncbi.nlm.nih.gov/7271457/

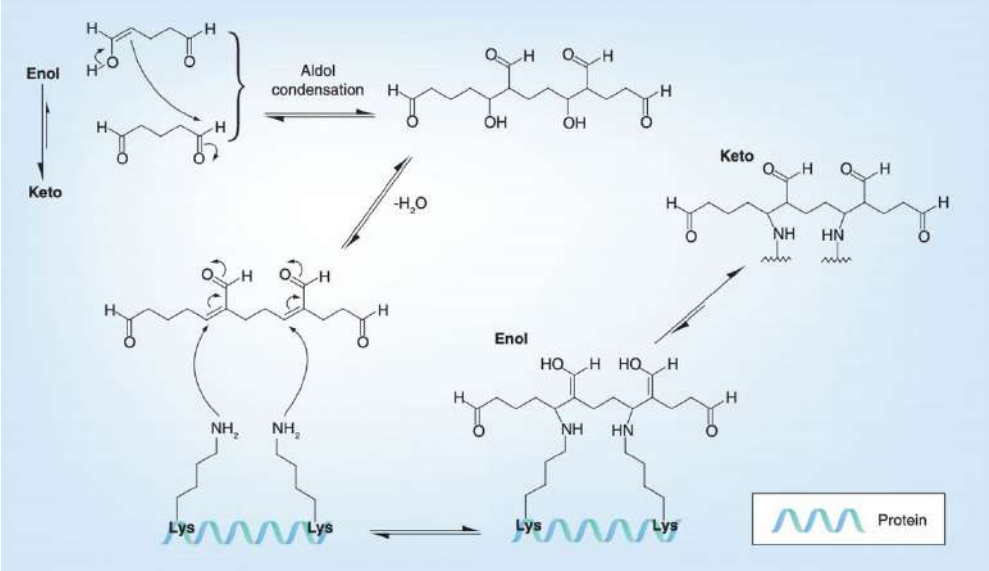
[156] Nims RW, Plavsic M.
Polyomavirus inactivation - a review.
Biologicals. 2013 Mar;41(2):63-70. doi: 10.1016/j.biologicals.2012.09.011. Epub 2012 Oct 30.
https://pubmed.ncbi.nlm.nih.gov/23116715/

[157] Stauffer F, El-Bacha T, Da Poian AT.
Advances in the development of inactivated virus vaccines.
Recent Pat Antiinfect Drug Discov. 2006 Nov;1(3):291-6. doi: 10.2174/157489106778777673.
https://pubmed.ncbi.nlm.nih.gov/18221154/

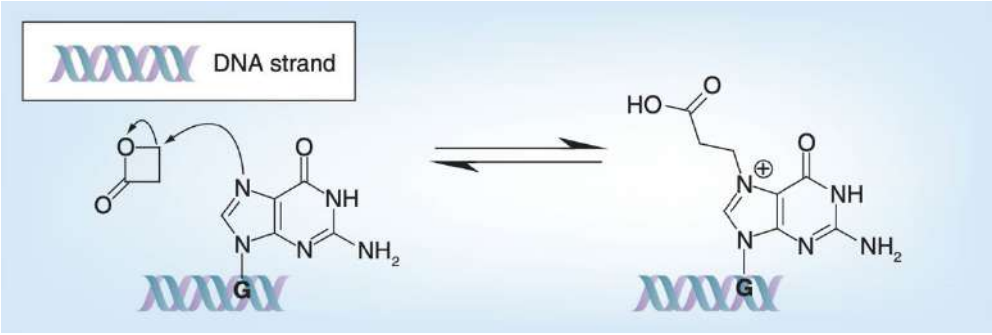
[158] Herrera-Rodriguez J, Signorazzi A, Holtrop M, de Vries-Idema J, Huckriede A.
Inactivated or damaged? Comparing the effect of inactivation methods on influenza virions to optimize vaccine production.
Vaccine. 2019;37(12):1630-1637. doi:10.1016/j.vaccine.2019.01.086
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7115651/



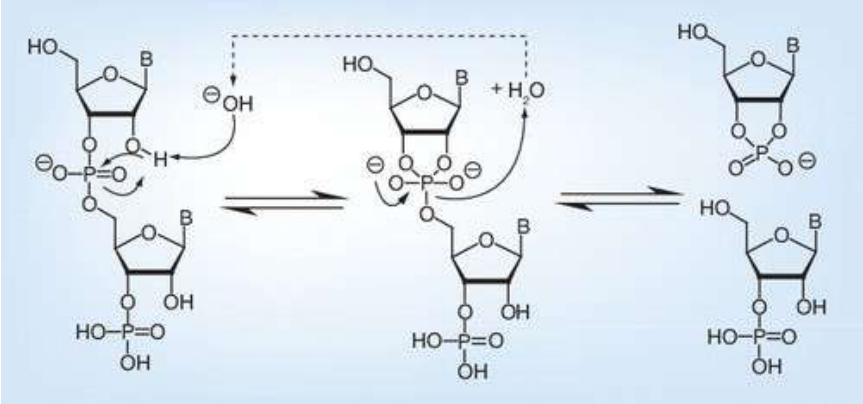
https://www.tandfonline.com/doi/full/10.1586/erv.12.38
Mechanism of reaction of formaldehyde with DNA/RNA or amino acids.
Reaction with (A) DNA or RNA (adenine) and, similarly, (B) amino acids (e.g., lysine) of proteins with a primary amine group: monohydroxy methylation and methylene bridge formation.
A: Adenine; Lys: Lysine.



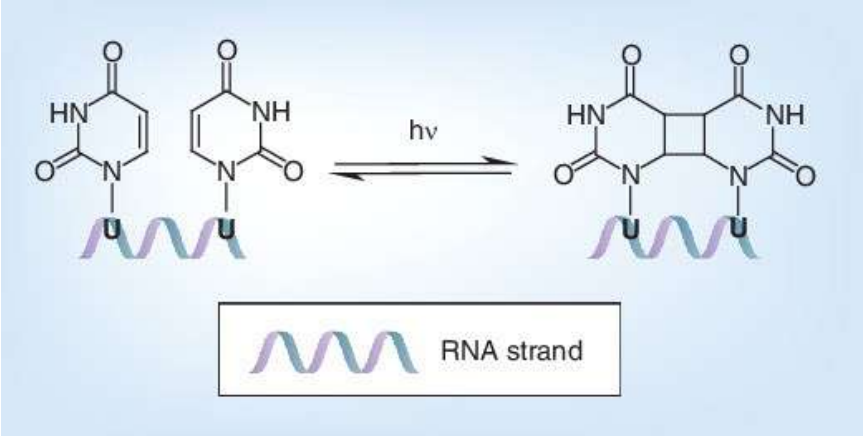
Reaction mechanism of glutaraldehyde with protein amino acids (e.g., with the primary amino group of lysine).
Lys: Lysine.



Reaction mechanism of β-propiolactone with DNA or RNA (guanine).



RNA degradation in an alkaline environment.
B: Nucleobase.



RNA degradation in an alkaline environment.
B: Nucleobase.

Historical events have shaped the way inactivated vaccines are currently developed and characterized today.
The **Cutter incident** in 1955 was one of the worst pharmaceutical disasters in U.S. history.
380,000 doses of inactivated virus vaccine (IPV), produced in Cutter Laboratories, were administered to healthy children.
However, these vaccines contained replication-competent poliovirus due to inadequate purification of the viral eluate during production.
The presence of cellular debris in the vaccine pools prevented adequate exposure of viral particles to formaldehyde and thus complete inactivation [159].
As a result, 40,000 children who received the vaccine contracted abortive polio, 51 were permanently paralyzed, and five died [160].

Federal requirements for vaccine manufacturers were immediately revised as a result of the Cutter incident, creating a better system of vaccine regulation. However, the legacy of this vaccine injury remains, and vaccine manufacturers should always exercise caution when inactivating pathogens to ensure complete inactivation.

Another serious reaction involves two inactivated vaccines that led to the development of enhanced disease and even death when vaccinated people became infected with the pathogen.
In fact, clinical trials **with formalin-inactivated respiratory syncytial virus (RSV)** in infants have had a disastrous outcome.

[159] Offit PA.
The Cutter incident, 50 years later.
N Engl J Med. 2005 Apr 7;352(14):1411-2. doi: 10.1056/NEJMp048180.
https://pubmed.ncbi.nlm.nih.gov/15814877/

[160] Nathanson N, Langmuir Ad.
The Cutter incident. Poliomyelitis following formaldehyde- inactivated poliovirus vaccination in the United States during the Spring of 1955. ii. relationship of poliomyelitis to Cutter vaccine.
Am J Hyg. 1963 Jul;78:29-60. doi: 10.1093/oxfordjournals.aje.a120328.
https://pubmed.ncbi.nlm.nih.gov/14043545/

Not only did the vaccine fail to prevent the disease, but 80 percent of vaccine recipients were hospitalized after being infected with circulating RSV compared with hospitalization of only 5 percent in the unvaccinated control group.
In addition, two vaccine recipients died as a result of vaccine-induced disease enhancement[161].

The enhanced disease was later attributed to an unfavorable immune response due to an impaired Th2 response and lack of antibody affinity maturation after vaccination [162]. In addition, a large portion of vaccine-induced antibodies were directed toward nonprotective epitopes because formalin treatment had altered epitopes that induce functional antibodies (neutralizing and fusion inhibitory) that are presumed to be necessary for protection [163].

A **formalin-inactivated measles** vaccine was licensed in 1963 and unfortunately had a similar, though less severe, outcome.
The vaccine induced neutralizing antibodies; however, immunity waned rapidly and recipients regained susceptibility to measles.
Once contracted, a more severe and atypical measles disease developed [164].
As with RSV, the enhanced disease was associated with a lack of cytolytic T-lymphocyte response and low avidity antibodies [165], related to formaldehyde-induced alteration of measles F protein. [166].

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[161] Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE.
An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine.
Am J Epidemiol. 1969 Apr;89(4):405-21. doi: 10.1093/oxfordjournals.aje.a120954.
https://pubmed.ncbi.nlm.nih.gov/4305197/

Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH.
Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine.
Am J Epidemiol. 1969 Apr;89(4):422-34. doi: 10.1093/oxfordjournals.aje.a120955.
https://pubmed.ncbi.nlm.nih.gov/4305198/

[162] Delgado MF, Coviello S, Monsalvo AC, et al.
Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease.
Nat Med. 2009;15(1):34-41. doi:10.1038/nm.1894
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2987729/

Johnson TR, Graham BS.
Contribution of respiratory syncytial virus G antigenicity to vaccine-enhanced illness and the implications for severe disease during primary respiratory syncytial virus infection.
Pediatr Infect Dis J. 2004 Jan;23(1 Suppl):S46-57. doi: 10.1097/01.inf.0000108192.94692.d2.
https://pubmed.ncbi.nlm.nih.gov/14730270/

[163] Murphy BR, Walsh EE.
Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity.
J Clin Microbiol. 1988;26(8):1595-1597. doi:10.1128/JCM.26.8.1595-1597.1988
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC266671/pdf/jcm00080-0171.pdf

Murphy BR, Prince GA, Walsh EE, et al.
Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine.
J Clin Microbiol. 1986;24(2):197-202. doi:10.1128/JCM.24.2.197-202.1986
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC268874/
[164] Griffin DE, Pan CH.
Measles: old vaccines, new vaccines.
Curr Top Microbiol Immunol. 2009;330:191-212. doi: 10.1007/978-3-540-70617-5_10.
https://pubmed.ncbi.nlm.nih.gov/19203111/

[165] Polack FP, Auwaerter PG, Lee SH, Nousari HC, Valsamakis A, Leiferman KM, Diwan A, Adams RJ, Griffin DE.
Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody.
Nat Med. 1999 Jun;5(6):629-34. doi: 10.1038/9473.
https://pubmed.ncbi.nlm.nih.gov/10371500/

[166] Annunziato D, Kaplan MH, Hall WW, Ichinose H, Lin JH, Balsam D, Paladino VS.
Atypical measles syndrome: pathologic and serologic findings.
Pediatrics. 1982 Aug;70(2):203-9.
https://pubmed.ncbi.nlm.nih.gov/7099785/

It has been suggested that carbonyl groups on vaccine antigens introduced by formaldehyde treatment induce profound effects on immunogenicity, which may tip the balance between protection and adverse effects or enhanced disease [167].

These unfortunate events serve as a warning to all vaccine developers; inactivation of a pathogen does not necessarily result in a vaccine that by definition elicits protective immunity, and the viral epitopes necessary for the induction of protective immunity should be preserved after inactivation.

To achieve such a high level of safety, analyses of virus inactivation are critical to the production of an inactivated vaccine.
Inactivation kinetics (KOI) must be fully acquired, and to ascertain the completeness of inactivation, the effective inactivation assay must be validated and well characterized with respect to sensitivity and robustness.
KOI will differ by pathogen and inactivation method, so to ensure the safety of the inactivated vaccine mass, the inactivation process should be extensively studied, and observation of reproducible KOI is essential.
Quantification of viral infectivity in bulk vaccine or in-process intermediates is usually achieved by an in vitro assay based on cell cultures, however, this can also be done in vivo.

Generally, the cell line used to propagate the virus or an alternative cell line shown to be equally susceptible is inoculated with the (inactivated) virus sample to amplify any potential infectious units present.
The presence or absence of viruses in in vitro cultures can be detected by various methods; for lytic viruses this is made possible by cytopathic effect monitoring (CPE), for nonlytic viruses methods based on genome amplification (PCR) or antigen detection (immunofluorescence or ELISA) can be used.
In addition, a second step involving inoculation of amplified material into an appropriate in vivo model followed by monitoring the onset of disease symptoms can be performed.

Since the test for effective inactivation depends on the sensitivity of the assay, sample volume, and the absence of interference from inactivated particles, the assay used to confirm **completeness of inactivation (COI)** must be fine-tuned for assay sensitivity and matrix effects, particularly with the use of positive controls comprising samples fortified with a known concentration of virus to confirm cell culture susceptibility, while negative matrix controls are used to ensure that no other components in the formulation induce cell death or interfere with assay sensitivity.

When making an inference about COI, two variables come into play. The first concerns the sensitivity of the COI test, which must be characterized to assign a minimum number of infectious units (i.e., lower limit of detection) detected using the test. The second relates to sampling size; the larger the test sample volume, the greater the chance of detecting a potential infectious unit in the entire batch.
The combination of these two variables allows the manufacturer to specify a maximum tolerable level of output infectivity [168] or to adhere to a predefined criterion.

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[167] Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, Openshaw PJ.
A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines.
Nat Med. 2006 Aug;12(8):905-7. doi: 10.1038/nm1456. Epub 2006 Jul 23.
https://spiral.imperial.ac.uk/handle/10044/1/21962

[168] Cornfield J, Halperin M, Moore F.
Some statistical aspects of safety testing the Salk polyomyelitis vaccine. Public Health Rep. 1956;71(10):1045-1056. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2031000/pdf/pubhealthreporig00154-0095.pdf

Annex 5 Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed)
https://www.who.int/biologicals/vaccines/Tetanus_Recommendations_TRS_980_Annex_5.pdf

Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines

The World Health Organization and the European Pharmacopoeia (Ph. Eur.) have established guidelines, for each type of inactivated vaccine antigen, [169] for testing effective 'inactivation with a minimum sample size, expressed in volume or number of doses, to be tested.
In addition, cell types, incubation duration, and dilution of the vaccine sample before inoculation are parameters that can affect the sensitivity of COI and therefore must be carefully optimized. [170]

Once inactivated, the viral mass is further purified to remove contaminants; this can be achieved using various techniques such as ultrafiltration, size exclusion chromatography (SEC), and sucrose gradient centrifugation.
In addition, tests are needed to assess the purity of the vaccine product, such as tests for the absence of contaminants from the manufacturing process such as host cell proteins and DNA.

In addition, to ensure that changes in epitopes do not occur during inactivation, as has been observed in the past with RSV and measles, the immunogenic potency of the inactivated viral particle needs to be measured.
This can be achieved by measuring the immune response before and after immunization in vivo, through, for example, the rat potency assay, which is used to measure poliovirus-neutralizing antibodies after immunization of rats with an inactivated virus vaccine [171].

The in vivo immunogenicity test can also be related to in vitro cell-based potency tests; such as the D-antigen ELISA test, which quantifies the antigenic content of inactivated poliovirus particles and is consequently used for vaccine assay [172].
Of course, the development of such assays requires knowledge of the neutralizing epitopes necessary for adequate immune response and, hence, protection.

Not only do inactivated vaccines possess a higher safety profile than live vaccines, but they are also generally less reactogenic, relatively simple and technically feasible to produce with fewer regulatory hurdles for licensing [173].

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https://apps.who.int/iris/rest/bitstreams/284404/retrieve

Thaysen-Andersen M, Jørgensen SB, Wilhelmsen ES, Petersen JW, Højrup P. nvestigation of the detoxification mechanism of formaldehyde-treated tetanus toxin.
Vaccine. 2007 Mar 8;25(12):2213-27. doi: 10.1016/j.vaccine.2006.12.033. Epub 2007 Jan 2.
https://pubmed.ncbi.nlm.nih.gov/17240009/

[169] Sanders B, Koldijk M, Schuitemaker H.
Inactivated Viral Vaccines.
Vaccine Analysis: Strategies, Principles, and Control. 2014;45-80. Published 2014 Nov 28. doi:10.1007/978-3-662-45024-6_2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7189890/

[170] Markey K, Asokanathan C, Feavers I.
Assays for Determining Pertussis Toxin Activity in Acellular Pertussis Vaccines.
Toxins (Basel). 2019;11(7):417. Published 2019 Jul 17. doi:10.3390/toxins11070417
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6669641/

Yuen CT, Asokanathan C, Cook S, Lin N, Xing D.
Effect of different detoxification procedures on the residual pertussis toxin activities in vaccines.
Vaccine. 2016 Apr 19;34(18):2129-34. doi: 10.1016/j.vaccine.2016.03.007. Epub 2016 Mar 11.
https://pubmed.ncbi.nlm.nih.gov/26973066/

[171] van Steenis G, van Wezel AL, Sekhuis VM.
Potency testing of killed polio vaccine in rats.
Dev Biol Stand. 1981;47:119-28. PMID: 6262142.
https://pubmed.ncbi.nlm.nih.gov/6262142/

[172] Beale AJ.
The D-antigen content in poliovaccine as a measure of potency.
Lancet. 1961 Nov 25;2(7213):1166-8. doi: 10.1016/s0140-6736(61)90843-1
https://pubmed.ncbi.nlm.nih.gov/13866114/

[173] Zepp F.
Principles of vaccine design-Lessons from nature.
Vaccine. 2010 Aug 31;28 Suppl 3:C14-24. doi: 10.1016/j.vaccine.2010.07.020.

However, inactivated vaccines are typically associated with lower immunogenicity, which may imply the need for multiple doses or the addition of adjuvants that consequently raise the price of the vaccine.

Thus, the choice of an inactivated vaccine approach is generally a trade-off between increased safety (if inactivation is obviously complete) and a fast path to regulatory approval, and the risk of reduced antigenicity of the immunogen that often requires the addition of adjuvants and/or multiple doses, which not only increase production costs but also increase the complexity of formulation and administration.

SARS-COV-2 INACTIVATED VACCINE

An example of inactivated vaccine production against SARS-Cov-2 is that reported by Wang et al. of the Beijing Institute of Biological Products Company, from three strains of SARS-CoV-2 isolated from hospitalized patients, of which the HBO2 strain showed optimal replication and had the highest virus yield when cultured in vitro in Vero cells.

HBO2 was passaged ten times in Vero cells to induce adaptation to growth in culture; at the 10th passage it was sequenced in depth and showed 99.95 percent homology to the complete amino acid sequence and 100 percent homology to the S protein from the 7th passage, indicating that the virus had adapted and achieved a stable genetic sequence, making it suitable for production in culture. This strain was then mass-produced in Vero cells and inactivated by the addition of β- propionolactone. The resulting inactivated virus was finally mixed with aluminum hydroxide before administration. [174]

Other examples of inactivated vaccines whose preclinical studies are reported are PiCoVacc/CoronaVac (SARS-CoV-2 strains were isolated from bronchoalveolar lavage fluid (BALF) samples of 11 hospitalized patients (including 5 ICU patients), including 5 from China, 3 from Italy, 1 from Switzerland, 1 from the UK, and 1 from Spain) [175] and the BBiBp-CorV .[176]

All these strains were isolated from Vero cells, which have been certified by WHO for vaccine production. [177] Vero cells were infected through patients' pharyngeal swabs to prevent possible mutations during viral culture and isolation.

It is reported in the literature that inactivated SARS-CoV vaccine increases the pro-inflammatory eosinophilic pulmonary response upon reinfection (challenge test) [178], therefore, there is a possibility that the

https://pubmed.ncbi.nlm.nih.gov/20713252/

[174] Wang H, Zhang Y, Huang B, et al.
Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2.
Cell. 2020;182(3):713-721.e9. doi:10.1016/j.cell.2020.06.008
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7275151/

[175] Gao Q, Bao L, Mao H, et al.
Development of an inactivated vaccine candidate for SARS-CoV-2.
Science. 2020;369(6499):77-81. doi:10.1126/science.abc1932
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7202686/

[176] Wang H, Zhang Y, Huang B, et al.
Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2.
Cell. 2020;182(3):713-721.e9. doi:10.1016/j.cell.2020.06.008
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7275151/

[177] https://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO_TRS_878_A1Animalcells.pdf

[178] Bolles M, Deming D, Long K, et al.
A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge.
J Virol. 2011;85(23):12201-12215. doi:10.1128/JVI.06048-11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3209347/

Tseng CT, Sbrana E, Iwata-Yoshikawa N, et al.
Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus

vaccines against SARS-CoV-2 may cause similar vaccine-associated immunopathology, as will be discussed in more detail in the section on the mechanism of COVID-19 vaccine adverse reactions (part two). [179]

Studies have shown that vaccination with SARS-CoV protein N potentiates immunopathological changes in the lungs, [180] and that the induction of protein-specific T lymphocytes for SARS- CoV protein N and altered Th2 cytokine profile may be the cause of the adverse reactions. [181]

[published correction appears in PLoS One. 2012;7(8). doi:10.1371/annotation/2965cfae-b77d-4014-8b7b-236e01a35492]. PLoS One. 2012;7(4):e35421. doi:10.1371/journal.pone.0035421
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3335060/

[179] Simon HU, Karaulov AV, Bachmann MF.
Strategies to Prevent SARS-CoV-2-Mediated Eosinophilic Disease in Association with COVID-19 Vaccination and Infection.
Int Arch Allergy Immunol. 2020;181(8):624-628. doi: 10.1159/000509368. Epub 2020 Jun 16.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7360494/

[180] Yasui F, Kai C, et al.
Prior immunization with severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV.
J Immunol. 2008 Nov 1;181(9):6337-48. doi: 10.4049/jimmunol.181.9.6337.
https://www.jimmunol.org/content/jimmunol/181/9/6337.full.pdf

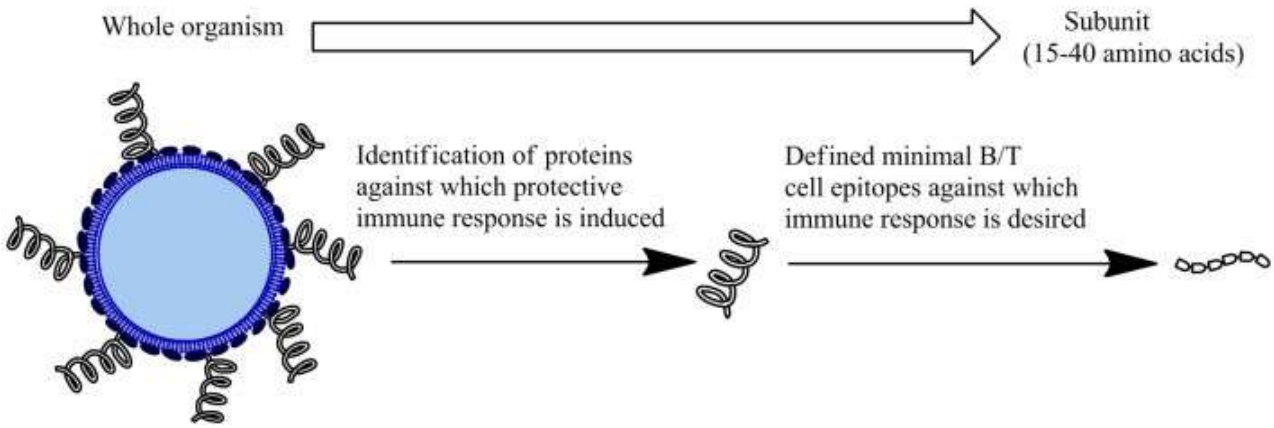
[181] Deming D, et al
Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants.
PLoS Med. 2006 Dec;3(12):e525. doi: 10.1371/journal.pmed.0030525. Erratum in: PLoS Med. 2007 Feb;4(2):e80.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1716185/.

Iwata-Yoshikawa N, Uda A, Suzuki T, et al.
Effects of Toll-like receptor stimulation on eosinophilic infiltration in lungs of BALB/c mice immunized with UV-inactivated severe acute respiratory syndrome-related coronavirus vaccine.
J Virol. 2014;88(15):8597-8614. doi:10.1128/JVI.00983-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4135953/

PROTEIN SUBUNIT VACCINES

oSARS-CoV-2 rS/Matrix M1-Adjuvant (Full length recombinant SARS CoV-2 glycoprotein nanoparticle vaccine adjuvanted with Matrix M) - NVX-CoV2373 / *Novavax*
oRecombinant SARS-CoV-2 vaccine (CHO Cell) / *Anhui Zhifei Longcom Biopharmaceutical + Institute of Microbiology, Chinese Academy of Sciences*
oKBP-COVID-19 (RBD-based) / *Kentucky Bioprocessing Inc.*
oSARS-CoV-2 vaccine formulation 1 with adjuvant 1 (S protein (baculovirus production) / *Sanofi Pasteur + GSK*
oSCB-2019 + AS03 or CpG 1018 adjuvant plus Alum adjuvant (Native like Trimeric subunit Spike Protein vaccine) / *Clover Biopharmaceuticals Inc./GSK/Dynavax*
oCOVID19 vaccine/ *Vaxine Pty Ltd. + Medytox*
oMF59 adjuvanted SARS-CoV-2 Sclamp vaccine / CSL Ltd. + Seqirus + University of Queensland
oMVC-COV1901 (S-2P protein + CpG 1018) / *Medigen Vaccine Biologics + Dynavax + National Institute of Allergy and Infectious Diseases (NIAID)*
oFINLAY-FR anti-SARS-CoV-2 Vaccine (RBD + adjuvant) / *Instituto Finlay de Vacunas*
oEpiVacCorona (EpiVacCorona vaccine based on peptide antigens for the prevention of COVID-19) / *Federal Budgetary Research Institution State Research Center of Virology and Biotechnology "Vector"*
oRBD (baculovirus production expressed in Sf9 cells) Recombinant SARS-CoV-2 vaccine (Sf9 Cell) / *West China Hospital + Sichuan University*
oIMP CoVac-1 (SARS-CoV-2 HLA-DR peptides) / *University Hospital Tuebingen*
oUB-612 (Multitope peptide based S1-RBD-protein based vaccine) / *COVAXX + United Biomedical Inc*
oAdimrSC-2f (recombinant RBD +/- Aluminium) / *Adimmune Corporation*
oCIGB-669 (RBD+AgnHB) / *Center for Genetic Engineering and Biotechnology (CIGB)*
oCIGB-66 (RBD+aluminium hydroxide) / *Center for Genetic Engineering and Biotechnology (CIGB)*
oBECOV2 / *Biological ELimited*
oRecombinant Sars-CoV-2 Spike protein, Aluminum adjuvanted / *Nanogen Pharmaceutical Biotechnology*
oRecombinant protein vaccine S-268019 (using Baculovirus expression vector system) / *Shionogi*

Rather than injecting a whole pathogen to trigger an immune response, subunit vaccines (sometimes called acellular vaccines) contain purified pieces of it that have been specially selected for their ability to stimulate immune cells. [182]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3793171/
Subunit vaccines. An alternative to inactivated or attenuated whole pathogen vaccines. Composed of fragment(s) of the microorganism to generate a protective immune response.

[182] Zaman M, Toth I.
Immunostimulation by synthetic lipopeptide-based vaccine candidates: structure-activity relationships.
Front Immunol. 2013;4:318. Published 2013 Oct 9. doi:10.3389/fimmu.2013.00318
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3793171/

Because these fragments are not capable of causing disease, subunit vaccines are considered very safe. There are several types:

- protein subunit vaccines contain specific proteins isolated from viral or bacterial pathogens;
- polysaccharide vaccines contain chains of sugar molecules (polysaccharides) found in the cell walls of some bacteria;
- subunit conjugate vaccines bind a polysaccharide chain to a carrier protein to enhance the immune response.

Subunit vaccines that are already widely used include the hepatitis B and acellular pertussis vaccines (protein subunit), the pneumococcal polysaccharide vaccine (polysaccharide), and the MenACWY vaccine, which contains polysaccharides from the surface of four types of bacteria that cause meningococcal disease combined with diphtheria or tetanus toxoid (conjugated subunit).

Only protein subunit vaccines have been developed against the virus that causes COVID-19.

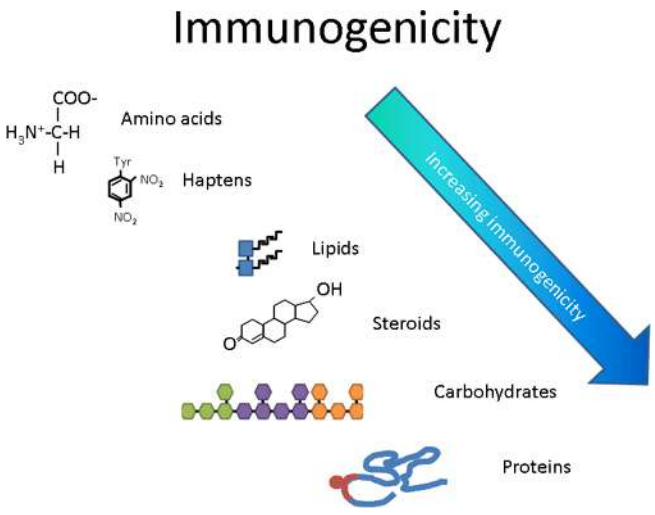
Peptide subunit vaccines are generally composed of 30-60 amino acids representing the antigen(s) of interest (immunogenic peptide sequences). [183]

IN-DEPTH STUDY: THE ANTIGEN-ANTIBODY INTERACTION

IMMUNODOMINANCE [184]

Any substance capable of eliciting an immune response is called **immunogenic**, and is called **immunogenic**.
Antigen is that substance capable of binding to a specific antibody (or to a T lymphocyte): all antigens have the potential to stimulate the production of specific antibodies, but only a few are able to actually do so, because most behave as **haptens**, that is, they succeed only when bound to a molecule (**carrier**) that makes them immunogenic.

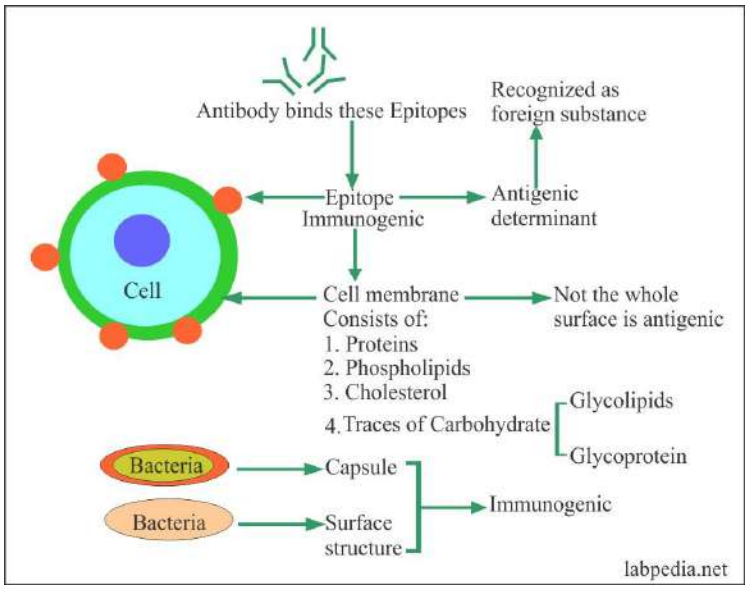
Thus, a molecule can be antigenic (i.e., it can react with products or components of the immune system) but not be immunogenic (i.e., not be able by itself to induce an immune response). It follows that all immunogens are antigens, but not all antigens are immunogens.



[183] Rueckert C, Guzmán CA.
Vaccines: from empirical development to rational design.
PLoS Pathog. 2012;8(11):e1003001. doi:10.1371/journal.ppat.1003001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3493475/

[184] https://didattica-2000.archived.uniroma2.it/immunology/deposito/Antigeni.pdf

https://www.labpedia.net/elementary-immunology/chapter-3-immunogen-and-antigen/



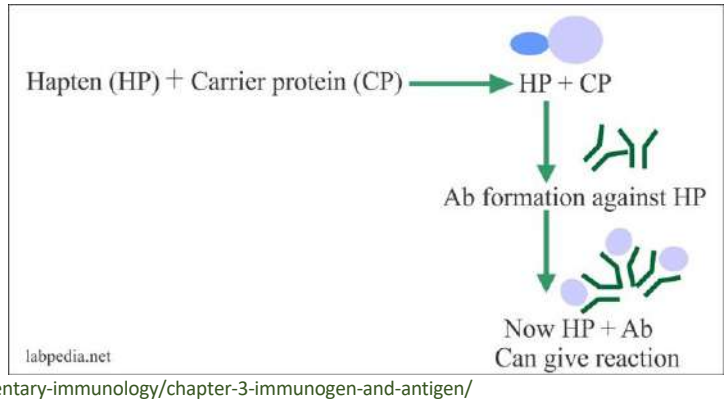
https://www.labpedia.net/elementary-immunology/chapter-3-immunogen-and-antigen/
https://www.microbiologybook.org/mayer/antigens2000.htm

EPITHOPUS or ANTIGENIC DETERMINANT.

Portion of an antigen that contacts the binding site of an antibody or the antigen receptor of T cells. Epitopes are thus the most important portions of the antigen, capable of evoking the immune response.

APTENE

Low-molecular-weight molecule (< 4 kD) capable of acting as an epitope but which itself is not capable of evoking an antibody response. As already seen, haptene is an antigenic but not immunogenic molecule unless it is bound to a carrier; it induces an immune response only if the immune system has previously come in contact with the haptene-carrier complex.



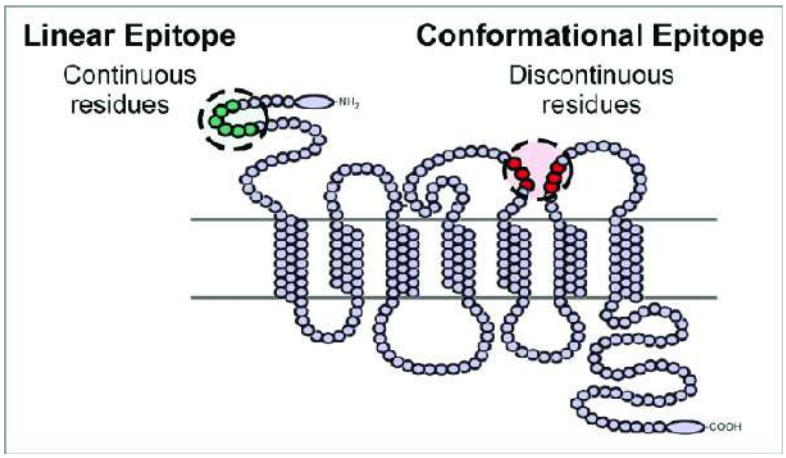
https://www.labpedia.net/elementary-immunology/chapter-3-immunogen-and-antigen/

Antigenic determinants possess some residues that are more important than others (**immune-dominant epitopes**).
An antigen normally possesses more than one epitope, and the response it will induce will be polyclonal, that is, multiple lymphocyte clones will be activated.

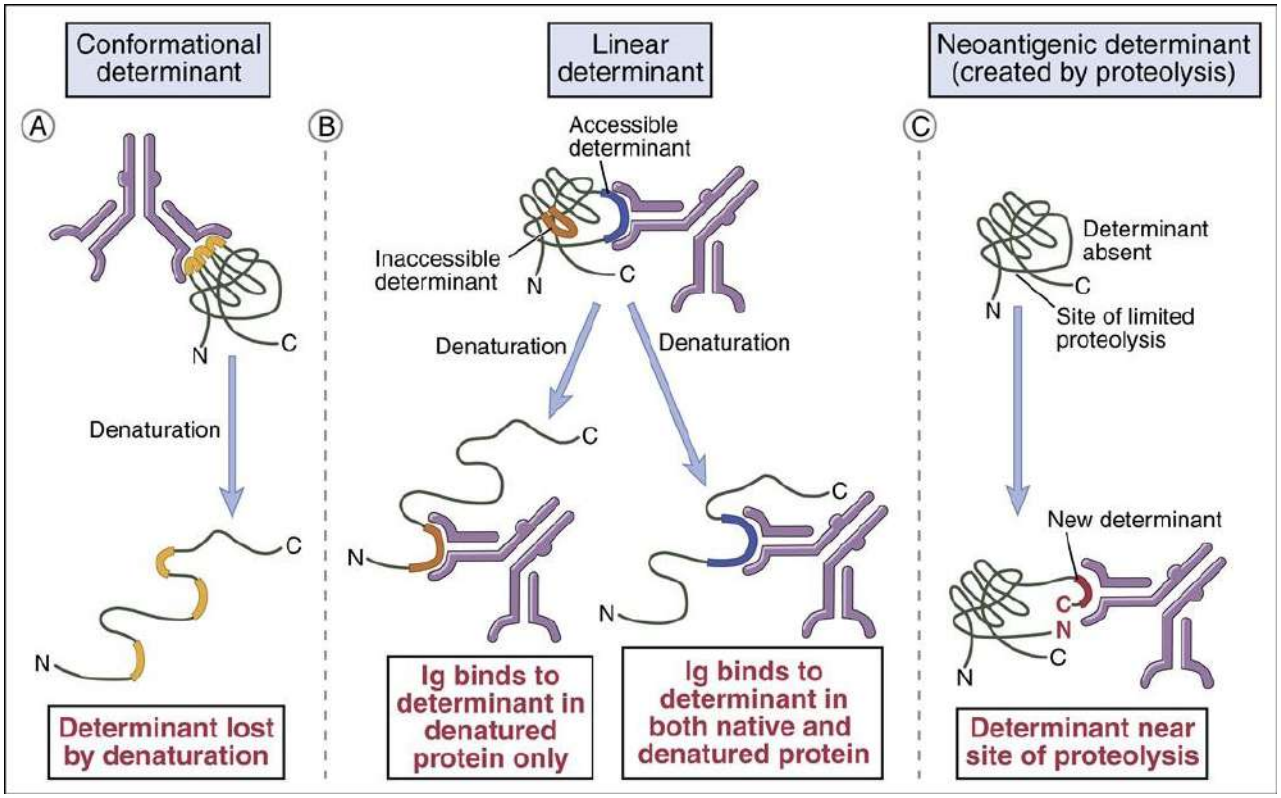
There are, however, some antigenic molecules that possess one or a few immunodominant epitopes, and are thus capable of inducing a monoclonal (if only one clone is activated) or oligoclonal (when only a few clones are activated) response. Within an antigenic molecule there may be a hierarchy in the immunodominance of epitopes, resulting in preferential activation of some clones compared to others.

These types of **antigenic determinants** are called **continuous (or linear)** because they are given by amino acids arranged linearly, that is, one after the other in the primary sequence of the protein.

There are, however, other types of **determinants, the** existence of which has been demonstrated using lysozyme molecules, called **discontinuous (or conformational)** formed by amino acids that are discontinuous in the primary sequence but become contiguous in the tertiary structure because they are brought together by the three-dimensional folding of the protein. (e.g., 2 regions linked by disulfide bridges) [185]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5825199/
Linear and conformational epitopes. Linear epitopes consist of residues that are continuous on a protein sequence. Conformational epitopes consist of residues that are discontinuous on the protein sequence but are in close proximity to form an antigenic surface on the three-dimensional structure of the protein. Conformational epitopes are less obvious and more patent than linear epitopes.



https://oncohemakey.com/immunogenicity-and-antigenicity/

[185] Immunogenicity and Antigenicity
https://oncohemakey.com/immunogenicity-and-antigenicity/

Neil S. Greenspan, Lisa A. Cavacini,
15 - Immunoglobulin Function,
Editor(s): Robert R. Rich, Thomas A. Fleisher, William T. Shearer, Harry W. Schroeder, Anthony J. Frew, Cornelia M. Weyand, Clinical Immunology (Fifth Edition), Elsevier, 2019, Pages 223-233.e1, ISBN 9780702068966,
https://doi.org/10.1016/B978-0-7020-6896-6.00015-6.
https://www.sciencedirect.com/science/article/pii/B9780702068966000156

The nature of antigenic determinants. Antigenic determinants (shown in orange, red and blue) may depend on protein folding (conformation) and primary structure. Some determinants are accessible in native proteins and are lost during denaturation (A), while others are exposed only during protein unfolding (B). Neodeterminants arise from postsynthetic modifications such as peptide bond cleavage (C). (From Abbas AK, Lichtman AH, Pillai S: Cellular and molecular immunology, ed 6 [updated edition], Philadelphia, 2010, Saunders.)

Linear epitopes, in particular, are recognized by both T and B lymphocytes, while conformational epitopes are recognized only by B lymphocytes.

During somatic hypermutation, higher affinity antibodies are those directed against dominant epitopes (immune-dominant epitopes) while less affinity antibodies against more hidden epitopes.
In the course of an immune response, therefore, there is a kind of "Darwinian selection" among the most important epitopes, toward which more related antibodies are produced.

On the basis of this evidence, the accepted theory is that immune-dominant epitopes are found on hydrophilic portions of antigens (because they are more easily reached by antibodies) rather than hydrophobic, and that these epitopes are the ones that antibodies are most likely to react against, even in different individuals, and therefore are usually used in the preparation of vaccines.

IMMUNOGENICITY AND IMMUNOTOLERANCE: THE HYDROPHOBICITY PARADIGM

The hypothesis that immunotolerance may result from a process of negative selection of self-reactive lymphocytes has recently been questioned [186].
In fact, immunoproteomic analyses show that almost all epitopes that have been experimentally validated as immunoreactive in the human host and are derived from pathogens such as hepatitis C virus (HCV), Epstein-Barr virus, and human papillomavirus, are composed of amino acid (aa) sequences shared with human proteins [[187] and further references therein].

The peptide sharing between viral epitopes and human proteins documents that deletion of autoreactive lymphocytes does not occur and, beyond the pathological implications in terms of cross-reactivity and consequent tumors and autoimmune diseases, reintroduces the debated question of what immunotolerance is and what are the molecular mechanisms that make an antigen tolerated (or,

Thus, the observation that immunotolerance toward an antigen can be defined by the frequency of the Peptide determinants of the antigen [188] is relevant.

[186] Cohn M, Mitchison NA, Paul WE, Silverstein AM, Talmage DW, Weigert M.
Reflections on the clonal-selection theory.
Nat Rev Immunol. 2007 Oct;7(10):823-30. doi: 10.1038/nri2177.
https://pubmed.ncbi.nlm.nih.gov/17893695/

Rose NR.
Negative selection, epitope mimicry and autoimmunity.
Curr Opin Immunol. 2017 Dec;49:51-55. doi: 10.1016/j.coi.2017.08.014. Epub 2017 Nov 3.
https://pubmed.ncbi.nlm.nih.gov/29102863/

[187] Kanduc D.
From hepatitis C virus immunoproteomics to rheumatology via cross-reactivity in one table. Curr Opin Rheumatol. 2019 Sep;31(5):488-492. doi: 10.1097/BOR.000000000606.
https://pubmed.ncbi.nlm.nih.gov/31356379/

Kanduc D, Shoenfeld Y. Human Papillomavirus Epitope Mimicry and Autoimmunity: The Molecular Truth of Peptide Sharing. Pathobiology. 2019;86(5-6):285-295. doi: 10.1159/000502889. Epub 2019 Oct 8.
https://pubmed.ncbi.nlm.nih.gov/31593963/

Kanduc D, Shoenfeld Y.
From Anti-EBV Immune Responses to the EBV Diseasome via Cross-reactivity.
Glob Med Genet. 2020 Aug;7(2):51-63. doi: 10.1055/s-0040-1715641. Epub 2020 Aug 31.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7490125/

[188] Kanduc D.
Immunogenicity, Immunopathogenicity, and Immunotolerance in One Graph.
Anticancer Agents Med Chem. 2015;15(10):1264-8. doi: 10.2174/1871520615666150716105543.

This concept stems from the fact that frequent peptide sequences are mostly unable to evoke an immune response, whereas rare peptide sequences are specifically targeted by immune responses and appear to be associated with immunogenicity.
In addition, from the physicochemical point of view, specific properties such as hydrophilicity and hydrophobicity have been shown to characterize tolerated frequent peptide sequences and immunogenic rare peptide, respectively.[189]

In the recent publication "*Hydrophobicity and the Physico-Chemical Basis of Immunotolerance*" [190] Dr. Kanduc suggests that the question of self versus non-self recognition (i.e., immunotolerance vs. immunogenicity) finds a logical scientific explanation in the **hydrophobicity paradigm**.

In particular, this study points to hydrophobicity as the primary motive for the generation of immune responses.

In fact, it was found that hydrophobicity:

(1) *Characterizes rare/never occurring immunogenic pentapeptide determinants*.
Antibody generation begins with a weak electrostatic interaction between the membrane-bound BCR and the antigen [191]. The weak primary interaction may decay or, alternatively, evolve into a stronger secondary interaction that triggers a cascade of intracellular B-cell signaling and a succession of events (i.e., antigen internalization and presentation to helper T cells, VDJ recombination, somatic hypermutation, and isotype change by recombination [192]) leading to the generation of high-affinity antibodies. [193].

Specifically, 2 factors govern the first BCR-antigen encounter and its evolution into productive binding:

(1) *the small size of the contact surface between BCR and antigen* (analysis of the binding energy of antigen-antibody complexes, site-directed mutagenesis, and epitope mapping experiments have conclusively validated **pentapeptide as the minimum unit of measurement of immune recognition** [194] . In parallel, the maximum size of polysaccharide epitopes was also found to be close to that of penta- or hexasaccharides).

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[189] Capone G, Novello G, Fasano C, et al.
The oligodeoxynucleotide sequences corresponding to never-expressed peptide motifs are mainly located in the non-coding strand.
BMC Bioinformatics. 2010;11:383. Published 2010 Jul 20. doi:10.1186/1471-2105-11-383
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919516/

[190] Kanduc D.
Hydrophobicity and the Physico-Chemical Basis of Immunotolerance.
Pathobiology. 2020;87(4):268-276. doi: 10.1159/000508903. Epub 2020 Jul 29.
https://pubmed.ncbi.nlm.nih.gov/32726789/

[191] van Oss CJ.
Nature of specific ligand-receptor bonds, in particular the antigen-antibody bond. J Immunoassay. 2000 May-Aug;21(2-3):109-42. doi: 10.1080/01971520009349531.
https://pubmed.ncbi.nlm.nih.gov/10929884/

Van Oss CJ.
Hydrophobic, hydrophilic and other interactions in epitope-paratope binding.
Mol Immunol. 1995 Feb;32(3):199-211. doi: 10.1016/0161-5890(94)00124-j.
https://pubmed.ncbi.nlm.nih.gov/7534869/
[192] http://atlasgeneticsoncology.org/Educ/PolyIGID30013IS.html

[193] Kwak K, Akkaya M, Pierce SK.
B cell signaling in context.
Nat Immunol. 2019 Aug;20(8):963-969. doi: 10.1038/s41590-019-0427-9. Epub 2019 Jul 8.
https://pubmed.ncbi.nlm.nih.gov/31285625/

[194] Reddehase MJ, Rothbard JB, Koszinowski UH.
A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes.
Nature. 1989 Feb 16;337(6208):651-3. doi: 10.1038/337651a0.
https://core.ac.uk/reader/12167563?utm_source=linkout

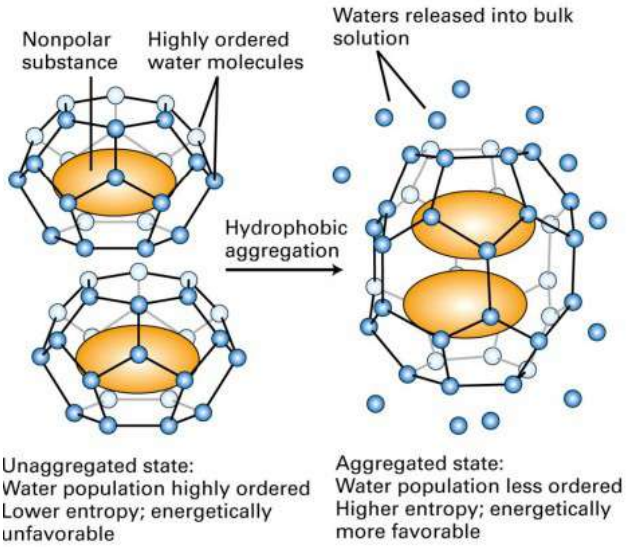
(2) *the weakness of the primary interaction between BCR and antigen*. The human proteome consists of ~2.4 million pentapeptides, about half a million of which occur only once, while the remaining
recur repeatedly in human proteins even hundreds of times. Including multiple occurrences, the human proteome consists of ~16 million pentapeptides [195].

Such pentapeptide redundancy further reduces the likelihood of a primary BCR-pentapeptide interaction proceeding to a stronger productive secondary interaction. Pentapeptide crowding on the BCR is obviously greater for frequent pentapeptides, so the greater the frequency of a pentapeptide antigen, the lower the chance of durable and productive binding to the BCR. Immunologically, the end result will be BCR nonreactivity, that is, a state of anergy or immunotolerance.

(2) is the chemical and physical factor by which the apolar van der Waals force of attraction can transform the random, weak and nonspecific primary electrostatic interaction into a stronger and more specific secondary interaction.

Although the apolar van der Waals attractive force is weak (~0.5 to 1 kcal/mol) [196], it can overcome the local hydration force and cause the expulsion of water molecules from the surface contact between the apolar entities (Fig. below) because the expulsion of water is accompanied by an increase in entropy (i.e., it is an energetically favored process) due to the transition of water molecules from an ordered to a random disordered state.

Extrusion of water from the BCR-pentapeptide surface interaction brings t h e BCR and pentapeptide antigen closer together, thereby strengthening the primary electrostatic interaction.
This leads to the assumption that, in general, water molecules at the BCR-pentapeptide interface will be ejected to an extent and with a strength proportional to the hydrophobicity and rarity of the pentapeptide antigen.



http://lbam.pwr.edu.pl/FILES/Lecture_12_Biophysics_Hydrophobic_effect.pdf
The hydrophobic effect. Van der Waals attractive force leads to aggregation of apolar groups and extrusion of water molecules (gray dots) from the contact surface.

As a result, polar forces (or hydrogen bonds) may intervene and help strengthen the primary interaction.

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[195] Capone G, De Marinis A, Simone S, Kusalik A, Kanduc D.
Mapping the human proteome for non-redundant peptide islands.
Amino Acids. 2008 Jun;35(1):209-16. doi: 10.1007/s00726-007-0563-7. Epub 2007 Aug 15.
https://pubmed.ncbi.nlm.nih.gov/17701099/

196 Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: WH Freeman. Section 1.3, Chemical Bonds in Biochemistry, 2002.

Hydrogen bonds are due to the acid-base interaction between a hydroxyl group and another polar molecule and are weak, with energies between 1 and 3 kcal/mol and, unlike electrostatic forces, have a length of ~0.2 nm.

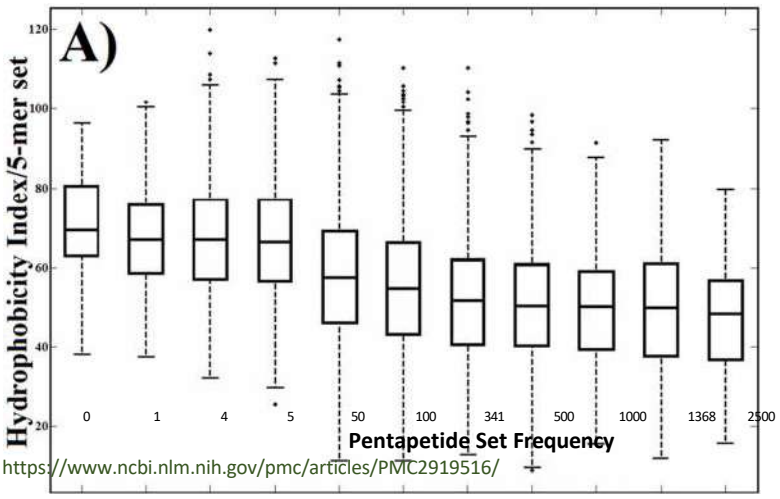
In practice, the hydrogen bond cannot exert its attraction at distances that exceed 0.2 nm and cannot play a major role in primary BCR-antigen pentapeptide interactions, but when BCR and antigen pentapeptide approach each other under the effect of van der Waals attraction force, the neighboring chains of BCR and antigen pentapeptide also approach each other.
Under these circumstances, direct hydrogen bonds may contribute to the formation of secondary bonds, either between BCR and antigen pentapeptide or between opposite chains in the vicinity of BCR and the antigen pentapeptide.

Thus, the proximity between BCR and antigen pentapeptide can lead to conformational changes that produce a final optimal stereochemical adaptation that is the precondition for the generation of specific high-energy binding between antibody and antigen.

(3) is the filter by which the terminal sequence of the germline BCR avoids frequent sequences and positively selects and binds rare/never hydrophobic pentapeptide determinants, thereby discriminating self from non-self.

Compared with frequent pentapeptides, immunogenic low/zero-frequency pentapeptides generally have higher levels of hydrophobicity, as shown in the following Figure [197].

In particular, hydrophobic methionine (M), phenylalanine (F), tryptophan (W), and tyrosine (Y) are the main components of immunogenic, low/zero frequency antigenic pentapeptides, while, in contrast, non-immunogenic, frequent pentapeptides are composed mainly of hydrophilic amino acid residues.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919516/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919516/
Statistical characterization of hydrophobicity with different frequencies in the universal proteome.
The boxplots show the distribution of values of each physical-biochemical factor for each set of pentapeptides. The line within each box represents the median value. The top and bottom of each box represent the 75th and 25th percentiles, respectively. The whiskers show the range of values that are not considered outliers.
Outliers are plotted individually as plus signs.
The p-values among the different classes of 5-mers for hydrophobicity were all less than 0.001, indicating in each case that the averages of the different sets are different.

Hydrophobicity outlines a scientific context for understanding protective immunity and autoimmunity and also provides a new research perspective for yet unexplained immunological phenomena such as the adjuvant effect of alum.

[197] Capone G, De Marinis A, Simone S, Kusalik A, Kanduc D.
Mapping the human proteome for non-redundant peptide islands. Amino Acids. 2008 Jun;35(1):209-16. doi: 10.1007/s00726-007-0563-7. Epub 2007 Aug 15.
https://pubmed.ncbi.nlm.nih.gov/17701099/

In fact, as early as 1997, Naim et al. [198], in describing the properties of alum, pointed out that cations can neutralize all or part of negatively charged compounds, causing such compounds to become more hydrophobic.
Therefore, it can be assumed that Al ions^{3+} of alum, at neutral pH, can have a hydrophobizing effect on negatively charged proteins, [199] with the immunogenic effects described above.

In conclusion, the affinity of the antibodies generated depends largely on the number and type of amino acids that form the CDR folds and determine the surface topography of the binding site [200].

The hydrophobicity paradigm may therefore represent a radical change in the way we study and use the immune system in cancer and infectious diseases, which remain the most crucial health problems for the human population.

Antibodies and autoimmunity

As already seen, the antigen binding region (paratope) and the antigenic determinant (epitope) are held together by noncovalent forces, which also determine the affinity of these antibodies [201].
This interaction is also orchestrated by complementarity in charge and shape of the paratope-epitope.[202]

In general, an antibody should discriminate between self-molecules (produced by the body) and exogenous foreign antigens (such as viruses and bacteria) to limit its function against external antigens and demonstrate the necessary tolerance to self-antigens.
The inability to distinguish between these interacting processes could explain several autoimmune diseases.

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[198] Naim JO, van Oss CJ, Wu W, Giese RF, Nickerson PA.
Mechanisms of adjuvancy: I--Metal oxides as adjuvants.
Vaccine. 1997 Aug;15(11):1183-93. doi: 10.1016/s0264-410x(97)00016-9.
https://pubmed.ncbi.nlm.nih.gov/9286042/

[199] al-Shakhshir RH, Regnier FE, White JL, Hem SL.
Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminum-containing adjuvants.
Vaccine. 1995 Jan;13(1):41-4. doi: 10.1016/0264-410x(95)80009-3.
https://pubmed.ncbi.nlm.nih.gov/7762276/

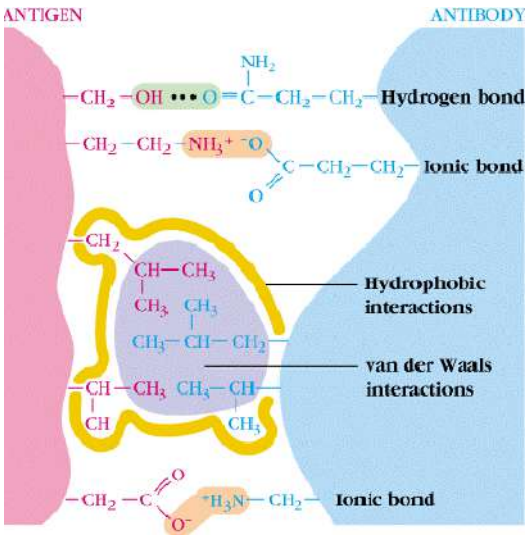
Subunit Vaccine Delivery
Camilla Foged
January 2015 Publisher: Springer ISBN: 978-1-4939-1417-3 Editor: Foged, C., Rades, Th., Perrie, Y., Hook, S

[200] Kaur H, Salunke DM.
Antibody promiscuity: Understanding the paradigm shift in antigen recognition.
IUBMB Life. 2015 Jul;67(7):498-505. doi: 10.1002/iub.1397. Epub 2015 Jul 15.
https://iubmb.onlinelibrary.wiley.com/doi/full/10.1002/iub.1397

[201] Van Oss CJ.
Hydrophobic, hydrophilic and other interactions in epitope-paratope binding.
Mol Immunol. 1995 Feb;32(3):199-211. doi: 10.1016/0161-5890(94)00124-j.
https://pubmed.ncbi.nlm.nih.gov/7534869/

[202] Al Qaraghuli, M.M., et al.
Defining the complementarities between antibodies and haptens to refine our understanding and aid the prediction of a successful binding interaction.
BMC Biotechnol 15, 99 (2015). https://doi.org/10.1186/s12896-015-0217-x
https://bmcbiotechnol.biomedcentral.com/articles/10.1186/s12896-015-0217-x

Kim, Su & Shin, Ki-Roo & Zhang, Byoung-Tak. (2004).
Molecular immunocomputing with application to alphabetical pattern recognition mimics the characterization of ABO blood type. 2003 Congress on Evolutionary Computation, CEC 2003 - Proceedings. 4. 2549 - 2556 Vol.4. 10.1109/CEC.2003.1299409.
https://www.researchgate.net/publication/4074726_Molecular_immunocomputing_with_application_to_alphabetical_pattern_recognition_mimics_the_characterization_of_ABO_blood_type



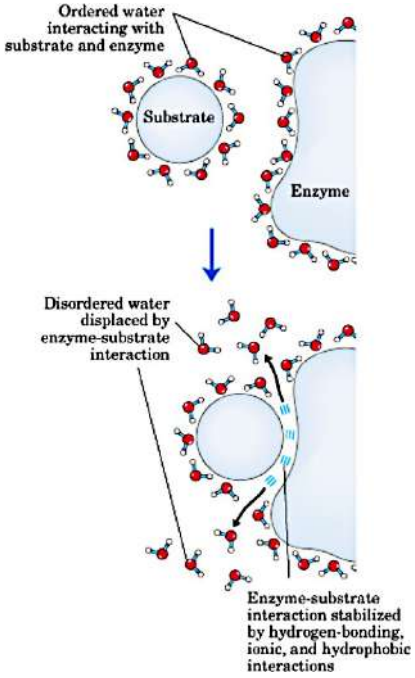


Fig. 1Fig . 2

Fig. 1
https://www.researchgate.net/publication/4074726_Molecular_immunocomputing_with_application_to_alphabetical_pattern_recognition_mimics_the_characterization_of_ABO_blood_typeThe noncovalent interactions that form the basis of antigen-antibody (Ag-Ab) binding
Fig.2 http://lbam.pwr.edu.pl/FILES/Lecture_12_Biophysics_Hydrophobic_effect.pdf

In her recent article "*On the Inexistence of a Negative Selection Process*" [203] Dr. Kanduc reiterates the following concepts:

- *the fact that pathogen-derived immunoreactive epitopes consist mainly of fragments of peptides common to human proteins is indisputable proof that "negative selection" of autoreactive lymphocytes does not exist.*

Abnormal peptide sharing between immunoreactive microbial epitopes and human proteins invalidates the current model of self-tolerance based on a process of negative selection whereby lymphocytes with specificity for sequences expressed in the host are deleted from the immunologic repertoire to avoid self-reactivity and subsequent autoimmunity.

- *As a corollary to the self-reactivity of the antimicrobial immune response, the unambiguous defensive role attributed to antibodies is also lost.*

Currently, antibodies are defined as the main defense against infection. In contrast, the massive sharing of peptides between human proteins and pathogen-derived immunoreactive epitopes indicates that self-reactivity and subsequent autoimmunity characterize the immune response to infection.
A prime example is offered by the symmetric correspondence linking EBV epitopes to EBV disease, from lymphomas to heart disease, through peptide sharing. [204]

- *In more or less serious forms, cross-reactivity and autoimmunity seem to be a constant consequence following infection or active immunization, thus conferring a pathogenic character to the immune response against infectious agents.*[205]

[203] Darja Kanduc
Immunobiology: On the Inexistence of a Negative Selection Process
Advanced Studies in Biology, Vol. 12, 2020, no. 1, 19 - 28 https://doi.org/10.12988/asb.2020.91221
http://www.m-hikari.com/asb/asb2020/asb1-2020/p/kanducASB1-2020.pdf

[204] Kanduc D, Shoenfeld Y.
From Anti-EBV Immune Responses to the EBV Diseasome via Cross-reactivity.
Glob Med Genet. 2020;7(2):51-63. doi:10.1055/s-0040-1715641
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7490125/

[205] Ray SK, Putterman C, Diamond B.
Pathogenic autoantibodies are routinely generated during the response to foreign antigen: a paradigm for autoimmune disease.

The potentially infinite antibody universe resulting from gene recombination and affinity maturation has led to the assumption that each individual's antibody repertoire is unique.
Similarly, the number of potential target epitopes on pathogen antigens has led to the assumption that the generation of specific anti-pathogen antibody patterns must follow each encounter with a foreign antigen.

- *Actually, the terms "infinite" and "universe" should be deleted, 5-6 aa residues being sufficient to delineate an antigenic immune determinant. This yields a finite number (between 20^5 and 20^6) of antigenic immune sequences and related antibodies.*

The clinical observation that the first encounter with a pathogen shapes and conditions the way the immune system reacts to subsequent pathogen exposures led to the study of the phenomenon called "*original antigenic sin*" or "immunological imprinting," first described in the 1940s and remaining unexplained until recently, until it found a logical explanation in the massive sharing of peptides between infectious pathogens and the human host.

- *In fact, according to Kanduc and Shoenfeld [206] , preexisting immune responses against the immune determinants of a first pathogen can be enhanced by subsequent exposure to the same identical immune determinants present in a second similar or different pathogen.*
  This means that the primary response to one pathogen is transformed into a secondary response to a different pathogen previously encountered.

An anamnestic, high-avidity, high-affinity, and quantitatively abnormal secondary response is triggered against the previous sensitizing pathogen no longer present in the body, while no immune response is elicited against the pathogen last encountered following infection or active immunization.

Translated into the process of immunological maturation, the immunogenic encounters during an individual's early life form a pattern of immune responses (which at the cellular level correspond to a set of reactive lymphocytes) that will determine, control, and dominate the immune system in adulthood.
Such early imprinting or "immunological memory" becomes firmly fixed in the individual's early immune system and cannot be forgotten, conditioning future immune responses in the adult body, and during any active infection or immunization, the immune system will prioritize the production of lymphocytes reactive against immune determinants already encountered.

- *This may explain why efforts to activate the lymphocyte population in order to protect with active immunization are bound to remain unsuccessful.* [207]

Proc Natl Acad Sci U S A. 1996 Mar 5;93(5):2019-24. doi: 10.1073/pnas.93.5.2019.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC39902/

[206] Kanduc, Darja & Shoenfeld, Yehuda
Inter-Pathogen Peptide Sharing and the Original Antigenic Sin: Solving a Paradox.
The Open Immunology Journal (2018). 08. 16-27. 10.2174/1874226201808010016.
https://cdn.onb.it/2018/10/Kanduc_Shoenfeld-2018.pdf
http://www.onb.it/wp-content/uploads/2018/10/Kanduc_Shoenfeld_tradClaudioAndreini.pdf

[207] Ladhani S., Heath P.T., Slack M.P. et al,
Haemophilus influenzae serotype b conjugate vaccine failure in twelve countries with established national childhood immunization programs,
Clin. Microbiol. Infect., 16 (2010), 948-954. https://doi.org/10.1111/j.1469-0691.2009.02945.x
https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(14)61756-0/fulltext

Mahalingam S., Herring B.L., Halstead S.B.,
Call to action for dengue vaccine failure,
Emerg. Infect. Dis., 19 (2013), 1335-1337. https://doi.org/10.3201/eid1908.121864
https://wwwnc.cdc.gov/eid/article/19/8/12-1864_article

Ramsay M., Brown K.,
The public health implications of secondary measles vaccine failure,
J. Prim. Health Care, 5 (2013), 92. https://doi.org/10.1071/hc13092
https://www.publish.csiro.au/hc/pdf/HC13092

This evidence underscores the need to review the mechanisms governing immune responses in light of the extensive sharing of inter- and intra-proteomic peptides between microbial entities and human proteins.

According to the author, these data indicate that there is no negative a priori selection of autoreactive lymphocytes according to Burnet, but rather positive a posteriori selection of reactive lymphocytes occurs as a mechanism that drives the memory underlying the maturation and activity of the immune system.

ANTIGEN RECOGNITION BY T AND B LYMPHOCYTES

T and B lymphocytes have the antigen receptor structurally related and encoded by a similar gene family (supergene family). Despite this, their antigen recognition mechanism varies considerably.

A first difference is that the T lymphocyte recognizes the antigenic determinant associated with MHC-encoded molecules on the surface of accessory cells, whereas the B lymphocyte recognizes the antigenic determinant alone and does not strictly require the presence of an accessory cell.

T lymphocytes, unlike B lymphocytes, in order to recognize the antigen on an accessory cell require that the antigen be processed or degraded or, as more recently demonstrated, at least have its conformation changed.
One difference that follows is that the T lymphocyte, unlike the B lymphocyte, does not discriminate between native and denatured conformations of the antigen.

https://mainebiotechnology.com/antigen-presentation-gift-keeps-giving/
There are three main types of immune receptors that are specifically intended to bind protein antigens: B-cell receptors (and antibodies), T-cell receptors, and Major Histocompatibility Complex (MHC). T cells have a T cell receptor (TCR) that is structurally related to a BCR, but unlike BCRs, TCRs do not interact with native antigens. Rather, TCRs have antigens presented by MHC as a peptide fragment (TCR epitope) and specifically recognize both MHC and peptide. The MHC shows the peptide fragment in the context that is recognizable for the TCR to interact with the epitope. Therefore, protein antigens must be digested, processed and bound by MHC to be presented to T lymphocytes. MHC I will present intracellular antigens and MHC II will present extracellular antigens. In both cases, the antigens are processed and bound by MHC and the antigen-MHC peptide complex is displayed on the cell surface to enable T-cell surveillance. All nucleated cells have the ability to process intracellular proteins (antigens) and present peptides through MHC I on the cell surface. A defined population of cells, called antigen-presenting cells (APCs), has the ability to import extracellular antigens, process them into peptides and present them via MHC II molecules on their cell surface.

Michael A, Picker L.J., Moore J.P., Burton D.R.,
Another HIV vaccine failure: where to next?
Nat. Med., 19 (2013), 1576-1577. https://doi.org/10.1038/nm.3413
https://www.nature.com/articles/nm.3413

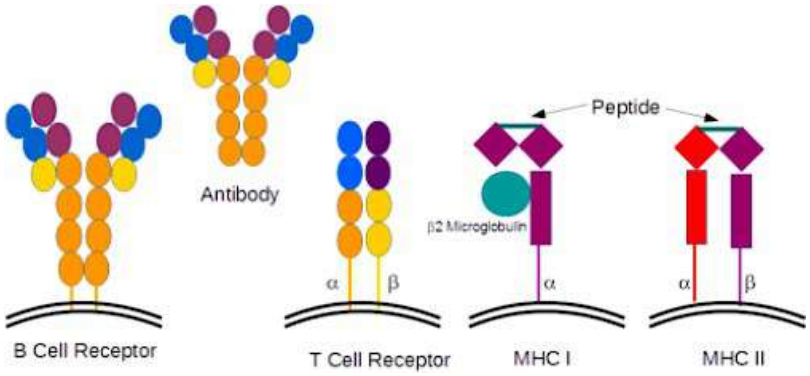
Cherry J.D.,
Epidemic pertussis and acellular pertussis vaccine failure in the 21st century,
Pediatrics, 135 (2015), 1130-1132. https://doi.org/10.1542/peds.2014- 4118
https://escholarship.org/uc/item/3q93v54z

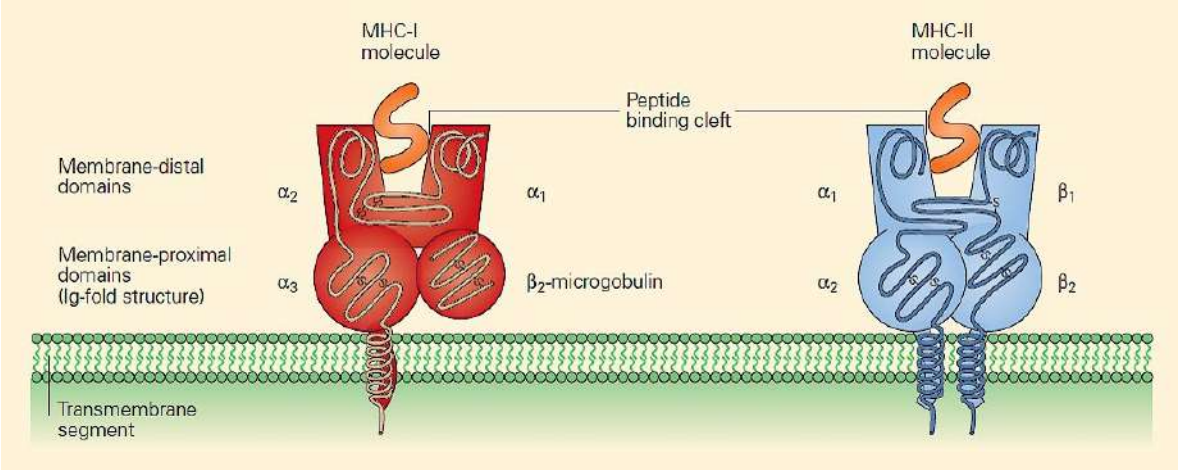
Lopez-Lacort M., Collado S., Díez-Gandía A., Díez-Domingo J.,
Rotavirus, vaccine failure or diagnostic error?
Vaccine, 34 (2016), 5912-5915. https://doi.org/10.1016/j.vaccine.2016.10.032
https://www.sciencedirect.com/science/article/pii/S0264410X16309537?via%3Dihub

Wiedermann U., Garner-Spitzer E., Wagner A.,
Primary vaccine failure to routine vaccines: Why and what to do?
Hum, Vaccin, Immunother, 12 (2016), 239-243. https://doi.org/10.1080/21645515.2015.1093263
https://www.tandfonline.com/doi/full/10.1080/21645515.2015.1093263

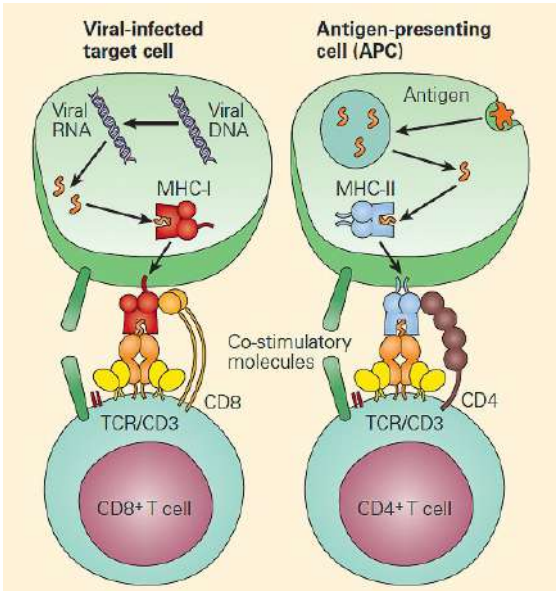
Poland G.A.,
Influenza vaccine failure: failure to protect or failure to understand?
Expert Rev. Vaccines, 17 (2018), 495-502. https://doi.org/10.1080/14760584.2018.1484284
https://www.tandfonline.com/doi/full/10.1080/14760584.2018.1484284

Masters N.B., Wagner A.L., Ding Y., Zhang Y., Boulton M.L.,
Assessing measles vaccine failure in Tianjin, China,
Vaccine, 37 (2019), 3251-3254. https://doi.org/10.1016/j.vaccine.2019.05.005
https://www.sciencedirect.com/science/article/pii/S0264410X19305985?via%3Dihub





https://www.immunopaedia.org.za/immunology/basics/4-mhc-antigen-presentation/



https://www.immunopaedia.org.za/immunology/basics/4-mhc-antigen-presentation/
Endogenous antigens are generally presented to CD8 T cells⁺ (left panel) and exogenous antigens are generally presented to CD4 T cells⁺ (right panel).

T lymphocytes, and more specifically their receptor, recognize a linear sequence of amino acids (about 5-12 amino acids of the primary structure of a protein) present in a secondary structure (alpha helix).

In contrast, B lymphocytes, or more properly their immunoglobulin receptor, bind both primary or secondary (sequential) and tertiary (conformational) amino acid sequences.
Further analysis using synthetic peptides showed that T lymphocytes can discriminate differences at the level of even one amino acid.

This great specificity of T lymphocytes to discriminate a few amino acids is further enhanced by association with MHC molecules: for example, it has been observed that the substitution of a single

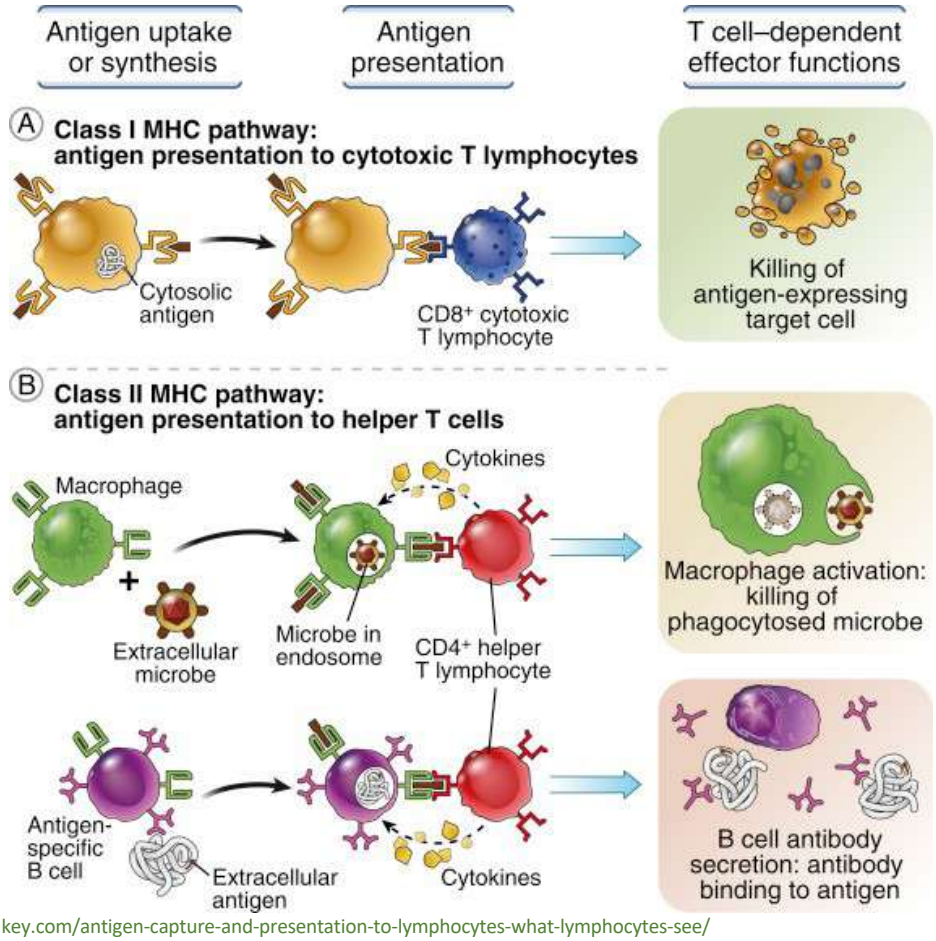
amino acid on the alpha chain of DR (a human MHC class II molecule) alters associative recognition. This demonstrates the fine discriminative specificity of the T lymphocyte receptor and gives insight into the variability of its receptor repertoire.

When the immune system encounters a conventional T-dependent antigen, only a small fraction (1 in 10 - 10^{45}) of the T-cell population is able to recognize the antigen and activate (monoclonal/oligoclonal response). However, there are some antigens that polyclonally activate a large fraction of T cells (up to 25%) called **superantigens.**

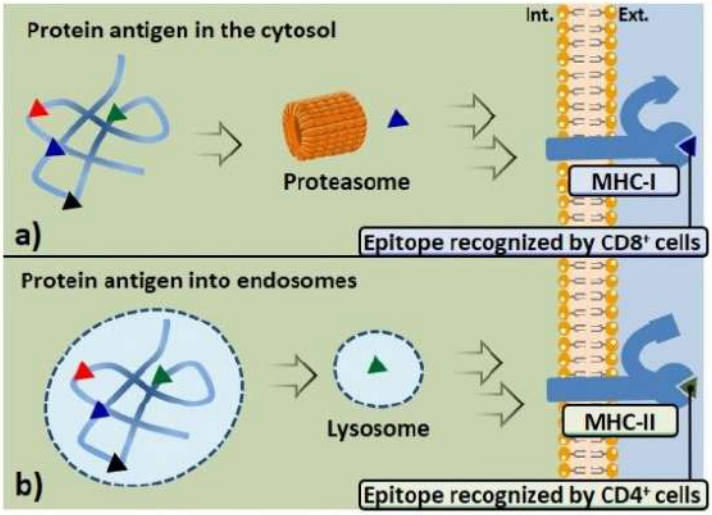
The first step in stimulating adaptive responses requires the proper capture, processing, and display of antigens to lymphocytes by antigen-presenting cells (APCs).

Dendritic cells (DCs) represent the most specialized cell type in this regard: they capture antigens from an external environment to present them, in secondary lymphoid organs, to naïve T cells.
Other cell types can act as APCs at different stages of the immune response (e.g., macrophages and B cells).
Capture of microbes and subsequent digestion of their proteins is followed by surface expression of microbial peptides (≈ 10 amino acid residues) in association with MHC molecules.
Antigen processing mechanisms are predisposed to generate peptide fragments suitable for their co-expression with MHC molecules on the cell surface.

MHC presentation of both self and foreign antigens enables the immune system to prevent targeting of its own cells and recognition of infected or mutated cells.
Epitopes obtained from the MHC-I pathway will interact with CD8-expressing lymphocytes (Figure a).
Antigens that undergo internalization from the extracellular environment through the vesicles of APCs are generally processed in endo-lysosomes; this pathway leads to the expression of peptide epitopes on MHC-II molecules, which are recognized by CD4 cells^{+} (Figure b).

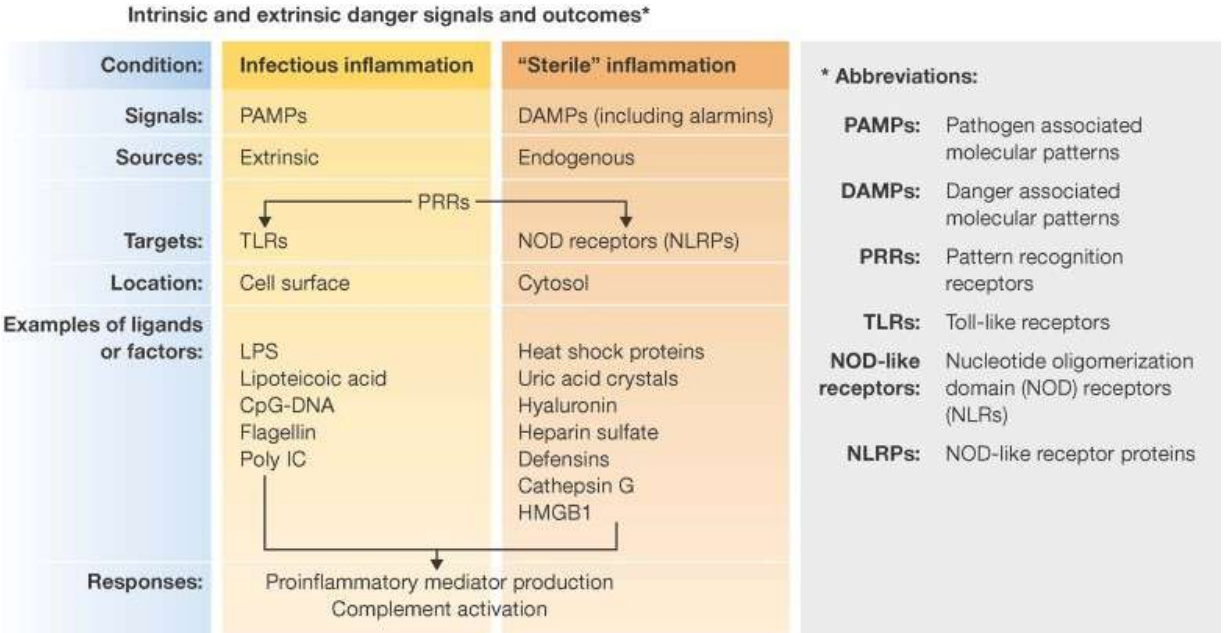


https://basicmedicalkey.com/antigen-capture-and-presentation-to-lymphocytes-what-lymphocytes-see/



http://www.glycopedia.eu/e-chapters/Overview-of-Immune-Responses-A-Primer-72/Antigen-presentation
Routes of processing and presentation of protein antigens. a) MHC-I pathway. b) MHC-II pathway. It is interesting to note that if the same protein passes through different pathways, the resulting MHC-associated peptide epitope could be different in terms of peptide sequence

Antigen-presenting cells such as macrophages, but especially DCs, express several surface receptors (PRRs) capable of recognizing structures shared by many pathogens (PAMPs); these receptors can efficiently bind and internalize a wide variety of antigens, thus promoting their presentation on class II MHCs. B cells through its surface receptors (BCRs) can also be ascribed among APCs. [208]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3531600/
Intrinsic (DAMPs) and extrinsic (PAMPs) signals develop during an infectious condition (e.g., bacterial pneumonia) that causes inflammation and sepsis that is often associated with the development of SIRS, accumulation of ROS and RNS in tissues, multi-organ failure (MOF), and lethality. The receptors (PRRs) for these signals involve both TLRs and NOD-like receptors. The list of ligands that interact with TLRs and NOD receptors is somewhat artificial. For example, while HMGB1 (considered a DAMP) interacts with TLR4, it also interacts with TLR2 and the receptor for advanced glycation products (RAGE). Heat shock proteins (DAMPs) react with TLR2, TLR4 and receptors on antigen-presenting cells (CD36, a scavenger receptor). "Sterile" inflammation occurs after hemorrhagic shock, polytrauma, ischemia/reperfusion and is usually not associated with the presence of an infectious agent. In all cases, the same downstream cascade of events appears to occur.

DCs carry the antigenic load to draining lymph nodes, where naïve T cells recirculate, increasing the likelihood of their interaction. According to **the clonal selection hypothesis** [209] even before exposure

[208] Ward PA.
New approaches to the study of sepsis.
EMBO Mol Med. 2012;4(12):1234-1243. doi:10.1002/emmm.201201375
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3531600/

[209] Cohn M, Mitchison NA, Paul WE, Silverstein AM, Talmage DW, Weigert M.

to antigens, there are a large number of lymphocytes with different specificities for a panel of antigens in secondary lymphoid organs; each lymphocyte (and its progeny) with a common specificity is referred to as a clone. Upon maturation, expansion of the clones that best interact with the antigen is observed, increasing the magnitude of action and the efficacy of the response.

This selection hypothesis will have to be revised in light of the new paradigm on hydrophobicity discussed above and the holobiont concept, in relation to both the development of immunotolerance/autoimmunity but also to vaccine design. [210]

In fact, the production of antibodies targeted to vaccine immunogenic epitopes designed on the basis of classical clonal selection theory could explain the occurrence of autoimmune-based adverse reactions to both eukaryotic cells and the microbiota.

Vaccines against SARS-Cov-2 were largely made from an in-depth bioinformatic study of immunogenic epitopes both to assess their molecular mimicry (see below) and to predict their potential protective capacity. Please refer to the cited literature for an in-depth discussion of the methods and results obtained. [211]

Reflections on the clonal-selection theory.
Nat Rev Immunol. 2007 Oct;7(10):823-30. doi: 10.1038/nri2177.
https://pubmed.ncbi.nlm.nih.gov/17893695/

Chen, Jun. (2021). Biologically Inspired Optimisation Algorithms for Transparent Knowledge Extraction
Allied to Engineering Materials Processing.
https://www.researchgate.net/publication/42424453_Biologically_Inspired_Optimisation_Algorithms_for_Transparent_Knowledge_Extraction_Allied_to_Engineering_Materials_Processing

[210] Segal Y, Shoenfeld Y.
Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction.
Cell Mol Immunol. 2018;15(6):586-594. doi:10.1038/cmi.2017.151
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6078966/

Rojas Met al
Molecular mimicry and autoimmunity.
J Autoimmun. 2018 Dec;95:100-123. doi: 10.1016/j.jaut.2018.10.012. Epub 2018 Oct
26. https://www.sciencedirect.com/science/article/pii/S0896841118305365?via%3Dihub

[211] Tahir Ul Qamar M et al.
Reverse vaccinology assisted design of multiepitope-based subunit vaccine against SARS-CoV-2.
Infect Dis Poverty. 2020 Sep 16;9(1):132. doi: 10.1186/s40249-020-00752-w.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7492789/

Baruah V, Bose S.
Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-nCoV.

J Med Virol. 2020;92(5):495-500. doi:10.1002/jmv.25698
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7166505/

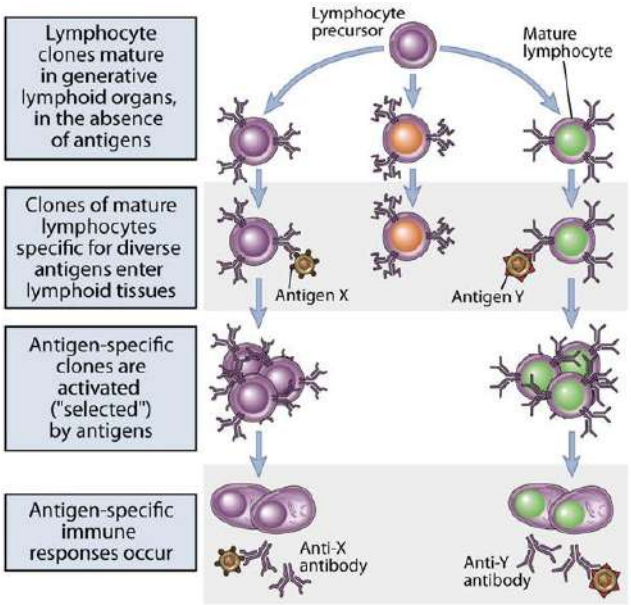
Chen Z, Ruan P, Wang L, Nie X, Ma X, Tan Y.
T and B cell Epitope analysis of SARS-CoV-2 S protein based on immunoinformatics and experimental research.
J Cell Mol Med. 2020 Dec 15. doi: 10.1111/jcmm.16200.
https://onlinelibrary.wiley.com/doi/epdf/10.1111/jcmm.16200

Ashik AI, Hasan M, Tasnim AT, Chowdhury MB, Hossain T, Ahmed S.
An immunoinformatics study on the spike protein of SARS-CoV-2 revealing potential epitopes as vaccine candidates.
Heliyon. 2020 Sep;6(9):e04865. doi: 10.1016/j.heliyon.2020.e04865. Epub 2020 Sep 4.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7472982/

Poran A et al
Sequence-based prediction of SARS-CoV-2 vaccine targets using a mass spectrometry-based bioinformatics predictor identifies immunogenic T cell epitopes.
Genome Med. 2020 Aug 13;12(1):70. doi: 10.1186/s13073-020-00767-w.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7425796/

Kim A et al
Divergent paths for the selection of immunodominant epitopes from distinct antigenic sources.
Nat Commun. 2014 Nov 21;5:5369. doi: 10.1038/ncomms6369.
https://www.nature.com/articles/ncomms6369

Dar HA, Waheed Y, Najmi MH, et al.
Multiepitope Subunit Vaccine Design against COVID-19 Based on the Spike Protein of SARS-CoV-2: An In Silico Analysis.



https://biology-forums.com/gallery/5195_05_08_11_11_28_29.jpeg

IMMUNOTOLERANCE, AUTOIMMUNITY, AND THE HOLOBIONT

While not pertinent to this in-depth study, it is worth mentioning that studies on the microbiome and virobiome are increasingly solidifying the concept that bacteria, viruses, and eukaryotic cells interact with each other according to a dynamic, symbiotic equilibrium referred to as the holobiome (the holobiome is the sum total of the component genomes in a eukaryotic organism; it comprises the genome of a single member of a given taxon (the host genome) and the microbiome (the genomes of the symbiotic microbiota [212])).

This means that even the classical concept of the immune system's function of discriminating between the self (antigens/immunogens produced by eukaryotic cells) and the non-self (antigens/immunogens produced foreign pathogens) is about to be overcome. [213]

J Immunol Res. 2020;2020:8893483. Published 2020 Nov 19. doi:10.1155/2020/8893483
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7678744/

Abd Albagi SO, Al-Nour MY, Elhag M, et al.
A multiple peptides vaccine against COVID-19 designed from the nucleocapsid phosphoprotein (N) and Spike Glycoprotein (S) via the immunoinformatics approach.
Inform Med Unlocked. 2020;21:100476. doi:10.1016/j.imu.2020.100476

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7654333/
[212] Guerrero R, Margulis L, Berlanga M.
Symbiogenesis: the holobiont as a unit of evolution.
Int Microbiol. 2013 Sep;16(3):133-43. doi: 10.2436/20.1501.01.188.
http://revistes.iec.cat/index.php/IM/article/viewFile/74108/73862

[213] Postler TS, Ghosh S.
Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System.
Cell Metab. 2017;26(1):110-130. doi:10.1016/j.cmet.2017.05.008
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5535818/

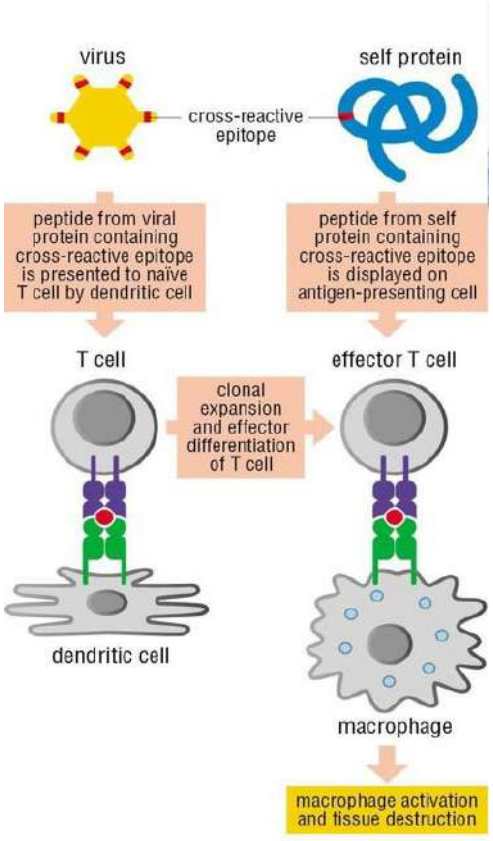
Thomas-Vaslin V.
Individuation and the Organization in Complex Living Ecosystem: Recursive Integration and Self-assertion by Holon-Lymphocytes.
Acta Biotheor. 2020 Mar;68(1):171-199. doi: 10.1007/s10441-019-09364-w. Epub 2019 Sep 20.
https://pubmed.ncbi.nlm.nih.gov/31541308/

Gilbert SF, Sapp J, Tauber AI.
A symbiotic view of life: we have never been individuals.
Q Rev Biol. 2012 Dec;87(4):325-41. doi: 10.1086/668166.
https://works.swarthmore.edu/cgi/viewcontent.cgi?article=1164&context=fac-biology

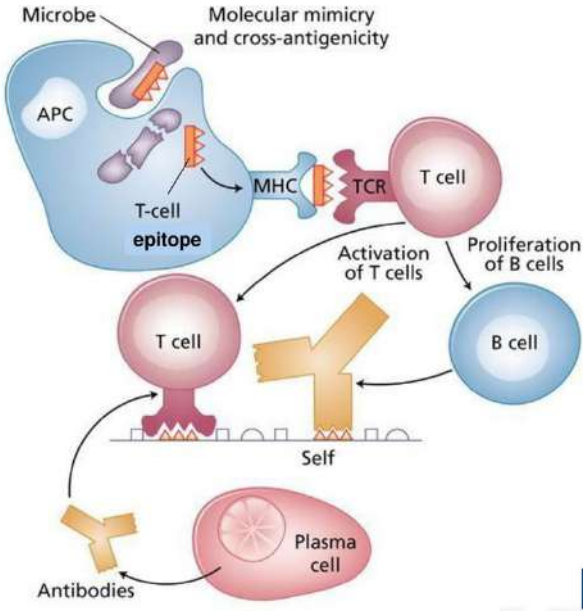
Gilbert SF.
Symbiosis as the way of eukaryotic life: the dependent co-origination of the body.
J Biosci. 2014 Apr;39(2):201-9. doi: 10.1007/s12038-013-9343-6.
https://www.ias.ac.in/article/fulltext/jbsc/039/02/0201-0209

An interesting hypothesis suggests that molecular mimicry * [214], a condition in which several organisms share common antigens, is a mechanism for establishing tolerance between commensals and their hosts (**holo-immunity**). [215]

***Molecular mimicry**: is one of the main mechanisms by which infectious or chemical agents can induce autoimmunity. It occurs when similarities between foreign peptides and self-peptides promote an activation of autoreactive T or B cells by a foreign-derived antigen in a susceptible individual. However, molecular mimicry is unlikely to be the only mechanism underlying autoimmune responses; other factors such as violation of central tolerance, nonspecific activation by bystander cells, or persistent antigenic stimuli may contribute to the development of autoimmune disease. Host genetics, exposure to microbiota and environmental chemicals or iatrogens (drugs and vaccines) are further links to our understanding of molecular mimicry.
[216]



https://slideplayer.com/slide/16159576/
Immune-Mediated Diseases and Autoimmunity
Fig. 1 If there is a cross-reaction between the antigen of the infectious agent and a component of the self, the cross-reacting antibody or T cells can initiate autoimmune disease
If a viral protein and a self-protein contain peptide epitopes recognized by the same T cell, the virus-specific T cell can attack not only virus-infected cells but also uninfected cells displaying the same self-epitope



. 2

Gilbert SF.
Evolutionary transitions revisited: holobiont evo-devo.
J Exp Zool B Mol Dev Evol. 2019 Dec;332(8):307-314. doi: 10.1002/jez.b.22903. Epub 2019 Sep 29.
https://pubmed.ncbi.nlm.nih.gov/31565856/

Triviño V, Suárez J.
Holobionts: Ecological communities, hybrids, or biological individuals? A metaphysical perspective on multispecies systems.
Stud Hist Philos Biol Biomed Sci. 2020 Dec;84:101323. doi: 10.1016/j.shpsc.2020.101323. Epub 2020 Aug 9.
https://pubmed.ncbi.nlm.nih.gov/32788054/

[214] Fujinami RS, von Herrath MG, Christen U, Whitton JL.
Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease.
Clin Microbiol Rev. 2006;19(1):80-94. doi:10.1128/CMR.19.1.80-94.2006
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1360274/

[215] Land WG.
How evolution tells us to induce allotolerance.
Exp Clin Transplant. 2015 Apr;13 Suppl 1:46-54.
https://pubmed.ncbi.nlm.nih.gov/25894127/

[216] Rojas Met al
Molecular mimicry and autoimmunity.
J Autoimmun. 2018 Dec;95:100-123. doi: 10.1016/j.jaut.2018.10.012. Epub 2018 Oct 26.
https://www.sciencedirect.com/science/article/pii/S0896841118305365?via%3Dihub

Fig. 2 In molecular mimicry, the cell surface antigens of some microbial pathogens resemble self-proteins. When the immune system recognizes these pathogens, the self-proteins are attacked by "friendly fire" (a case of mistaken identity). The connection between "sore throat" and rheumatic heart disease is one of the most emblematic examples of such cross-antigenicity.
Self tissue cells can be induced to express the major histocompatibility complex (MHC) when stimulated with γ-interferon. Excessive cytokine release during infection or inflammation can awaken self-tolerant T cells, a theory consistent with autoimmune disease flare-ups observed after viral infections. APC: antigen-presenting cell; TCR - T cell receptor.
This mechanism may also be plausible following vaccination

microbiome-host mimicry also implies that on the one hand autoimmunity directed at host antigens will also attack components of the microbiome, and on the other hand that an immunological attack on the microbiome can cross-react with host antigens producing "**holo-autoimmunity.**" [217]
COVID-19 can also be placed in this context as its clinical manifestations include a large case history of autoimmune diseases as a result of an autoimmune process due to molecular mimicry between SARS-Cov-2 and human proteins. [218]

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[217] Root-Bernstein R.
Autoimmunity and the microbiome: T-cell receptor mimicry of "self" and microbial antigens mediates self-tolerance in holobionts: The concepts of "holoimmunity" (TcR-mediated tolerance for the holobiont) and "holoautoimmunity" (loss of tolerance for the holobiont) are introduced.
Bioessays. 2016;38(11):1068-1083. doi:10.1002/bies.201600083
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7161894/

Swiatczak B, Tauber AI.
Holoimmunity Revisited.
Bioessays. 2018 Nov;40(11):e1800117. doi: 10.1002/bies.201800117. Epub 2018 Sep 28.
https://pubmed.ncbi.nlm.nih.gov/30264468/

Root-Bernstein, Robert.
A General Theory of Autoimmune Disease Causation: Integrating Innate and Adaptive Immunity, Altered Antigen Processing, Sex and Genetic Predispositions, and Microbiome Effects.
Preprints (2019). 10.13140/RG.2.2.14654.54089.
https://www.researchgate.net/publication/333481656_A_General_Theory_of_Autoimmune_Disease_Causation_Integrating_Innate_and_Adaptive_Immunity_Altered_Antigen_Processing_Sex_and_Genetic_Predispositions_and_Microbiome_Effects

Root-Bernstein R.
Synergistic Activation of Toll-Like and NOD Receptors by Complementary Antigens as Facilitators of Autoimmune Disease: Review, Model and Novel Predictions. Int J Mol Sci. 2020;21(13):4645. Published 2020 Jun 30. doi:10.3390/ijms21134645
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7369971/

[218] Halpert G, Shoenfeld Y. SARS-CoV-2, the autoimmune virus.
Autoimmun Rev. 2020;19(12):102695. doi:10.1016/j.autrev.2020.102695
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7598743/

Kanduc D, Shoenfeld Y.
On the molecular determinants of the SARS-CoV-2 attack.
Clin Immunol. 2020;215:108426. doi:10.1016/j.clim.2020.108426
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7165084/

Kanduc D.
From Anti-SARS-CoV-2 Immune Responses to COVID-19 via Molecular Mimicry.
Antibodies (Basel). 2020;9(3):33. Published 2020 Jul 16. doi:10.3390/antib9030033
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7551747/

Kanduc D, Shoenfeld Y.
Molecular mimicry between SARS-CoV-2 spike glycoprotein and mammalian proteomes: implications for the vaccine.
Immunol Res. 2020;68(5):310-313. doi:10.1007/s12026-020-09152-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7499017/

Vojdani A, Kharrazian D.
Potential antigenic cross-reactivity between SARS-CoV-2 and human tissue with a possible link to an increase in autoimmune diseases.
Clin Immunol. 2020;217:108480. doi:10.1016/j.clim.2020.108480
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7246018/

Ehrenfeld M, Tincani A, Andreoli L, et al.
Covid-19 and autoimmunity.
Autoimmun Rev. 2020;19(8):102597. doi:10.1016/j.autrev.2020.102597
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7289100/

Angileri F, Legare S, Marino Gammazza A, Conway de Macario E, Jl Macario A, Cappello F.
Molecular mimicry may explain multi-organ damage in COVID-19.
Autoimmun Rev. 2020;19(8):102591. doi:10.1016/j.autrev.2020.102591
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7289093/

Cappello F, Gammazza AM, Dieli F, de Macario, Macario AJ.

TYPE OF SUBUNIT VACCINES

Subunit vaccines contain protein fragments and/or polysaccharides of the pathogen, which have been carefully studied to identify which combinations of these molecules can produce a strong and effective immune response. Such vaccines are believed to be safer, relatively cheap and easy to produce, and more stable than those containing whole viruses or bacteria.[219]

A disadvantage of this precision is that the antigens used to elicit an immune response may lack PAMPs. These structures are read by immune cells and recognized as danger signals, so their absence may result in a weaker immune response.

Moreover, because antigens do not infect cells, subunit vaccines primarily trigger only antibody-mediated immune responses. Again, this means that the immune response may be weaker than in other types of vaccines.

To overcome this problem, subunit vaccines are sometimes given together with adjuvants (agents that stimulate the immune system), and booster doses may be necessary.

All subunit vaccines are made using living organisms, such as bacteria and yeasts, which require substrates on which to grow them and strict hygiene to avoid contamination with other microorganisms.
The production line diagram for flu vaccine is shown below as an example: [220]

Does SARS-CoV-2 Trigger Stress-InducedAutoimmunity by Molecular Mimicry? A Hypothesis.
J Clin Med. 2020;9(7):2038. Published 2020 Jun 29. doi:10.3390/jcm9072038
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7408943/

Venkatakrishnan, et al.
Benchmarking evolutionary tinkering underlying human-viral molecular mimicry shows multiple host pulmonary-arterial peptides mimicked by SARS- CoV-2.
Cell Death Discov. 6, 96 (2020). https://doi.org/10.1038/s41420-020-00321-y
https://www.nature.com/articles/s41420-020-00321-y

Lucchese, G., Flöel, A.
SARS-CoV-2 and Guillain-Barré syndrome: molecular mimicry with human heat shock proteins as potential pathogenic mechanism.
Cell Stress and Chaperones 25, 731-735 (2020). https://doi.org/10.1007/s12192-020-01145-6
https://link.springer.com/article/10.1007/s12192-020-01145-6

Molecular Mimicry Map (3M) of SARS-CoV-2: Prediction of potentially immunopathogenic SARS-CoV-2 epitopes via a novel immunoinformatic approach
Hyunsu An, Jihwan Park
bioRxiv 2020.11.12.344424; doi: https://doi.org/10.1101/2020.11.12.344424
https://www.biorxiv.org/content/10.1101/2020.11.12.344424v1.full

Lyons-Weiler J.
Pathogenic priming likely contributes to serious and critical illness and mortality in COVID-19 via autoimmunity.
J Transl Autoimmun. 2020 Apr 9;3:100051. doi: 10.1016/j.jtauto.2020.100051.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7142689/

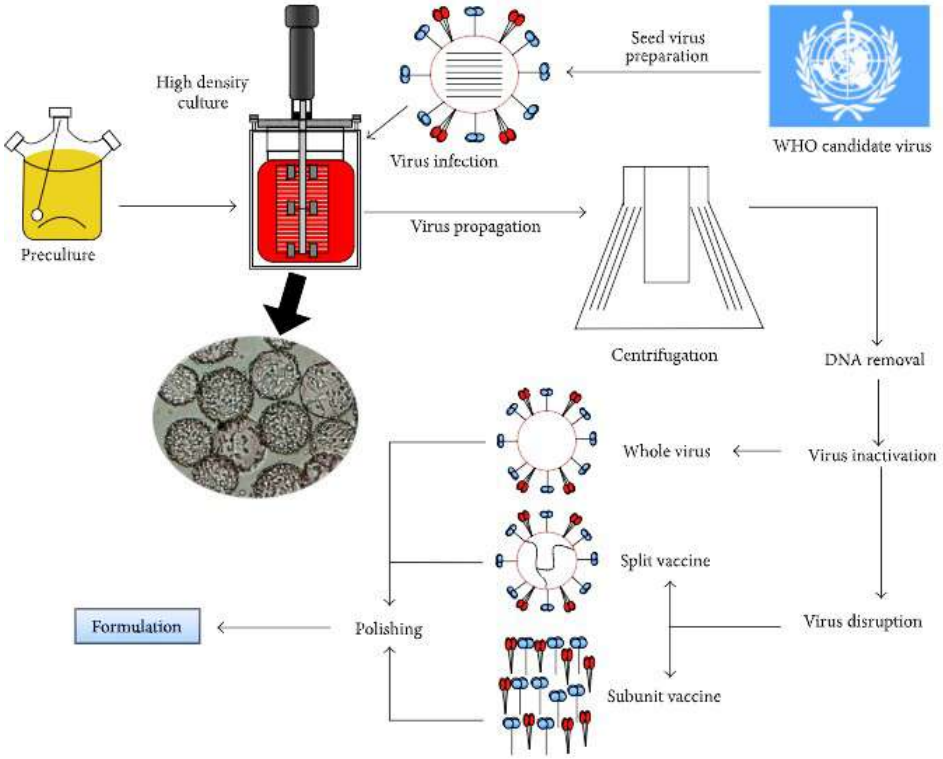
[219] Gupta V, Sengupta M, Prakash J, Tripathy BC.
Production of Recombinant Pharmaceutical Proteins.
Basic and Applied Aspects of Biotechnology. 2016;77-101. Published 2016 Oct 23. doi:10.1007/978-981-10-0875-7_4
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7120688/

Vartak A, Sucheck SJ.
Recent Advances in Subunit Vaccine Carriers.
Vaccines (Basel). 2016;4(2):12. Published 2016 Apr 19. doi:10.3390/vaccines4020012
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4931629/

Production and delivery of recombinant subunit vaccines
http://www.diva-portal.org/smash/get/diva2:8775/FULLTEXT01.pdf

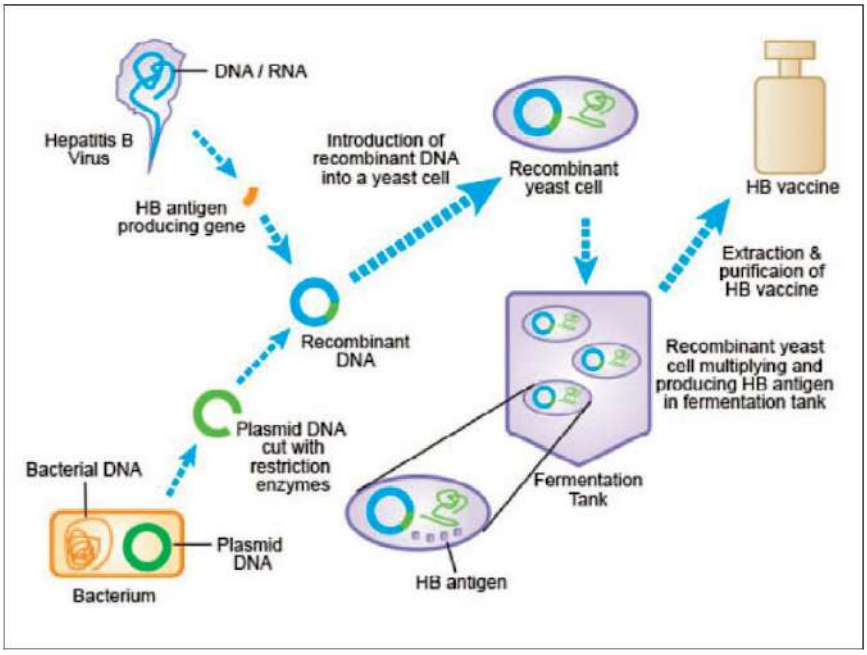
Hansson, M., Nygren, P.-Å., & Ståhl, S.
Design and production of recombinant subunit vaccines.
Biotechnology and Applied Biochemistry, (2000).32(2), 95. doi:10.1042/ba20000034
https://pubmed.ncbi.nlm.nih.gov/11001870/

[220] Milián E, Kamen AA.



https://www.hindawi.com/journals/bmri/2015/504831/

The method of production depends on the type of subunit vaccine: **protein subunit vaccines**, such as recombinant hepatitis B vaccine, are produced by inserting the genetic code of the antigen into yeast cells, which are relatively easy to grow and capable of synthesizing large amounts of protein.
The yeast is grown in large fermentation tanks and then lysed, allowing the antigen to be harvested. This purified protein is then added to other components of the vaccine, such as preservatives to keep it stable and adjuvants to enhance the immune response, in this case adjuvant aluminum.



https://www.ddw-online.com/creating-vaccines-drop-by-drop-1273-202008/

Current and emerging cell culture manufacturing technologies for influenza vaccines.
Biomed Res Int. 2015;2015:504831. doi:10.1155/2015/504831
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4359798/

Schematic representation of hepatitis B vaccine production. A gene that produces the hepatitis B virus antigen vinee inserted into a bacterial plasmid vector. A yeast cell is then transformed using this vector and grown in a fermentation reactor to produce the hepatitis B antigens, which are then isolated and purified to produce the vaccine.

For **polysaccharide or conjugate vaccines**, the manufacturing technologies are complex, multi-step processes. In the example shown in the figure below of the *Haemophilus influenzae* type B vaccine, they include:

(i) The separate cultivation of bacterial strains that produce the polysaccharide antigens and the transport protein,

(ii) Separate purification of polysaccharide antigen and carrier protein,

(iii) Chemical cleavage of LPS polysaccharides from lipid A followed by a second purification step,

(iv) Chemical coupling of polysaccharides to the carrier protein, and

(v) A third purification step to obtain the final product. [221]

Considerable losses occur at each step, and due to the random nature of chemical coupling, the final products are ill-defined.
The processes are time-consuming and expensive, and large-scale cultivation of pathogenic bacteria is often required for polysaccharide biosynthesis, making conjugate vaccines too expensive for vaccination campaigns in developing countries.

Therefore, methods for the production of conjugated antigens in bacterial cell lines (protein-glycan coupling technology) have also been developed, which are based on an N-glycosylation system of *Campylobacter jejuni,* which can be functionally expressed in *E. coli,* and on the ability of *E. coli* to synthesize heterologous polysaccharides on its glycosyl-lipid carrier. [222]

[221] https://www.gavi.org/vaccineswork/what-are-protein-subunit-vaccines-and-how-could-they-be-used-against-covid-19

Foged, Camilla. (2015). Subunit Vaccine Delivery.
https://www.researchgate.net/publication/269311879_Subunit_Vaccine_Delivery/citation/download

Berti F , Adamo R .
Antimicrobial glycoconjugate vaccines: an overview of classic and modern approaches for protein modification.
Chem Soc Rev. 2018 Dec 10;47(24):9015-9025. doi: 10.1039/c8cs00495a.
https://pubs.rsc.org/en/content/articlehtml/2018/cs/c8cs00495a

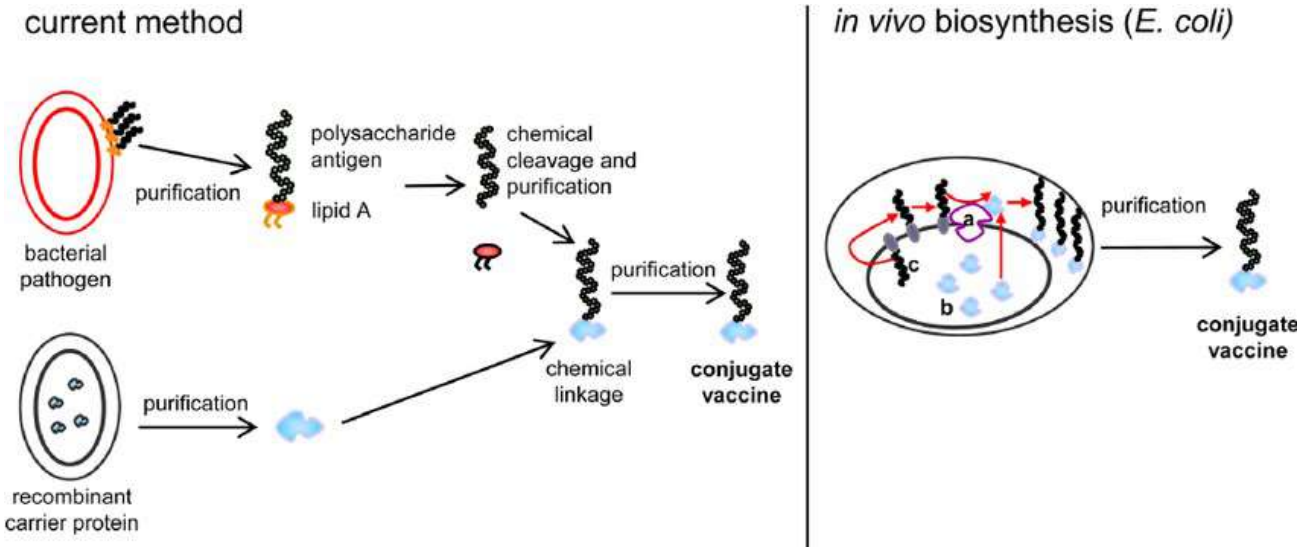
Lang S, Huang X.
Carbohydrate Conjugates in Vaccine Developments.
Front Chem. 2020;8:284. Published 2020 Apr 15. doi:10.3389/fchem.2020.00284
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7174737/

[222] Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thöny-Meyer L.
Production of glycoprotein vaccines in Escherichia coli.
Microb Cell Fact. 2010;9:61. Published 2010 Aug 11. doi:10.1186/1475-2859-9-61
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2927510/

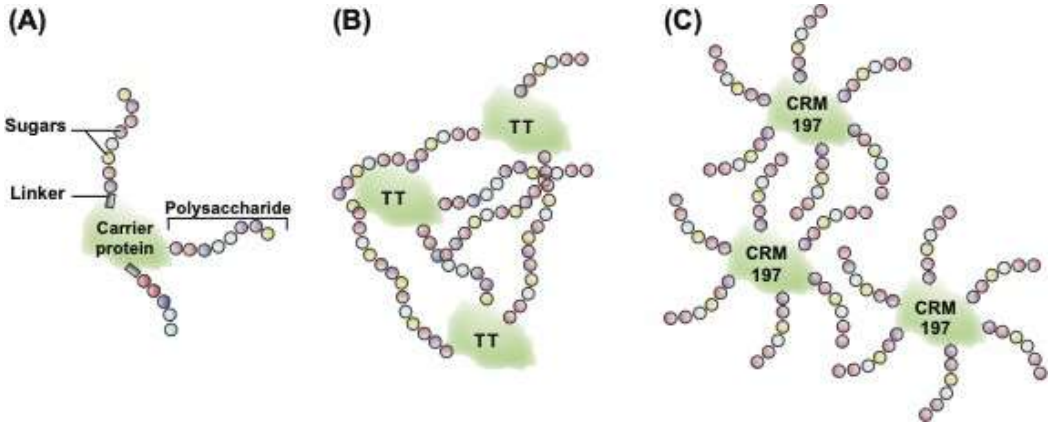
Feldman MF, Wacker M, Hernandez M, et al.
Engineering N-linked protein glycosylation with different O antigen lipopolysaccharide structures in Escherichia coli. Proc Natl Acad Sci U S A. 2005;102(8):3016-3021. doi:10.1073/pnas.0500044102
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC549450/

Yates LE, Mills DC, DeLisa MP.
Bacterial Glycoengineering as a Biosynthetic Route to Customized Glycomolecules.
Adv Biochem Eng Biotechnol. 2018 Aug 12. doi: 10.1007/10_2018_72.
https://www.biorxiv.org/content/10.1101/118224v1.full.pdf

Chapter 14 - Vaccination,
Editor(s): Tak W. Mak, Mary E. Saunders, Bradley D. Jett, Primer to the Immune Response (Second Edition),
Academic Cell, 2014, Pages 333-375, ISBN 9780123852458, https://doi.org/10.1016/B978-0-12-385245-8.00014-5.
http://www.sciencedirect.com/science/article/pii/B9780123852458000145



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2927510/
Current method for conjugate vaccine production and in vivo biosynthesis. a: PglB oligosaccharyltransferase, b: carrier protein with signal sequence for secretion into the periplasm, c: undecaprenylpyrophosphate-bound polysaccharides.



http://www.sciencedirect.com/science/article/pii/B9780123852458000145
Examples of conjugate vaccine structures
(A) Polysaccharide antigens derived from pathogens of various lengths and sugar compositions can be isolated from bacterial capsules and joined directly or via linkage molecules to a carrier protein to form a conjugate vaccine. **(B)** When tetanus toxoid (TT) is used as a carrier protein, random linkage of the polysaccharide protein and cross-linking between polysaccharide chains result in an inhomogeneous, meshed, ill-defined conjugate structure. **(C)** To form a more homogeneous, well-defined conjugate structure, the polysaccharide chains can be linked to the transport protein CRM197

CRITICALITY OF PROTEIN SUBUNIT VACCINES

Subunit vaccines mainly induce $CD4^+$ $_{TH}$ cells and antibody responses; therefore, most of these vaccines contain the full-length S protein of SARS-CoV-2 or parts of it with the aim of inducing neutralizing antibodies.

Subunit vaccines can be designed to focus the immune response toward neutralizing epitopes, to avoid the production of nonneutralizing antibodies that can promote disease enhancement (ADE) [223]. However, unlike nucleic acid-based or viral vector vaccines, recombinant S proteins in subunit vaccines may have improper epitope conformation unless they are produced in mammalian cells [224].

[223] Oscherwitz J.
The promise and challenge of epitope-focused vaccines.
Hum Vaccin Immunother. 2016;12(8):2113-2116. doi:10.1080/21645515.2016.1160977
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4994726/

[224] Du L, Zhao G, Chan CC, Sun S, Chen M, Liu Z, Guo H, He Y, Zhou Y, Zheng BJ, Jiang S.
Recombinant receptor-binding domain of SARS-CoV spike protein expressed in mammalian, insect and E. coli cells elicits potent neutralizing antibody and protective immunity.

Proteins or peptides alone are poorly immunogenic and generally require not only the addition of an adjuvant but also repeated administration, are poor activators of the CD8 T-cell response+ and are not suitable for respiratory mucosal vaccination.

As with inactivated viral vaccines, the use of aluminum as an adjuvant alters the immune response toward Th2 cell-induced Th2-like responses (eosinophilic immunopathology) [225], which is undesirable for host defense against SARS-CoV-2 and may play a role in disease potentiation [226].
To overcome this critical issue, the subunit vaccines for COVID-19 developed by GlaxoSmithKline and Novavax use AS03 and Matrix-M adjuvants, respectively. [227].

Name	Components	Receptor/pathway	Disease target tested in the clinic
Alum[a]	Aluminum salts (aluminum hydroxide, aluminum phosphate)	NLRP3 uric acid, DNA	Anthrax[a], Diphtheria[a], Tetanus[a], Pneumococcus[a], hepatitis A[a] Hepatitis B[a], Japanese Encephalitis[a], Meningococcal B[a] and C[a], human papillomavirus[a], SARS, COVID-19
MF59[a], AS03[a]	Oil-in-water emulsion squalene oil plus surfactants	MyD88, ASC, ATP	Influenza[a], COVID-19
CpG 1018[a]	Synthetic DNA alone or formulated with Alum	TLR9	Hepatitis B[a], Malaria, Influenza, Anthrax, Cancer, COVID-19
Matrix M/IscoMatrix	Saponin	Unknown	Hepatitis C, Influenza, HSV, human papillomavirus, Malaria, Cancer, COVID-19
Advax	polysaccharide particle made from delta inulin	Unknown	HIV, Influenza, Hepatitis B, COVID-19

Virology. 2009 Oct 10;393(1):144-50. doi: 10.1016/j.virol.2009.07.018. Epub 2009 Aug 15.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2753736/

[225] Bolles M, Deming D, Long K, et al.
A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge.
J Virol. 2011;85(23):12201-12215. doi:10.1128/JVI.06048-11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3209347/

[226] Diamond MS, Pierson TC.
The Challenges of Vaccine Development against a New Virus during a Pandemic.
Cell Host Microbe. 2020;27(5):699-703. doi:10.1016/j.chom.2020.04.021
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7219397/

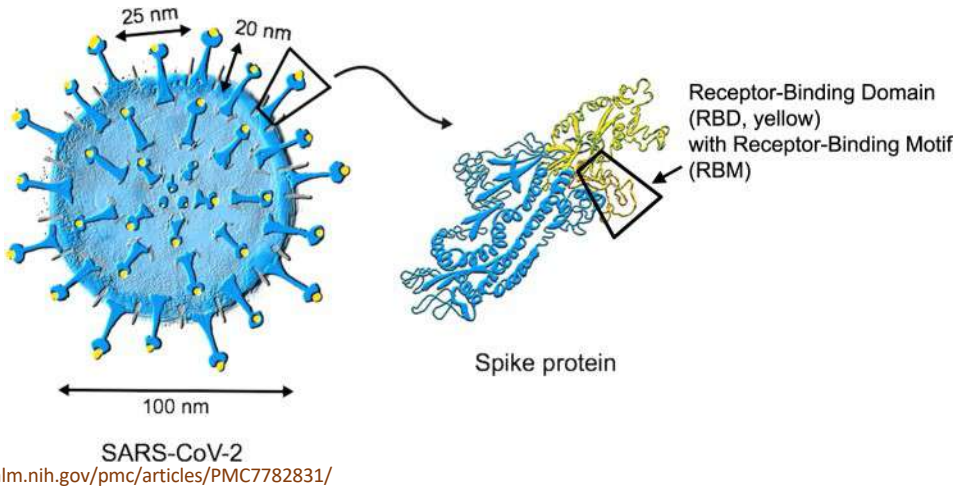
Del Giudice G, Rappuoli R, Didierlaurent AM.
Correlates of adjuvanticity: A review on adjuvants in licensed vaccines.
Semin Immunol. 2018 Oct;39:14-21. doi: 10.1016/j.smim.2018.05.001. Epub 2018 May 23.
https://www.sciencedirect.com/science/article/pii/S1044532318300514?via%3Dihub

[227] Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z.
Immunological considerations for COVID-19 vaccine strategies.
Nat Rev Immunol. 2020;20(10):615-632. doi:10.1038/s41577-020-00434-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7472682/

Pollet J, Chen WH, Strych U.
Recombinant protein vaccines, a proven approach against coronavirus pandemics
[published online ahead of print, 2021 Jan 7]. Adv Drug Deliv Rev. 2021;170:71-82. doi:10.1016/j.addr.2021.01.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/
List of adjuvants used in recombinant COVID-19 vaccine candidate protein currently tested in the clinic.

As is well known, SARS-CoV-2 shares extensive sequence homology as well as structural and functional homologies with previous coronaviruses, namely SARS, and MERS, and anti-SARS antibodies against S protein have been shown to inhibit the binding of SARS-CoV-2 to ACE-2.
These observations have focused vaccine development on antigens derived from the spike protein. While some groups have focused on the entire S1 subunit, others use RBD as their primary vaccine antigen candidate. [228]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7782831/
Structure of SARS-CoV-2.
Coronaviruses are named after the typical spikes formed by the spike protein (S) inserted into the virus' lipid bilayer membrane. The receptor binding domain (RBD) and its receptor binding motif (RBM) allow interaction with the cell surface receptor ACE2, which mediates virus entry into host cells. This can be blocked by neutralizing antibodies. Therefore, most of the neutralizing epitopes are found on RBD / RBM. In addition to the S protein, SARS-CoV-2 has two other viral surface proteins (not shown): envelope (E) and matrix (M).

One reason to focus on RBD lies in observations with the SARS protein S vaccine homolog in mice by Drs. Jiang and Tseng , who found lung pathology in mice due to antibody-dependent enhancement with full-length protein S as the vaccine antigen, but not with RBD. [229] In ADE, antibodies present in vaccinated individuals promote the entry of viral particles into the host cell through an additional mechanism using Fc receptor gamma. Notably, nonneutralizing antibodies that do not interfere with RBD binding to ACE-2 could therefore increase the risk of ADE.
Therefore, reducing antigen size to limit exposure to nonneutralizing epitopes could reduce the risk of unwanted immunopathology.

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[228] Lim HX, Lim J, Jazayeri SD, Poppema S, Poh CL.
Development of multi-epitope peptide-based vaccines against SARS-CoV-2
[published online ahead of print, 2020 Oct 1]. Biomed J. 2020;doi:10.1016/j.bj.2020.09.005
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7527307/

Lin L, Ting S, Yufei H, Wendong L, Yubo F, Jing Z.
Epitope-based peptide vaccines predicted against novel coronavirus disease caused by SARS-CoV-2.
Virus Res. 2020;288:198082. doi:10.1016/j.virusres.2020.198082
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7328648/

Bachmann MF, Mohsen MO, Zha L, Vogel M, Speiser DE.
SARS-CoV-2 structural features may explain limited neutralizing-antibody responses.
NPJ Vaccines. 2021;6(1):2. Published 2021 Jan 4. doi:10.1038/s41541-020-00264-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7782831/

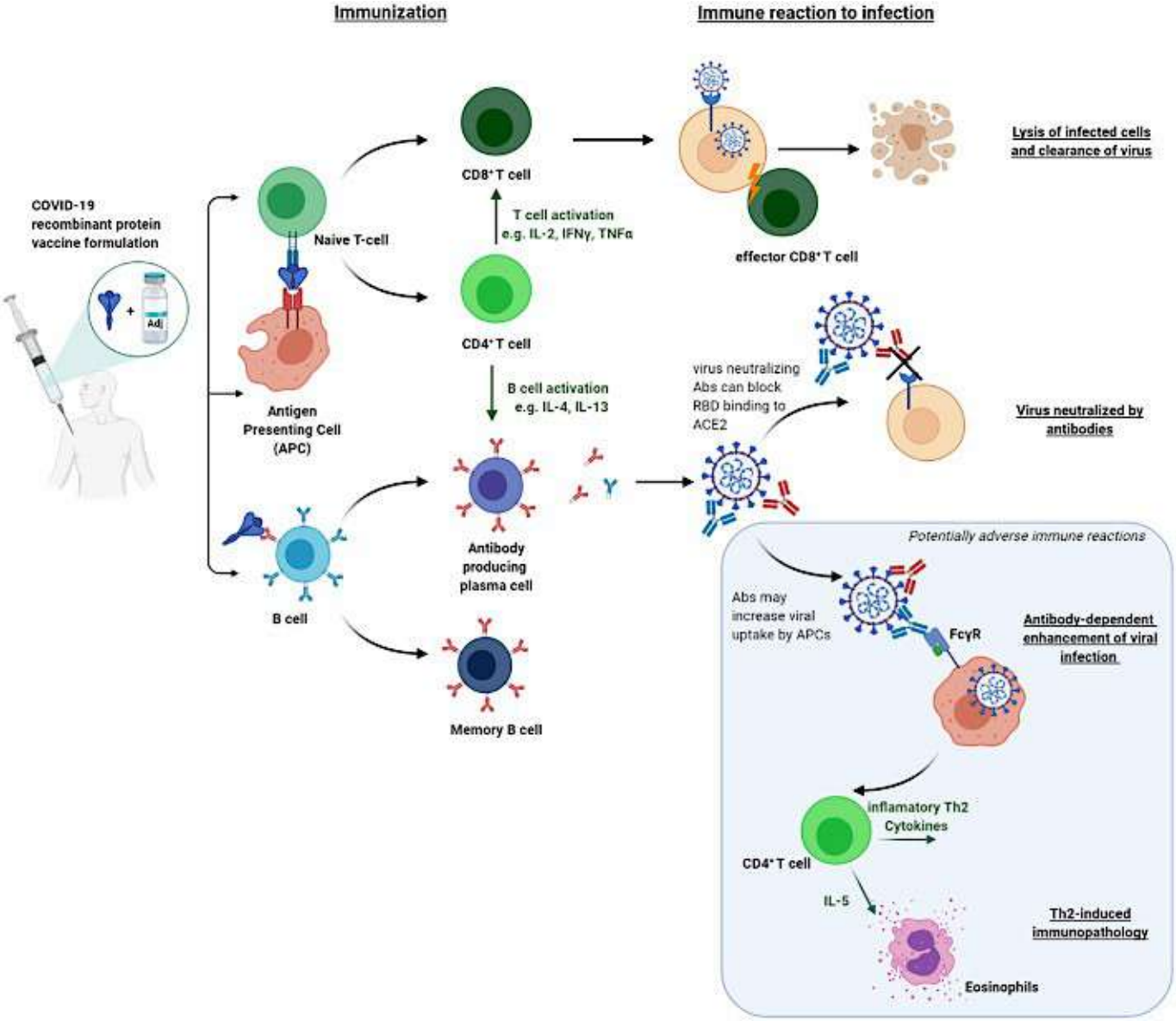
[229] Tseng CT, Sbrana E et al.
Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus
[published correction appears in PLoS One. 2012;7(8). doi:10.1371/annotation/2965cfae-b77d-4014-8b7b-236e01a35492]. PLoS One.
2012;7(4):e35421. doi:10.1371/journal.pone.0035421
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3335060/

Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S.
The spike protein of SARS-CoV--a target for vaccine and therapeutic development
Nat Rev Microbiol. 2009 Mar;7(3):226-36. doi: 10.1038/nrmicro2090. Epub 2009 Feb 9.
https://pubmed.ncbi.nlm.nih.gov/19198616/

Select recombinant protein vaccine candidates in clinical trials for COVID-19 as of December 8, 2020 [5]

Antigen	Vaccine developer	Platform/technology	Adjuvants
Full-length S-protein based vaccines			
Trimer	Novavax	Insect cells	Matrix M
S-protein	Sanofi Pasteur/GSK	Insect cells	2 different adjuvants (likely variants of AS03)
SCB-2019 trimer	Clover Biopharmaceuticals Inc./GSK/Dynavax	CHO cells	Alum+CpG 1018 or AS03
S-2P (MVC-COV1901)	Medigen Vaccine Biologics Corporation/NIAID/Dynavax	CHO cells	Alum+CpG1018
Covax-19	Vaxine Pty Ltd/Medytox	Insect cells	AdvaxCpG55.2
RBD-based vaccines			
AdimrSC-2f	Adimmune	Baculovirus/Sf9	Alum
SARS-CoV-2-RBDN1C1	Biological E/BCM	Yeast	Alum+CpG
FINLAY-FR-1/2	Instituto Finlay de Vacunas, Cuba		
KBP-201	Kentucky Bioprocessing, Inc	Plants	
RBD Dimer	Anhui Zhifei Longcom Biopharmaceutical/Institute of Microbiology, Chinese Academy of Sciences	CHO Cells	Aluminum preparation
RBD	West China Hospital, Sichuan University P	Insect Cells	Alum
Multi-epitope vaccines			
Multitope Peptide-based Vaccine (MPV)	COVAXX	Peptides	CpG and alum (AdjuPhos®)
EpiVacCoron	Vektor Laboratories, Russia	Chemical synthesis	Alum
CoVac-1	University Hospital Tübingen	Peptides	Montanide ISA51

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/pdf/main.pdf

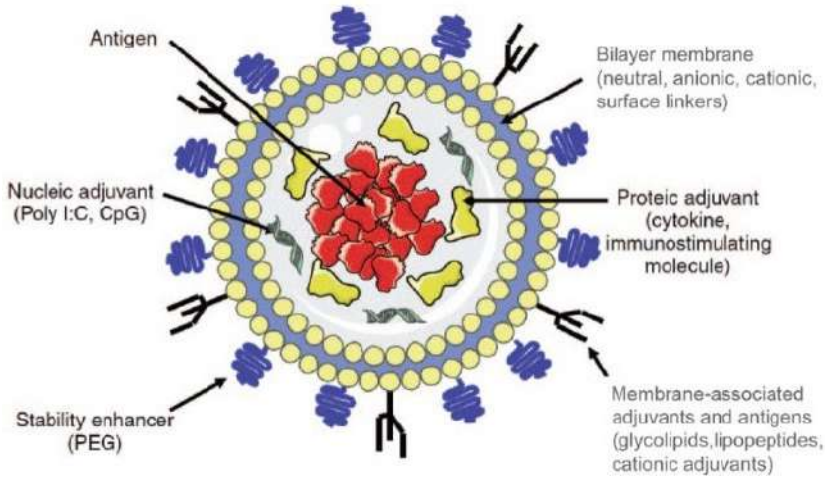


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/
Overview of immune reactions triggered by recombinant protein vaccines and their role in protection against COVID-19

VEHICLES FOR TRANSPORTING VACCINE ANTIGENS

Significant enhancement of vaccine-induced responses can be achieved by administering the vaccine antigen in a nontoxic delivery vehicle.
The vehicle protects vaccine components from protease or nuclease degradation (increasing their persistence in tissues) and may also act as adjuvants (inducing inflammation). Some vehicles facilitate the exposure of multiple molecules of the vaccine antigen on the vehicle surface, creating a polyvalent form of the antigen that increases its immunogenicity.
These properties have made transport vehicles critical for experimental vaccination for subunit and DNA vaccines.
Different types of transport vehicles have been devised, some of which are beginning to be used for human vaccination. [230]

Liposomes [231]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4212474/
Schematic representation of a small unilamellar liposome showing the versatility of incorporation of various compounds by encapsulation in the aqueous inner space or integration into the bilayer or surface attachment on the lipid bilayer membrane.
CpG, cytosine-phosphorothioate-guanine oligodeoxynucleotide;
PEG, poly (ethylene glycol)

Liposomes are prepared by mixing the vaccine antigen of interest with a phospholipid suspension under conditions that promote the formation of a spherical lipid bilayer structure.
The vaccine antigen is trapped in the aqueous center of the hydrophobic liposome, which is usually between 100 and 10,000 nm in size.
Liposomes are readily phagocytosed by DCs and macrophages, which means that the antigen is rapidly processed and used to initiate T-cell activation.
In addition, PRR ligands can be incorporated into liposomes to serve as adjuvants.

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[230] Chapter 14 - Vaccination, Editor(s): Tak W. Mak, Mary E. Saunders, Bradley D. Jett,
Primer to the Immune Response (Second Edition), Academic Cell, 2014, Pages 333-375, ISBN 9780123852458,
https://doi.org/10.1016/B978-0-12-385245-8.00014-5.
http://www.sciencedirect.com/science/article/pii/B9780123852458000145

Reshma J. Nevagi, Istvan Toth, Mariusz Skwarczynski,
12 - Peptide-based vaccines Editor(s): Sotirios Koutsopoulos,
Peptide Applications in Biomedicine, Biotechnology and Bioengineering,
Woodhead Publishing, 2018, Pages 327-358, ISBN 9780081007365,
https://doi.org/10.1016/B978-0-08-100736-5.00012-0.
http://www.sciencedirect.com/science/article/pii/B9780081007365000120

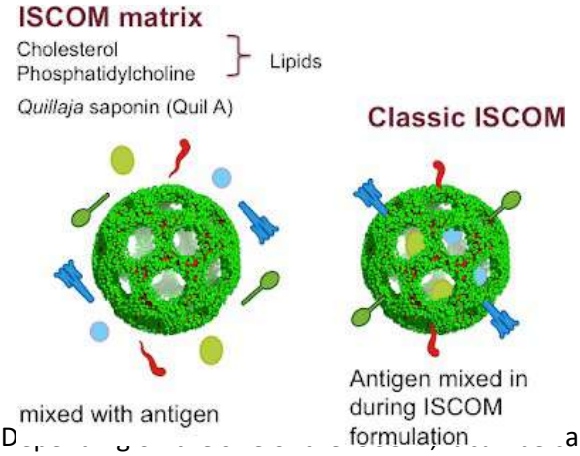
Foged, Camilla. (2015). Subunit Vaccine Delivery.
https://www.researchgate.net/publication/269311879_Subunit_Vaccine_Delivery/citation/download

[231] Wang N, Chen M, Wang T.
Liposomes used as a vaccine adjuvant-delivery system: From basics to clinical immunization.
J Control Release. 2019;303:130-150. doi:10.1016/j.jconrel.2019.04.025
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111479/

Schwendener RA.
Liposomes as vaccine delivery systems: a review of the recent advances.
Ther Adv Vaccines. 2014;2(6):159-182. doi:10.1177/2051013614541440
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4212474/

Liposomal vaccines for hepatitis A virus (HAV) and influenza are now licensed for use in some countries. A liposome-based malaria vaccine is currently being tested in clinical trials.

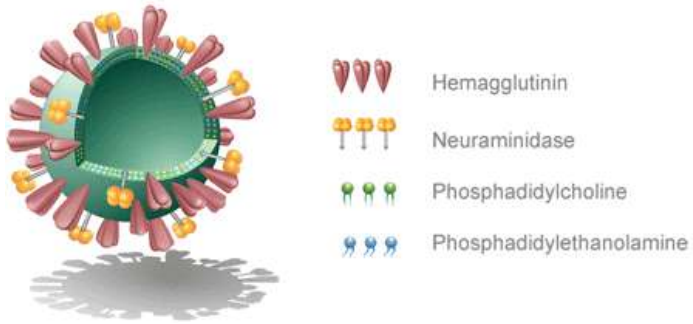
ISCOM [232]



"Immunostimulant complexes" are hollow spheres consisting of cholesterol, phospholipids and detergent. Relatively bulky protein immunogens can be easily inserted inside the sphere.
To the immune system, ISCOM resembles a multivalent antigen with a form that invites phagocytosis by APCs.
In addition, the detergent in ISCOM is a powerful adjuvant.

D alled a nanoparticle (50 nm) or a microparticle (1000-50,000 nm). Both types of particles fall within the size range of natural pathogens.

Virosomes [233]



Virosomes are like nonreplicating "artificial viruses" that can be used to deliver vaccine antigens directly into a host cell.
A virosome is basically a liposome coated with the envelope glycoproteins of a virus. Pathogen antigens of interest are captured within the lumen of the virosome or are chemically cross-linked to its surface.

https://www.biopharminternational.com/view/virosomes-novel-strategy-drug-delivery-and-targeting

Because of viral envelope proteins, a virosome can bind to and "infect" host cells and release antigen directly into the antigen processing pathway with class I MHCs.
Alternatively, the virosome can be phagocytosed by an APC.

A virosomal vaccine that incorporates the HA protein of influenza virus into liposomes is designed for immunization against seasonal influenza.

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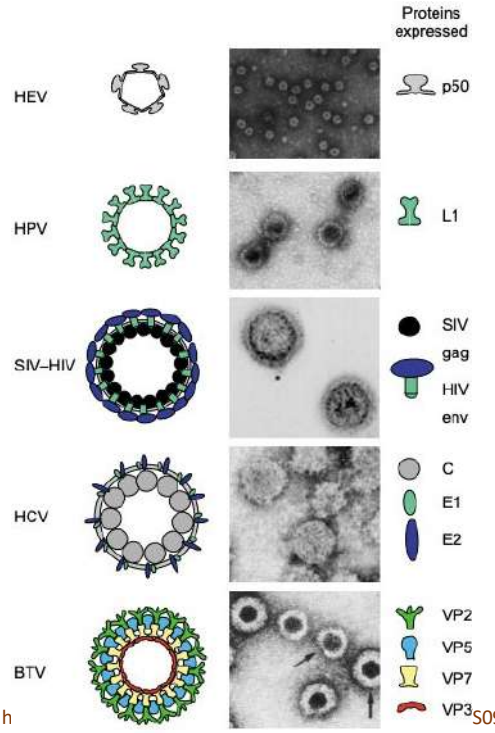
[232] Garcia A, Lema D.
An Updated Review of ISCOMSTM and ISCOMATRIXTM Vaccines.
Curr Pharm Des. 2016;22(41):6294-6299. doi: 10.2174/1381612822666160915161302.
https://www.researchgate.net/publication/308202867_An_Updated_Review_of_Iscoms_TM_and_Iscomatrix_TM_Vaccines

Pedersen JS, Oliveira CL, Hübschmann HB, et al.
Structure of immune stimulating complex matrices and immune stimulating complexes in suspension determined by small-angle x-ray scattering.
Biophys J. 2012;102(10):2372-2380. doi:10.1016/j.bpj.2012.03.071
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3353015/

[233] Basavaraj, K.N., et al.
Nanotechnology Based Virosomal Drug Delivery Systems.
J Nanotech Mater Sci (2014) 1(1):27-35. https://doi.org/10.15436/2377-1372.14.00
https://www.ommegaonline.org/article-details/Nanotechnology-Based-Virosomal-Drug-Delivery-Systems/59

Virus-like particles [234]



h S0966-842X(03)00208-7

Virus-like particles (VLPs) are 30-90 nm structures that result from the self-assembly of viral proteins without a nucleic acid genome or lipid envelope.
Sometimes it is the surface proteins of the virus of interest that form the rod-shaped or icosahedral VLP structure, or it is formed by the viral core proteins.
The surfaces of these VLPs have a repetitive structure that is ideal for inducing antibody production.
VLPs can also be constructed to contain PRR ligands as additional adjuvants.
Interestingly, although VLPs cannot replicate because they lack enzymes and nucleic acids, sometimes a VLP spontaneously assembles around an RNA or DNA fragment.
This capability offers vaccine developers the opportunity to manipulate a VLP and have it incorporate TLR7/9 ligands such as ssRNA or CpG sequences.

The storage of vaccines

Several vaccine formulations require constant refrigeration. The need for a cold chain makes their global distribution and application logistically difficult, and for underdeveloped and developing nations with tropical climates, nearly impossible. Indeed, one of the greatest challenges has been the dependence on refrigerated transport of vaccines in solution.

The World Health Organization estimated that about 2.8 million doses of vaccines were lost in five countries in 2011 because the cold chain was disrupted, and less than 10 percent of countries met WHO recommendations for effective vaccine management practices.
This included losses in countries such as Nigeria, where 41 percent of refrigerators were not working, and Ethiopia, which had about 30 percent of its cold chain equipment out of service.

The loss of millions of doses of COVID-19 vaccines could be disastrous for pandemic management.[235]

[234] Roldão A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM.
Virus-like particles in vaccine development.
Expert Rev Vaccines. 2010 Oct;9(10):1149-76. doi: 10.1586/erv.10.115. PMID: 20923267.
https://pubmed.ncbi.nlm.nih.gov/20923267/

Mohsen, M. O., Zha, L., Cabral-Miranda, G., & Bachmann, M. F.
Major findings and recent advances in virus-like particle (VLP)-based vaccines.
Seminars in Immunology (2017) 34, 123-132. doi:10.1016/j.smim.2017.08.014

Syomin BV, Ilyin YV.
Virus-Like Particles as an Instrument of Vaccine Production.
Mol Biol. 2019;53(3):323-334. doi:10.1134/S0026893319030154

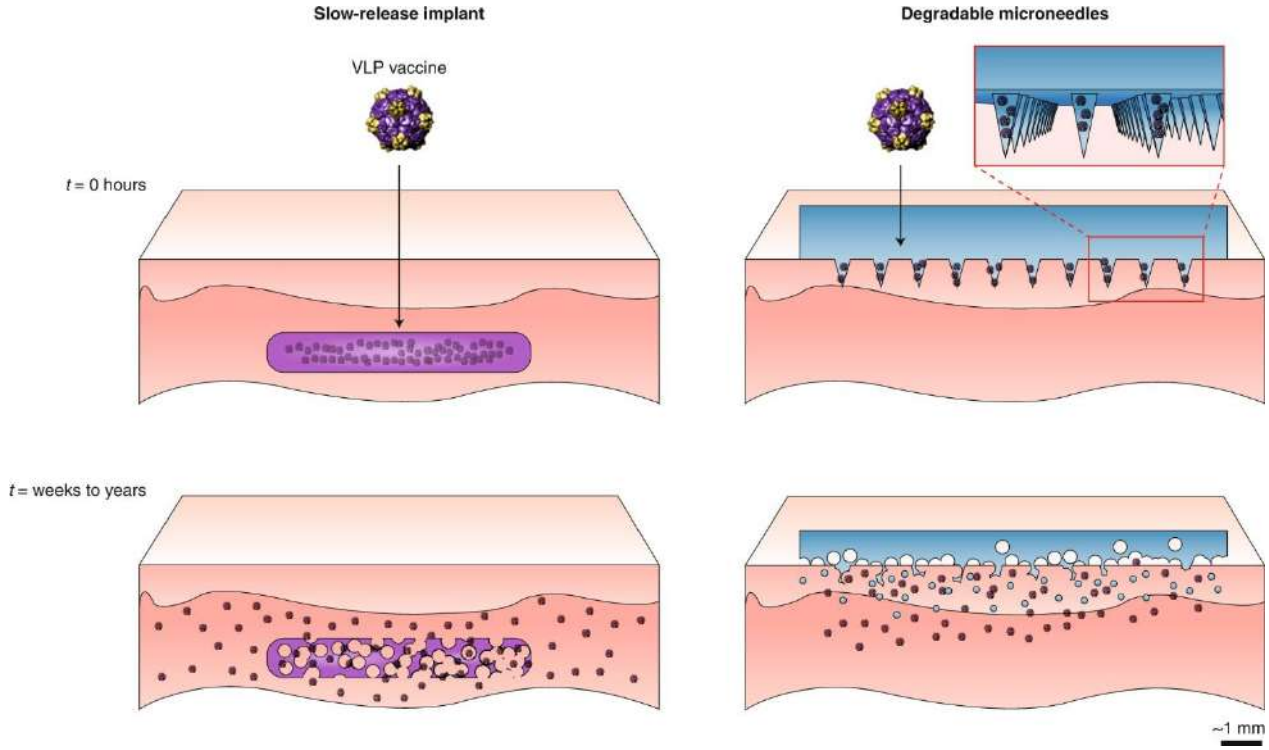
Noad, R., & Roy, P. (2003).
Virus-like particles as immunogens.
Trends in Microbiology, 11(9), 438-444. doi:10.1016/s0966-842x(03)00208-7
https://www.cell.com/trends/microbiology/comments/S0966-842X(03)00208-7

[235] https://www.who.int/immunization/call-to-action_ipac-iscl.pdf

Although some lyophilized vaccines are available that can be stored at room temperature, such solutions are difficult to produce and present challenges for health care providers who must reconstitute them on site. [236]

Vaccination campaigns to be efficient also require access to health care workers (HCPs), which is difficult in resource-poor or densely populated developing countries under normal circumstances, but poses a greater challenge during a global pandemic where the health care system is already under pressure.

Recently, modern alternatives to such distribution and access challenges have emerged, such as the use of slow-release single-dose implants[237], film-based vaccines [238] and micro-needle-based patches [239] that could reduce dependence on the cold chain and ensure vaccination even in situations where trained health workers are rare or in high demand.



https://www.nature.com/articles/s41565-020-0737-y
Vaccines are encapsulated in polymer components in an implant or patch with micro-needles. Over time, the polymer will hydrolyze in the aqueous environment of the body and release the active vaccine. The rate of degradation of the device and the subsequent rate of vaccine release can be adjusted according to the material in which the vaccine is incorporated. The main difference between the two devices is the mode of administration. Implants are administered subcutaneously by a trained health care provider, while micro-needle patches can be applied painlessly on their own.

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[236] Kristensen DD, Lorenson T, Bartholomew K, Villadiego S.
Can thermostable vaccines help address cold-chain challenges? Results from stakeholder interviews in six low- and middle-income countries.
Vaccine. 2016 Feb 10;34(7):899-904. doi: 10.1016/j.vaccine.2016.01.001. Epub 2016 Jan 8.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4744085/

[237] Engert, Julia. (2015). Implants as Sustained Release Delivery Devices for Vaccine Antigens.
10.1007/978-1-4939-1417-3_12. In book: Subunit Vaccine Delivery (pp.221-241).
https://www.researchgate.net/publication/278681098_Implants_as_Sustained_Release_Delivery_Devices_for_Vaccine_Antigens

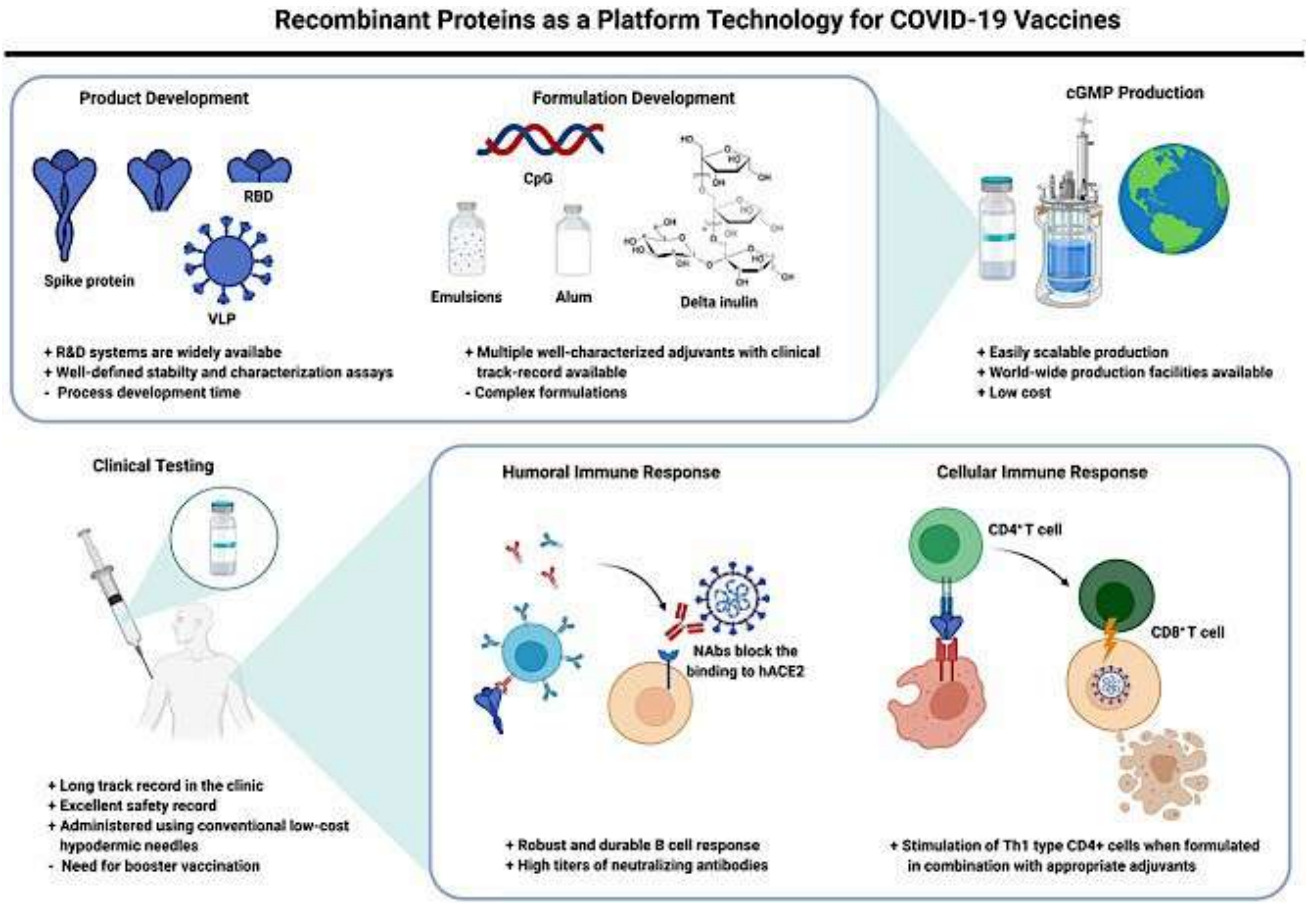
Bobbala S, Hook S.
Vaccine implants: current status and recent advances.
Emerg Top Life Sci. 2020 Dec 11;4(3):319-330. doi: 10.1042/ETLS20200164. PMID: 33231265.
https://pubmed.ncbi.nlm.nih.gov/33231265/

[238] Bajrovic I, Schafer SC, Romanovicz DK, Croyle MA.
Novel technology for storage and distribution of live vaccines and other biological medicines at ambient temperature.
Sci Adv. 2020;6(10):eaau4819. Published 2020 Mar 4. doi:10.1126/sciadv.aau4819
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7056310/

[239] Boopathy AV, Mandal A, Kulp DW, et al.
Enhancing humoral immunity via sustained-release implantable microneedle patch vaccination.
Proc Natl Acad Sci U S A. 2019;116(33):16473-16478. doi:10.1073/pnas.1902179116
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6697788/

VIRAL-LIKE PARTICLE VACCINES (VLP)

oRBD SARS-CoV-2 HBsAg VLP vaccine / *Serum Institute of India + Accelagen Pty*
oCoronavirus-Like Particle COVID-19 (CoVLP) / *Medicago Inc.*



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/

Virus-like particles (VLPs) are multi-protein nanostructures that mimic the organization and conformation of native wild-type viruses.
It is important to note that VLPs are natural byproducts that are generated during the infectious cycle of some viruses. [240]
Because these VLPs do not contain a viral genome, they are unable to replicate in cells and therefore should have an improved safety profile compared with attenuated viral vaccines. [241]

[240] Huang SN, Millman I, O'Connell A, Aronoff A, Gault H, Blumberg BS.
Virus-like particles in Australia antigen-associated hepatitis. An immunoelectron microscopic study of human liver.
Am J Pathol. 1972;67(3):453-470.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2032745/

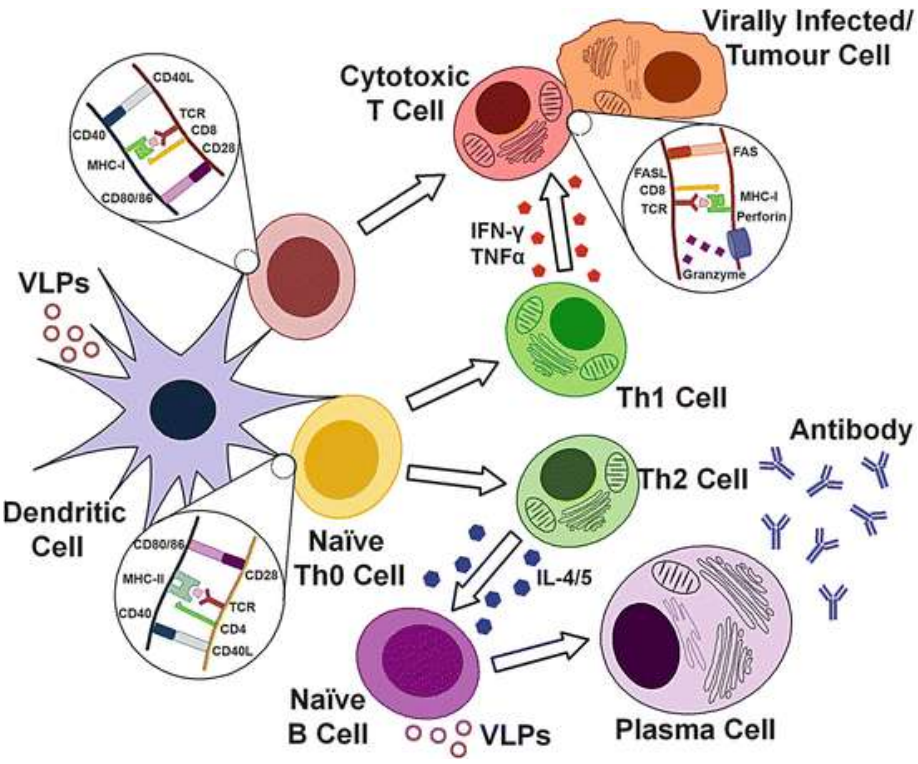
[241] Roldão A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM.
Virus-like particles in vaccine development.
Expert Rev Vaccines. 2010 Oct;9(10):1149-76. doi: 10.1586/erv.10.115.
https://www.tandfonline.com/doi/full/10.1586/erv.10.115?src=recsys

Mohsen MO, Zha L, Cabral-Miranda G, Bachmann MF.
Major findings and recent advances in virus-like particle (VLP)-based vaccines.
Semin Immunol. 2017 Dec;34:123-132. doi: 10.1016/j.smim.2017.08.014. Epub 2017 Sep 5.
https://pubmed.ncbi.nlm.nih.gov/28887001/

Brisse M, Vrba SM, Kirk N, Liang Y, Ly H.
Emerging Concepts and Technologies in Vaccine Development.
Front Immunol. 2020;11:583077. Published 2020 Sep 30. doi:10.3389/fimmu.2020.583077
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7554600/

The potency of these particles can be greatly improved if specific native viral proteins that have immunosuppressive function are excluded from the composition of VLPs.
Attenuation or inactivation is not required; this is especially important because epitopes are commonly modified by inactivation treatments. Compared with individual proteins or peptides, VLPs have conformational epitopes more similar to the native virus; therefore, antibody reactivity or immune system response should be significantly enhanced.

VLPs are taken up by dendritic cells, where they are processed and presented on MHC class I and II molecules to activate the adaptive immune response. Subsequent stimulation of CD8 T lymphocytes+ and CD4 helper T lymphocytes+ leads to activation of cell-mediated and B-cell responses, respectively (with antibody production) [242]. Consequently, VLP vaccines are considered highly immunogenic and can stimulate robust cellular and humoral immune responses due to the highly repetitive presence of antigenic epitopes. [243]



Foged, Camilla. (2015). Subunit Vaccine Delivery. p. 172
https://www.researchgate.net/publication/269311879_Subunit_Vaccine_Delivery/citation/download.
The immune response to VLPs. VLPs have the ability to stimulate both the cell-mediated and humoral immune systems. The desired immune response can be promoted through adjuvant selection

VLP vaccines use platforms capable of producing particles that mimic the structure of authentic viruses and can be produced by expressing antigenic proteins in a eukaryotic or prokaryotic system, resulting in particles with an intrinsic ability of the antigenic proteins to self-assemble. Alternatively, VLP vaccines can also be made by producing empty VLPs and chemically binding antigenic peptides onto the preformed particles. [244]

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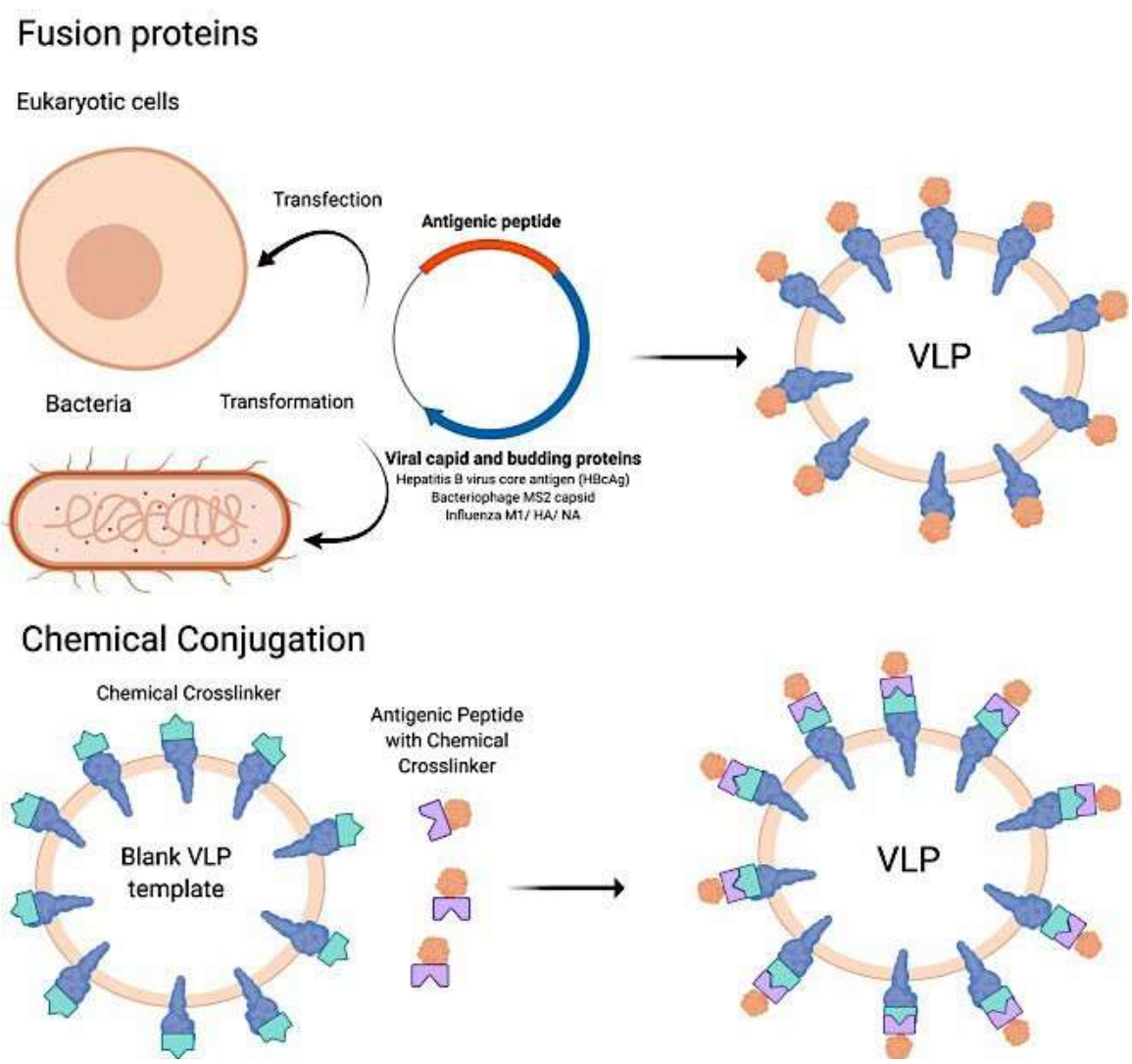
[242] Win SJ, Ward VK, Dunbar PR, Young SL, Baird MA.
Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway.
Immunol Cell Biol. 2011 Aug;89(6):681-8. doi: 10.1038/icb.2010.161. Epub 2011 Jan 11.
https://pubmed.ncbi.nlm.nih.gov/21221122/

Grgacic EV, Anderson DA.
Virus-like particles: passport to immune recognition.
Methods. 2006;40(1):60-65. doi:10.1016/j.ymeth.2006.07.018
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7128828/

[243] Noad R, Roy P.
Virus-like particles as immunogens.
Trends Microbiol. 2003 Sep;11(9):438-44. doi: 10.1016/s0966-842x(03)00208-7.
https://pubmed.ncbi.nlm.nih.gov/13678860/



Chackerian B.
Virus-like particles: flexible platforms for vaccine development.
Expert Rev Vaccines. 2007 Jun;6(3):381-90. doi: 10.1586/14760584.6.3.381.
https://pubmed.ncbi.nlm.nih.gov/17542753/

[244] Kushnir N, Streatfield SJ, Yusibov V.
Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development.
Vaccine. 2012;31(1):58-83. doi:10.1016/j.vaccine.2012.10.083
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7115575/

Ong HK, Tan WS, Ho KL.
Virus like particles as a platform for cancer vaccine development.
PeerJ. 2017;5:e4053. Published 2017 Nov 15. doi:10.7717/peerj.4053
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5694210/

Qian C, Liu X, Xu Q, et al.
Recent Progress on the Versatility of Virus-Like Particles.
Vaccines (Basel). 2020;8(1):139. Published 2020 Mar 20. doi:10.3390/vaccines8010139
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7157238/

Jeong H, Seong BL.
Exploiting virus-like particles as innovative vaccines against emerging viral infections.
J Microbiol. 2017;55(3):220-230. doi:10.1007/s12275-017-7058-3
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7090582/pdf/12275_2017_Article_7058.pdf

Liu J, Dai S, Wang M, Hu Z, Wang H, Deng F.
Virus like particle-based vaccines against emerging infectious disease viruses.
Virol Sin. 2016;31(4):279-287. doi:10.1007/s12250-016-3756-y
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7090901/pdf/12250_2016_Article_3756.pdf

Lua LH, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg AP.
Bioengineering virus-like particles as vaccines.
Biotechnol Bioeng. 2014 Mar;111(3):425-40. doi: 10.1002/bit.25159. Epub 2013 Dec 17.
https://pubmed.ncbi.nlm.nih.gov/24347238/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7554600/
Outline of the production of VLP vaccines. The methodology for producing VLP vaccines is summarized in this vignette. Briefly, VLP vaccines are produced by transfecting eukaryotic cells or transforming bacterial cells with a DNA plasmid that encodes an antigenic peptide attached to a viral capsid and/or other protein that is sufficient to form VLPs. The antigenic peptide is present outside the VLP that becomes available for interaction with the immune system. Antigens conjugated with a chemical crosslinker can also be attached to VLPs containing external proteins conjugated to a complementary chemical crosslinker, which will result in antigens attached to the VLP and presented on the outer edges.

In general, VLPs can be classified into **VLPs without casing and with casing**.
The former are self-assembled with at least one viral protein that is expressed using a suitable expression host system such as mammalian cells, insect cells, yeast, bacteria, and acellular systems without acquisition of any host component.
Yeast and mammalian cells are the most commonly used expression systems in the production of commercial VLP-based vaccines of HBV and HPV. [245]
The production of more structurally complex envelope-free chimeric VLPs allows the exposure of heterologous epitopes or peptides on the surface of VLPs created by genetic engineering (Fig. 1A) [246].
Envelope-less VLPs can also be chemically conjugated to a target antigen via a hetero-bifunctional chemical linker such as sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) and a nano-collant, in which the chimeric VLPs produced without extensive genetic alterations can be present, and this overcomes the limitation imposed on VLP formation (Fig. 1B) [247].

On the other hand, **enveloped VLPs** acquire part of host cell membranes as part of their lipid envelope, where a foreign epitope or peptide can be integrated and displayed on the surface [248].
Similar to VLPs without envelopes, VLPs with envelopes can be produced by expressing different viral structural proteins in a suitable expression system.
Alternatively, a protein transfer technique can be used to expose epitopes or heterologous peptides on the surface of enveloped VLPs.
This approach allows the spontaneous incorporation of glycosylphosphatidylinositol (GPI)-anchored protein or other immunostimulatory molecules into the lipid bilayer of enveloped VLPs via a simple incubation step (Fig. 1C) [249].
The main advantage of protein transfer technology in producing VLPs with chimeric envelope is its ability to maintain the functionality of the incorporated protein without extensive genetic modification. [250]

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[245] Kushnir N, Streatfield SJ, Yusibov V.
Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development.
Vaccine. 2012;31(1):58-83. doi:10.1016/j.vaccine.2012.10.083
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7115575/

[246] Yong CY, Yeap SK, Ho KL, Omar AR, Tan WS.
Potential recombinant vaccine against influenza A virus based on M2e displayed on nodaviral capsid nanoparticles.
Int J Nanomedicine. 2015;10:2751-2763. Published 2015 Apr 2. doi:10.2147/IJN.S77405
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4396508/

[247] Jemon K, Young V, Wilson M, et al.
An enhanced heterologous virus-like particle for human papillomavirus type 16 tumor immunotherapy.
PLoS One. 2013;8(6):e66866. Published 2013 Jun 14. doi:10.1371/journal.pone.0066866
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3682997/

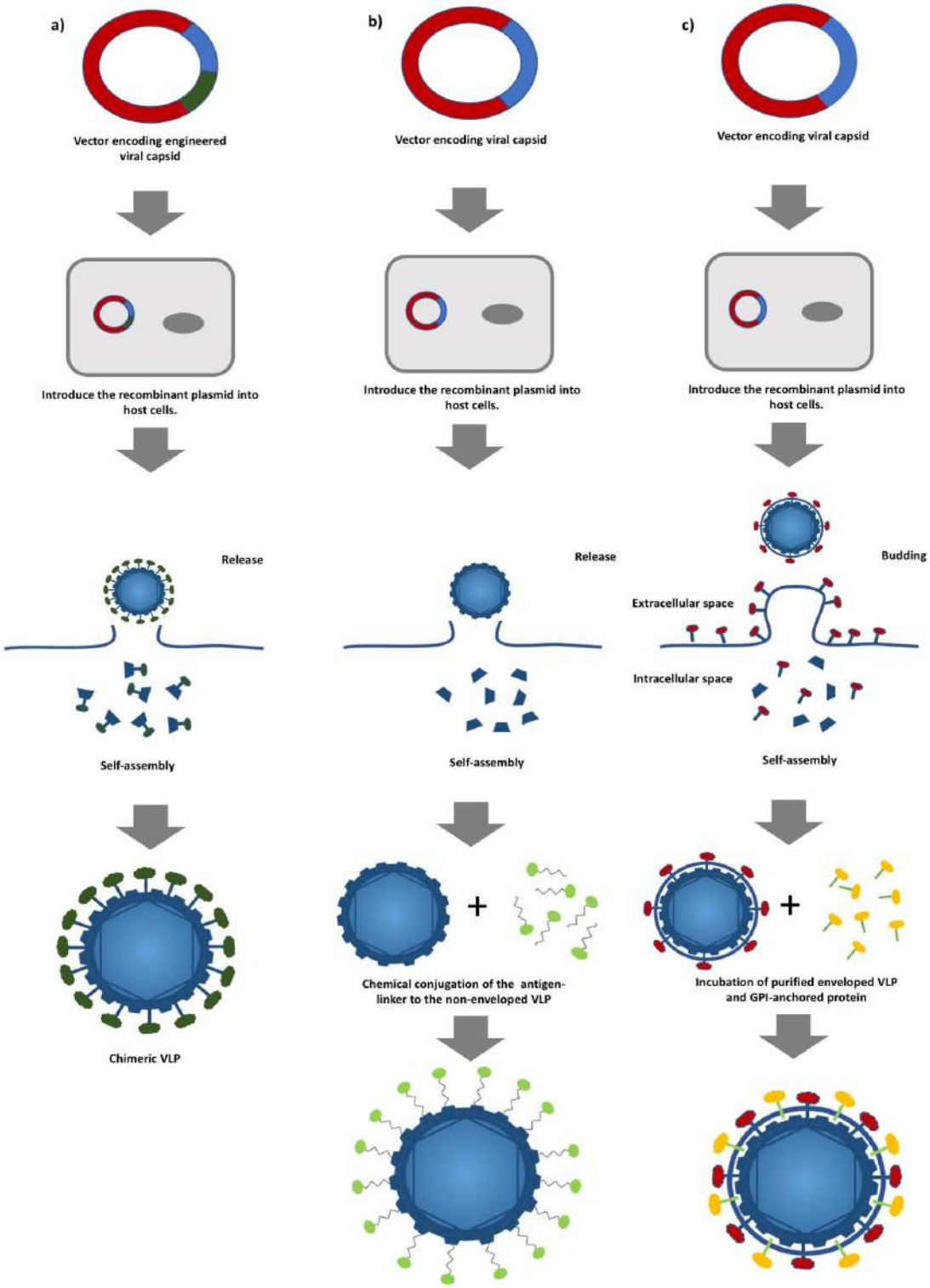
Lee KW, Tey BT, Ho KL, Tejo BA, Tan WS.
Nanoglue: an alternative way to display cell-internalizing peptide at the spikes of hepatitis B virus core nanoparticles for cell-targeting delivery.
Mol Pharm. 2012 Sep 4;9(9):2415-23. doi: 10.1021/mp200389t. Epub 2012 Jul 26.
https://pubmed.ncbi.nlm.nih.gov/22775561/

[248] Haynes JR.
Influenza virus-like particle vaccines.
Expert Rev Vaccines. 2009 Apr;8(4):435-45. doi: 10.1586/erv.09.8.
https://pubmed.ncbi.nlm.nih.gov/19348559/

[249] Patel JM et al.
Protein transfer-mediated surface engineering to adjuvant virus-like nanoparticles for enhanced anti-viral immune responses.
Nanomedicine. 2015;11(5):1097-1107. doi:10.1016/j.nano.2015.02.008
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4512837/

[250] Shashidharamurthy R, Bozeman EN, Patel J, Kaur R, Meganathan J, Selvaraj P. Immunotherapeutic strategies for cancer treatment: a novel protein transfer approach for cancer vaccine development.

In addition, due to the viral origin of VLPs, some of the VLP-based vaccines are self-adjuvanting in that they contain the pathogen-associated molecular pattern (PAMP) of viruses that are able to enhance activation of the innate immune system through Toll-like receptors and recognition pattern receptors. [251]

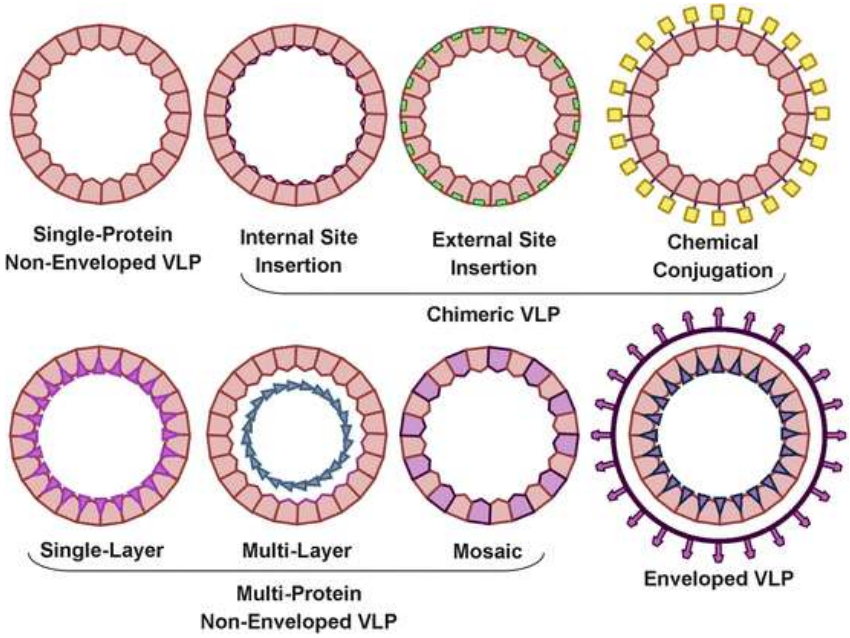


Med Res Rev. 2012 Nov;32(6):1197-219. doi: 10.1002/med.20237. Epub 2011 Jan 16. PMID: 23059764.
https://pubmed.ncbi.nlm.nih.gov/23059764/

[251] Crisci E, Bárcena J, Montoya M.
Virus-like particles: the new frontier of vaccines for animal viral infections.
Vet Immunol Immunopathol. 2012;148(3-4):211-225. doi:10.1016/j.vetimm.2012.04.026
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7112581/

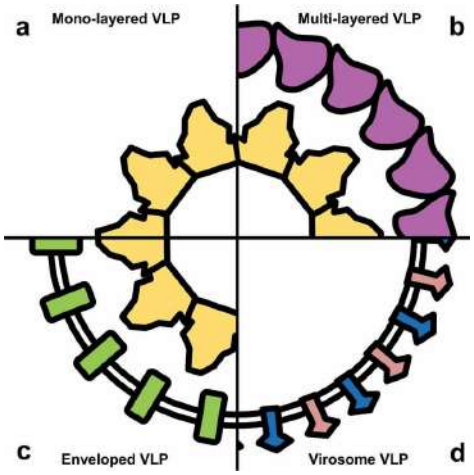
Rynda-Apple A, Patterson DP, Douglas T.
Virus-like particles as antigenic nanomaterials for inducing protective immune responses in the lung.
Nanomedicine (Lond). 2014;9(12):1857-1868. doi:10.2217/nnm.14.107
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4415509/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5694210/
A schematic diagram of the production of virus-like particles (VLPs) using different approaches.
(A) Production of chimeric VLPs without envelope by genetic engineering. The viral structural protein is fused with a foreign antigen by genetic engineering followed by expression of the chimeric protein in a suitable host system**. (B)** Production of chimeric VLPs without envelope by chemical conjugation. Envelope-free VLPs are produced by expressing a viral structural protein and surface decoration of VLPs is achieved by conjugating a foreign antigen to VLPs and **(C)** Production of chimeric VLPs with envelope by protein transfer approach. Enveloped VLPs are produced by expressing viral proteins in suitable host cells followed by incubation with glycosylphosphatidylinositol (GPI)-anchored proteins. Foreign antigens are then transferred to the lipid bilayer of VLPs.



Foged, Camilla. (2015). Subunit Vaccine Delivery. p. 162
https://www.researchgate.net/publication/269311879_Subunit_Vaccine_Delivery/citation/download.

VLP structure.
VLPs can be classified according to structural features such as capsid protein composition, encapsulation within a lipid bilayer envelope, and antigen incorporation b y recombinant insertion or chemical conjugation. Additional combinations are multiprotein chimeric VLPs and envelope mosaic or chimeric VLPs.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103734/
Structural biodiversity of VLPs.
VLP can be produced with a variety of structural morphologies defined by the structure of the parent virus. These morphologies include **(a)** monolayer VLPs, usually consisting of a single virus capsid protein; **(b)** multilayer VLPs, formed by multiple capsid proteins expressed simultaneously; **(c)** enveloped VLPs, with a lipid bilayer formed on the VLP capsid; and (**d)** virosomes, consisting of proteins embedded within a lipid bilayer envelope.

VLP EXPRESSION SYSTEMS

Various cell substrates including bacteria, yeast, insect, mammalian and plant expression systems have been used to produce VLPs.

Table 9.2 VLP expression systems

	Advantages	Limitations
Bacteria (e.g. *Escherichia coli*)	• Rapid cell growth	• No post-translational modification
	• Highest yield	• Limited applications for mammalian VLPs
	• Low production cost	• May form inclusion bodies
	• Scalable	• Requires removal of endotoxins
Yeast (e.g. *Saccharomyces cerevisiae*) 	• Rapid cell growth	• Limited post-translational modification
	• High yield	• May form inclusion bodies
	• Low production cost	
	• Scalable	
	• Already has some regulatory approval	
Insect cells/Baculovirus (e.g. *Spodoptera frugiperda*) 	• Average cell growth	• Requires removal of baculovirus proteins
	• High yield	• May form inclusion bodies
	• Scalable	
	• Complex post-translational modification	
	• Formation of multi-protein VLP	
Plant cells (e.g. *Nicotiana* sp.) 	• Rapid production	• Limited post-translational modification
	• Low production cost	• Relatively new system
	• Scalable	
Mammalian cells (e.g. Chinese hamster ovary cells) 	• Scalable	• Slow growth
	• Complex post-translational modification	• Low yield
	• Formation of multi-protein	• Demanding culture conditions
	• VLP	• High production cost
		• Potential infectious contamination
Cell free 	• Almost exclusive production of target protein	• Very high production cost
	• Limited cellular contaminants	• Limited scalability
	• Enables production of VLPs containing non-natural amino acids or toxic protein intermediates	• Relatively new system, not well characterised

Adapted from Rebeaud and Bachmann (2012)

Foged, Camilla. (2015). Subunit Vaccine Delivery. p. 159
https://www.researchgate.net/publication/269311879_Subunit_Vaccine_Delivery/citation/download.

Bacteria

Bacteria are the most widely used expression system for recombinant protein production, but they are not a preferred platform for VLP production due to a number of factors, including the absence of a post-translational modification (PTM) system as occurs in mammalian cells.

However, bacteria are used to produce VLPs without envelopes based on components of a pathogen with the ability to self-assemble in the bacterial host or as fusions of the vaccine target antigen with bacteriophage surface proteins (bacteriophage-based VLP vaccines).[252]

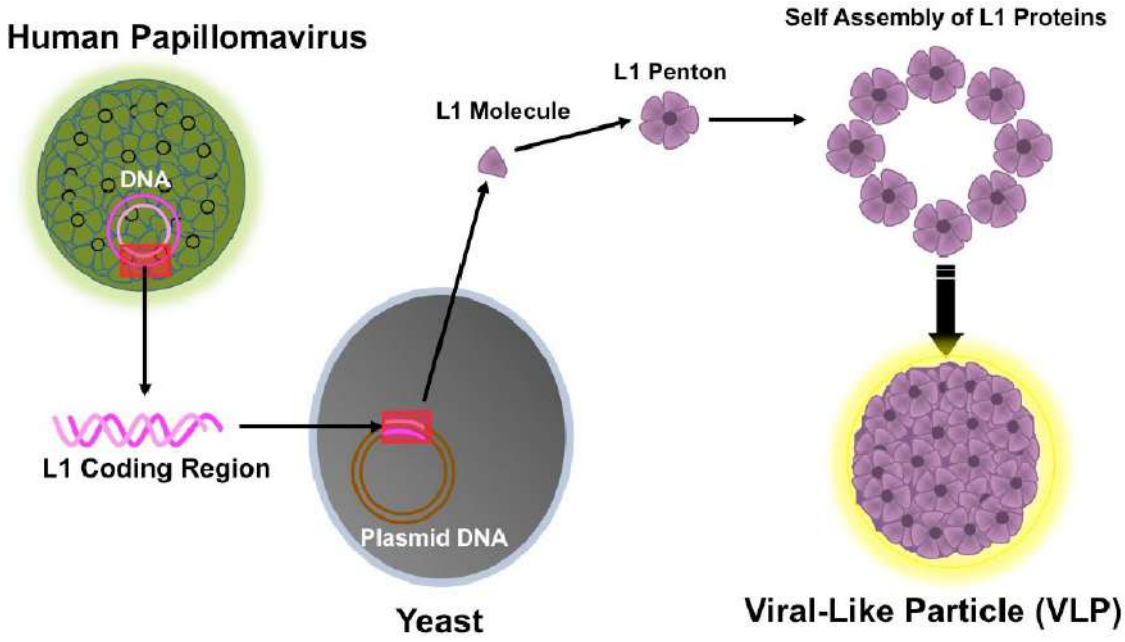
[252] Tissot AC, Renhofa R, Schmitz N, et al.
Versatile virus-like particle carrier for epitope-based vaccines.
PLoS One. 2010;5(3):e9809. Published 2010 Mar 23. doi:10.1371/journal.pone.0009809
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2843720/

Yeast

Yeast is an established platform for recombinant protein expression and continues to be used for VLP production.

Two VLP-based vaccines licensed by Merck and Co., Inc, Recombivax HB® and Gardasil®, have been produced using this system, which, however, has some limitations, as it differs from mammalian cells in protein PTM, particularly in glycosylation.

Because of this and other limitations, the system is generally used for the production of VLPs without casing. [253]



https://www.corvelva.it/speciale-corvelva/vaccinegate/approfondimenti-sulle-sequenze-di-acidi-nucleici-dna-e-rna-relativi-al-frammento-l1-del-genome-of-hpv-in-gardasil-9.html

From the quality survey on **GARDASIL 9 ®** conducted by Corvelva, the following contaminations were found to be present following interlaboratory analysis: [254]

metagenomic analyses:

- confirmation of the presence of L1 fragments 18, 16, 6 (RNA) present in higher copy numbers of HPV genome 1. The presence of L1 fragment DNA residues in HPV vaccine has already been reported in the studies of Prof. Lee, [255] who hypothesized that the presence of aluminum stabilizes the degradation of the

Middelberg AP, Rivera-Hernandez T, Wibowo N, Lua LH, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR.
A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines.
Vaccine. 2011 Sep 22;29(41):7154-62. doi: 10.1016/j.vaccine.2011.05.075. epub 2011 Jun 7. PMID: 21651936.
https://pubmed.ncbi.nlm.nih.gov/21651936/

Zhang W, Carmichael J, Ferguson J, Inglis S, Ashrafian H, Stanley M.
Expression of human papillomavirus type 16 L1 protein in Escherichia coli: denaturation, renaturation, and self-assembly of virus-like particles in vitro. Virology. 1998 Apr 10;243(2):423-31. doi: 10.1006/viro.1998.9050.
https://www.sciencedirect.com/science/article/pii/S004268229899050X?via%3Dihub

[253] Kim SN, Jeong HS, Park SN, Kim HJ.
Purification and immunogenicity study of human papillomavirus type 16 L1 protein in Saccharomyces cerevisiae.
J Virol Methods. 2007 Jan;139(1):24-30. doi: 10.1016/j.jviromet.2006.09.004. Epub 2006 Oct 10.
https://pubmed.ncbi.nlm.nih.gov/17034867/

[254] https://www.corvelva.it/speciale-corvelva/vaccinegate/sommario-delle-conferme-dei-dati-tramite-analisi-interlaboratorio.html

[255] Lee SH.
Detection of human papillomavirus (HPV) L1 gene DNA possibly bound to particulate aluminum adjuvant in the HPV vaccine Gardasil.
J Inorg Biochem. 2012 Dec;117:85-92. doi: 10.1016/j.jinorgbio.2012.08.015. Epub 2012 Aug 30.

viral fragments, enhancing their ability to activate a powerful long-term inflammatory response and to be transported through the lymphatic system into macrophages in various districts of the body.

- **Adventitious viruses and genetic material**: Molluscum contagious, viruses (Myoviridae), Phages, Retroviruses (DNA): Murine leukemia virus, Human endogenous retrovirus K; Retroviruses (RNA): Murine leukemia virus; Yeast and its viruses; Human and mouse DNA/RNA (trace a m o u n t s)

Insights into nucleic acid sequences related to the L1 fragment of the HPV genome in Gardasil 9

mass spectrometry analysis:
- high number of signals of chemical contaminants of which only 22% were known from the database comparison.
- Identification of **APDB** (illegal amphetamine) notified to NAS on May 23, 2019

Amazing APDB report in Gardasil 9 sample

Insect cells

Another system that has been widely used for VLP production is the expression system with **Baculovirus** in insect cells.

Similar to yeast, insect cells have been used to produce a number of VLP-based vaccines, most notably, one of the current HPV vaccines, Cervarix®.

Insect cells can be used for the production of VLPs either without envelopes or with envelopes.
VLP vaccines with envelopes are among the most advanced in clinical development because the insect cell system possesses eukaryotic-type PTMs including glycosylation, harbors high-level accumulation of foreign proteins, and lacks mammalian pathogens [256].

Contamination with coproduced envelope baculovirus particles [257] is the main limitation of this system, requiring the development of more complex schemes for VLP purification.

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https://pubmed.ncbi.nlm.nih.gov/23078778/
Lee, Sin. (2012). Detection of human papillomavirus L1 gene DNA fragments in postmortem blood and spleen after Gardasil® vaccination-A case report. Advances in Bioscience and Biotechnology. 03. 1214-1224. 10.4236/abb.2012.38148...
https://www.researchgate.net/publication/273751788_Detection_of_human_papillomavirus_L1_gene_DNA_fragments_in_postmortem_blood_and_spleen_after_GardasilR_vaccination-A_case_report

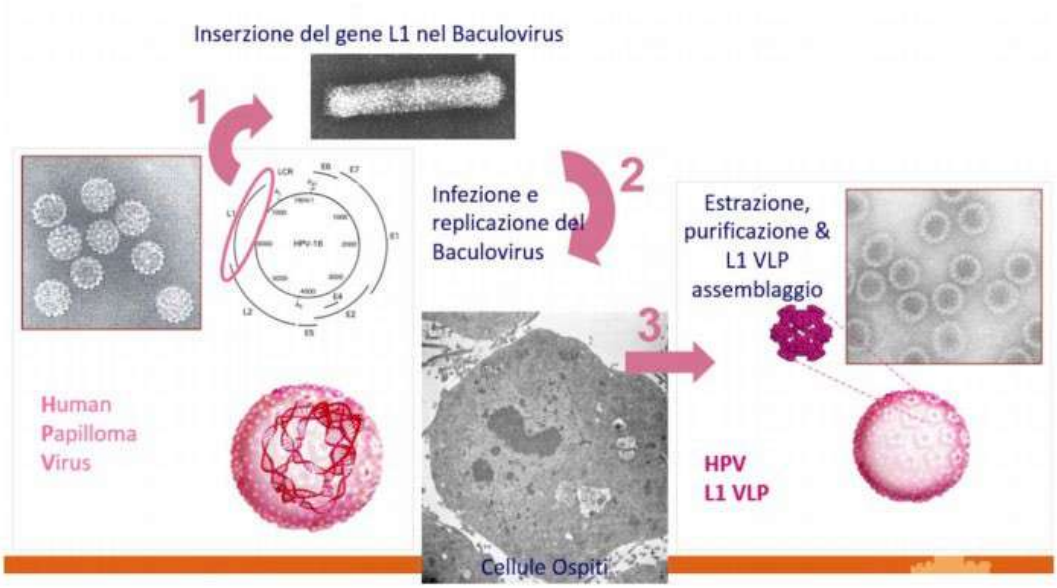
Lee, Sin. (2013). Topological conformational changes of human papillomavirus (HPV) DNA bound to an insoluble aluminum salt-A study by low temperature PCR. Advances in Biological Chemistry. 03. 76-85. 10.4236/abc.2013.31010.
https://www.researchgate.net/publication/273751597_Topological_conformational_changes_of_human_papillomavirus_HPV_DNA_bound_to_an_insoluble_aluminum_salt-A_study_by_low_temperature_PCR

Lee SH.
Melting profiles may affect detection of residual HPV L1 gene DNA fragments in Gardasil®.
Curr Med Chem. 2014 Mar;21(7):932-40. doi: 10.2174/09298673219991401021109 33.
https://pubmed.ncbi.nlm.nih.gov/24083601/

[256] Roy P, Noad R.
Virus-like particles as a vaccine delivery system: myths and facts.
Adv Exp Med Biol. 2009;655:145-158. doi:10.1007/978-1-4419-1132-2_11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7124136/pdf/978-1-4419-1132-2_Chapter_11.pdf

Zepeda-Cervantes J, Ramírez-Jarquín JO, Vaca L.
Interaction Between Virus-Like Particles (VLPs) and Pattern Recognition Receptors (PRRs) From Dendritic Cells (DCs): Toward Better Engineering of VLPs.
Front Immunol. 2020 Jun 9;11:1100. doi: 10.3389/fimmu.2020.01100.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7297083/

[257] Hervas-Stubbs S, Rueda P, Lopez L, Leclerc C.
Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN.
J Immunol. 2007 Feb 15;178(4):2361-9. doi: 10.4049/jimmunol.178.4.2361. Erratum in: J Immunol. 2007 May 15;178(10):6653.
https://www.jimmunol.org/content/178/4/2361.long



https://salute.regione.emilia-romagna.it/screening/tumori-femminili/documentazione/atti-relativi-a-convegni-e-seminari-regionali/la-sorveglianza-epidemiological-screening-of-neck-cancers-of2019utero-in-emilia-romagna-15-march-2018/Pascucci.pdf

Mammalian and bird cells

VLPs of HBsAg were successfully produced in mammalian cells (Chinese hamster ovary [CHO] cell line) and found to be similar in composition and glycosylation to plasma-derived VLPs of HBsAg proteins and to yeast-produced VLPs for lipid content.

They were larger in size and consisted of more monomers than yeast-derived VLPs, contributing to the higher immunogenicity of mammalian-derived VLPs.
Moreover, in contrast to yeast-derived HBsAg VLPs, HBsAg VLPs produced by CHO cells were shown to have a tubular structure.[258]

Plant cells

Over the past two decades, plants have shown great potential for producing recombinant proteins for industrial or pharmaceutical purposes, including vaccine development.

As production platforms, plant expression systems are rapid, highly scalable, inexpensive, and free of pathogens that may be present in mammals.
In addition, the structure, assembly, and PTMs of proteins in plants are similar to those in mammalian cells.
Recombinant protein expression in plants can be achieved by stable introduction of a transgene into the nuclear or plastid genome, or by transient transformation using plant viral vectors [259].

[258] Diminsky D, Schirmbeck R, Reimann J, Barenholz Y.
Comparison between hepatitis B surface antigen (HBsAg) particles derived from mammalian cells (CHO) and yeast cells (Hansenula polymorpha): composition, structure and immunogenicity.
Vaccine. 1997 Apr-May;15(6-7):637-47. doi: 10.1016/s0264-410x(96)00239-3.
https://pubmed.ncbi.nlm.nih.gov/9178464/

Patzer EJ, Nakamura GR, Simonsen CC, Levinson AD, Brands R.
Intracellular assembly and packaging of hepatitis B surface antigen particles occur in the endoplasmic reticulum. J Virol. 1986;58(3):884-892. doi:10.1128/JVI.58.3.884-892.1986
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC252996/

[259] Mett V, Farrance CE, Green BJ, Yusibov V.
Plants as biofactories. Biologicals. 2008 Nov;36(6):354-8. doi: 10.1016/j.biologicals.2008.09.001. Epub 2008 Oct 19.
https://pubmed.ncbi.nlm.nih.gov/18938088/

Yusibov V, Rabindran S.
Recent progress in the development of plant derived vaccines. Expert Rev Vaccines. 2008 Oct;7(8):1173-83. doi: 10.1586/14760584.7.8.1173.
https://pubmed.ncbi.nlm.nih.gov/18844592/

As with mammalian, insect and yeast cells, plants can be used to produce VLPs either without envelopes or with envelopes. [260]

Plant-made VLP vaccines have been produced on a large scale according to good manufacturing practice (cGMP) standards and have progressed in clinical development. [261]

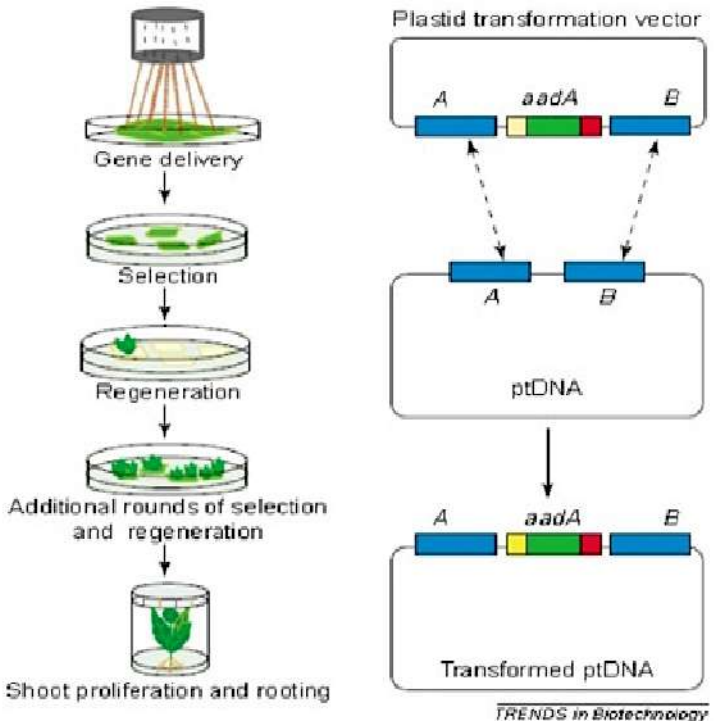


Fig.1

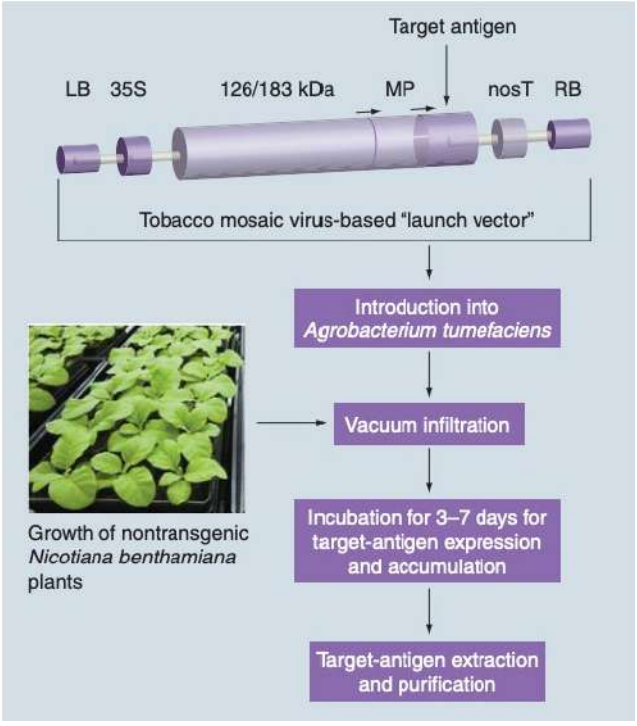


Fig.2

Fig.1 https://upbiotech.wordpress.com/2018/12/27/trasformazione-dei-plastidi/
Transformation within plastids is based on two homologous recombination events that occur between a region of a plasmid vector and the plastid genome. The vector used for transformation is an E.coli plasmid that contains, in addition to the two sequences homologous to the plastid genome required for recombination, the gene of interest and a marker gene useful for the efficient selection of transformed plants from nontransformed ones. One of the most widely used markers in this type of procedure is the aad gene that confers resistance to spectinomycin and streptomycin.

Fig.2 https://www.researchgate.net/publication/23308940_Recent_progress_in_the_development_of_plant-derived_vaccines
Schematic diagram of the tobacco mosaic virus-based launch vector system for the production of vaccine antigens in nontransgenic plants. Arrows indicate the positions of subgenomic mRNA promoters. The target antigen replaces the sequence encoding the coating protein. LB and RB refer to the left and right edges, respectively, of the T-DNA in the Agrobacterium tumefaciens binary vector; this T-DNA is transferred into the nuclei of plant cells following agroinfiltration; 35S: 35S promoter of cauliflower mosaic virus (a plant DNA virus) that drives transgene transcription; MP: movement protein required for cell-to-cell movement; nosT: nos transcriptional terminator from the nopaline synthase gene of A. tumefaciens; 126 / 183kDa: tobacco mosaic virus replicase protein required for virus replication.

Cell-free systems

VLPs can also be made in vitro under cell-free conditions[262] and be assembled into virosomes using membrane-like structures of VLPs with envelopes, or using polypeptide scaffolds to create nano-VLPs similar to VLPs without envelopes.

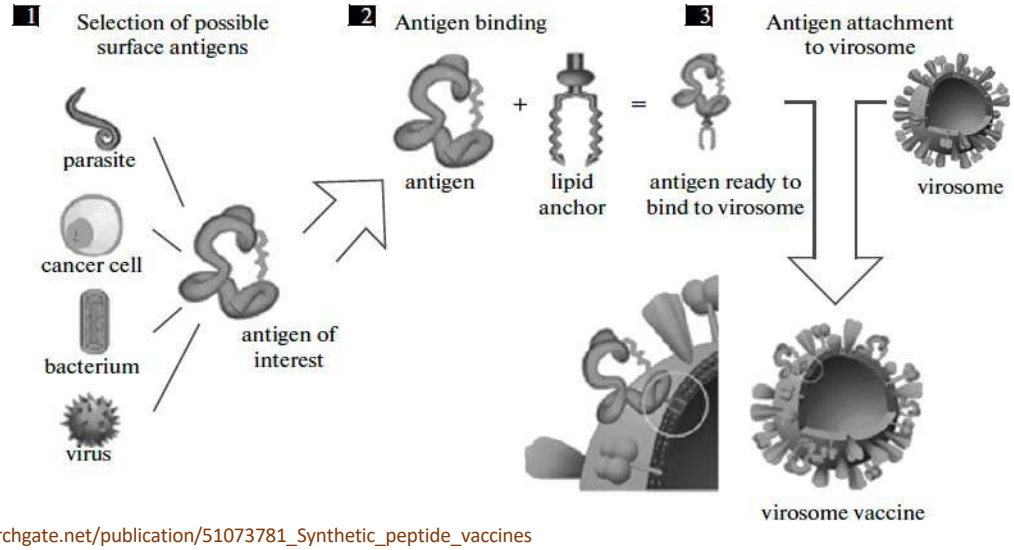
[260] D'Aoust MA, Lavoie PO, Couture MM, Trépanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vézina LP.
Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice.
Plant Biotechnol J. 2008 Dec;6(9):930-40. doi: 10.1111/j.1467-7652.2008.00384.x.
https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1467-7652.2008.00384.x

Santi L, Batchelor L, Huang Z, et al.
An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles.
Vaccine. 2008;26(15):1846-1854. doi:10.1016/j.vaccine.2008.01.053
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2744496/

[261] Landry N, Ward BJ, Trépanier S, et al.
Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza.
PLoS One. 2010;5(12):e15559. Published 2010 Dec 22. doi:10.1371/journal.pone.0015559
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3008737/

[262] Lua LH, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg AP.
Bioengineering virus-like particles as vaccines.

Nano-VLPs are one of the latest developments in this field and have only been tested in a pre-clinical setting.[263]
The approach uses the natural properties of a polypeptide's amino acids to self-assemble and allows the insertion of immunogenic epitopes. [264]
Virosomes essentially represent enveloped VLPs assembled in vitro rather than within a host cell.



https://www.researchgate.net/publication/51073781_Synthetic_peptide_vaccines
Virosome-based vaccine: stages of virosome vaccine development.

Fig.1 https://www.futuremedicine.com/doi/10.2217/ebo.11.8
(A) influenza virus. (B) influenza virosome without payload. (C) influenza virosome showing non-influenza antigen on its surface.

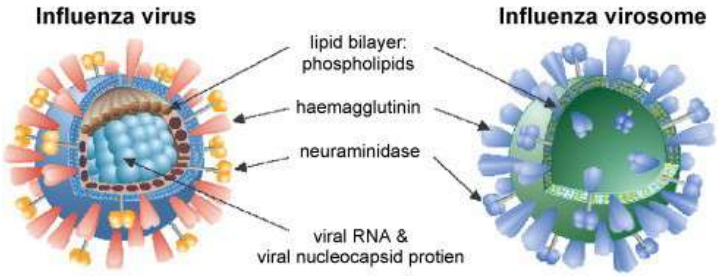


Fig. 2 https://www.sciencedirect.com/science/article/pii/S0264410X09007063
Virosomes are reconstituted influenza virus envelopes lacking internal nucleus and genetic information. Mimicking native influenza viruses, virosomes retain cellular entry and membrane fusion properties that allow presentation to the MHC class I and II pathway.

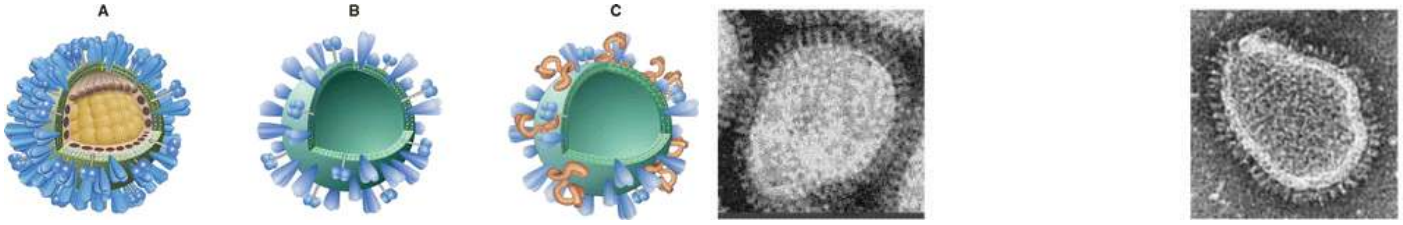


Fig.1Fig . 2

Fig.1 https://www.futuremedicine.com/doi/10.2217/ebo.11.8

Biotechnol Bioeng. 2014 Mar;111(3):425-40. doi: 10.1002/bit.25159. Epub 2013 Dec 17.
https://pubmed.ncbi.nlm.nih.gov/24347238/

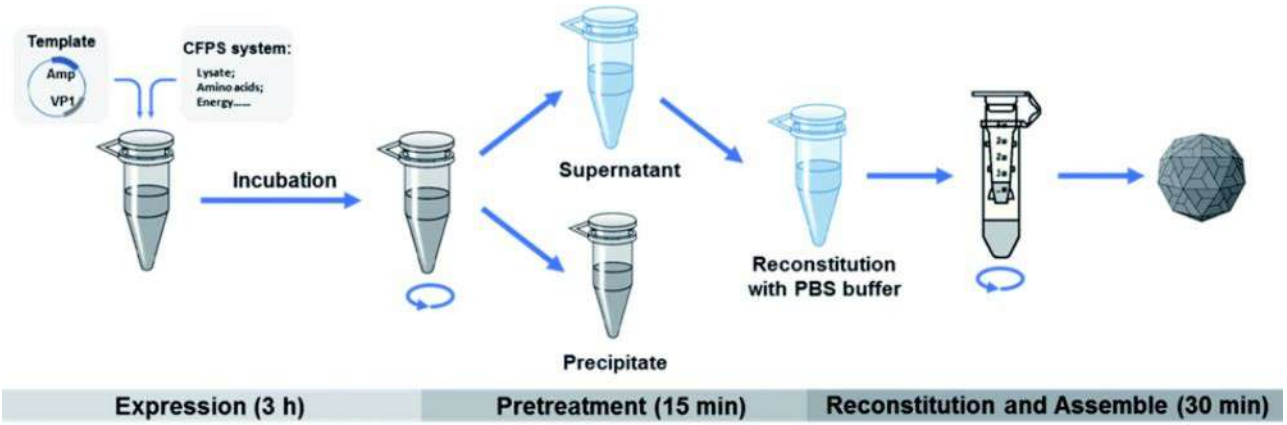
Jiayuan Sheng, Shaohua Lei, Lijuan Yuan and Xueyang Feng
Cell-free protein synthesis of norovirus virus-like particles
RSC Adv., 2017, **7**, 28837-28840 DOI: 10.1039/C7RA03742B
https://pubs.rsc.org/en/content/articlehtml/2017/ra/c7ra03742b

Spice AJ, Aw R, Bracewell DG, Polizzi KM.
Synthesis and Assembly of Hepatitis B Virus-Like Particles in a Pichia pastoris Cell-Free System.
Front Bioeng Biotechnol. 2020;8:72. Published 2020 Feb 14. doi:10.3389/fbioe.2020.00072
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7033515/

[263] Hashemzadeh A, Avan A, Ferns GA, Khazaei M.
Vaccines based on virus-like nano-particles for use against Middle East Respiratory Syndrome (MERS) coronavirus.
Vaccine. 2020 Aug 10;38(36):5742-5746. doi: 10.1016/j.vaccine.2020.07.003. Epub 2020 Jul 11.
https://pubmed.ncbi.nlm.nih.gov/32684497/

[264] Kushnir N, Streatfield SJ, Yusibov V.
Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development.
Vaccine. 2012;31(1):58-83. doi:10.1016/j.vaccine.2012.10.083
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7115575/

For virosome production, a purified and inactivated parent virus undergoes dissociation with detergent to separate the envelope fraction containing lipids and membrane-associated viral proteins from the central complex containing internal proteins and genetic material.
After detergent removal, the components of the envelope fraction assemble into empty virosomal particles.
Depending on the desired type of immune response to be induced by the virosomal vaccine, the recombinant or synthetic antigen can be displayed on the virosome surface (by integration or anchoring in the lipid bilayer, cross-binding with membrane-associated viral proteins via a lipid anchor, or adsorption to the bilayer surface) or incorporated into its lumen, stimulating antibody production or CTL responses, respectively. [265]



https://pubs.rsc.org/en/content/articlehtml/2017/ra/c7ra03742b

VLP vaccine formulation

A vaccine formulation refers to the components that make up the final administerable solution, including the vaccine carrier, adjuvants, and excipients.
For VLP vaccines, these excipients may include a variety of salts and compounds prepared as an aqueous solution or emulsion, which serve to maintain the physical stability of the VLP for longer vaccine shelf life.
Appropriate use of buffers can limit pH fluctuations, while protection from temperature fluctuations and desiccation can be provided by thermoprotectants and lyoprotectants, respectively.

Formulation science involves the study of the various components of a vaccine under different environmental conditions, with the intention of formulating a stable vaccine product suitable for the route of administration and with maximized immunogenicity.

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[265] Moser C, Amacker M, Zurbriggen R.
Influenza virosomes as a vaccine adjuvant and carrier system.
Expert Rev Vaccines. 2011 Apr;10(4):437-46. doi: 10.1586/erv.11.15.
https://pubmed.ncbi.nlm.nih.gov/21506642/

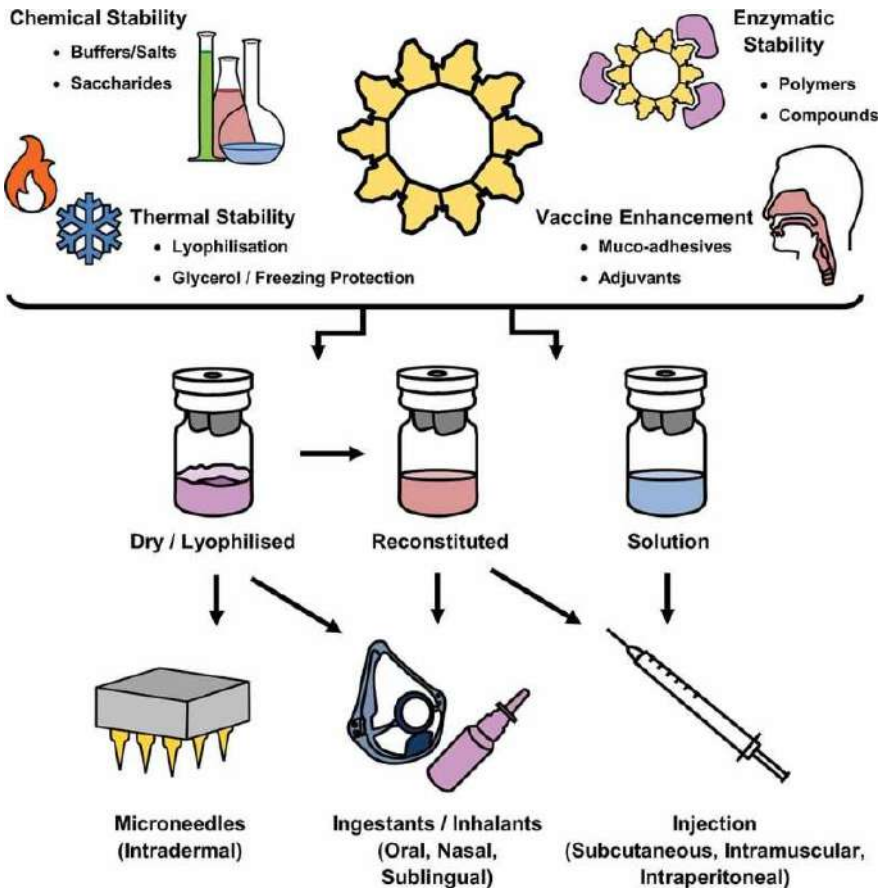
Herzog C, Hartmann K, Künzi V, Kürsteiner O, Mischler R, Lazar H, Glück R.
Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine.
Vaccine. 2009 Jul 16;27(33):4381-7. doi: 10.1016/j.vaccine.2009.05.029. Epub 2009 May 29.
https://pubmed.ncbi.nlm.nih.gov/19450630/

Moysa, Alexander & Kolesanova, Ekaterina. (2010).
Synthetic peptide vaccines.
Biomeditsinskaia khimiia. 57. 14-30. 10.1134/S1990750810040025.
https://www.researchgate.net/publication/51073781_Synthetic_peptide_vaccines

Sanjib Bhattacharya , Bhaskar Mazumder
Virosomes: A Novel Strategy for Drug Delivery and Targeting
BioPharm International, BioPharm International-01-02-2011, Volume 2011 Supplement, Issue 1
https://www.biopharminternational.com/view/virosomes-novel-strategy-drug-delivery-and-targeting

Singh, Narinder & Gautam, Surya & Kumari, Neelam & Kaur, Rupinder & Kaur, Manpreet. (2017).
Virosomes as Novel drug delivery System: An Overview. 5.
https://www.researchgate.net/publication/322118262_Virosomes_as_Novel_drug_delivery_System_An_Overview

The combination of formulation components, VLP vaccine preparation states and routes of administration are outlined in the following figure.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103734/
The roles of formulation science in VLP vaccines.
The role of formulation science in the production of VLP vaccines includes the chemical composition of buffers, preservatives, additives, and other stabilizing compounds to keep the VLP intact. This includes protecting the VLP from chemical or physical instability and enzymatic degradation. Formulations may also include targeted delivery compounds, such as muco-adhesives and immunogenic components as adjuvants. Storage and delivery of VLP vaccines and the subsequent route of administration are also important considerations in formulation science that are critical in determining vaccine efficacy and immunogenicity.

Most VLP vaccines currently on the market and under clinical evaluation are liquid suspensions, ready for administration.
This places strict limitations on the storage and distribution of VLP vaccine for safe and compliant administration. For example, commercial HPV vaccine Gardasil (Merck) must be refrigerated at 2-8°C and protected from light.
Gardasil cannot be frozen, and guidelines for the use of Gardasil recommend using the vaccine within 72 hours if removed from the refrigerator at temperatures below 25°C or if stored at 0-2°C. These guidelines mirror those of many other commercial VLP vaccines and outline the major stability, storage, and distribution challenges that formulation science needs to investigate.[266]

Vaccines against coronaviruses [267]

In the case of coronaviruses with envelopes, VLPs are formed when viral proteins S, M, and E, with or without N, are co-expressed in eukaryotic producer cells [268] .

[266] Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK.
Virus-like particle vaccines: immunology and formulation for clinical translation.
Expert Rev Vaccines. 2018;17(9):833-849. doi:10.1080/14760584.2018.1516552
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103734/

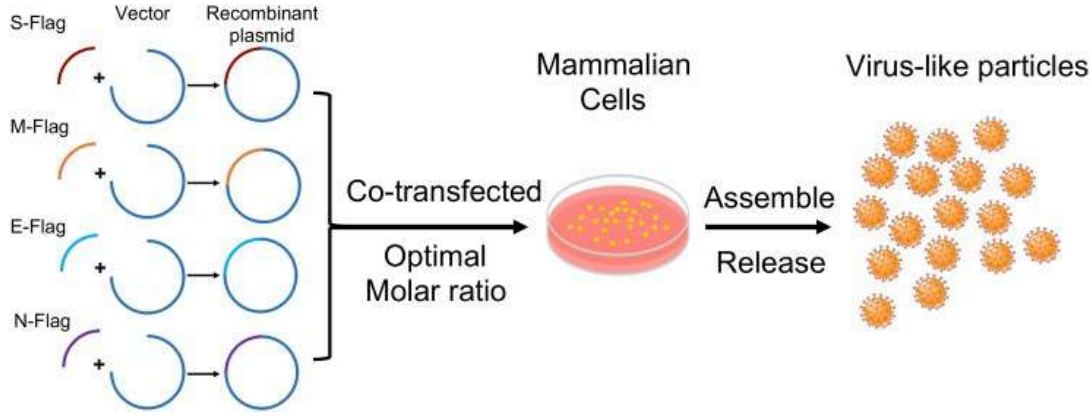
[267] Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z.
Immunological considerations for COVID-19 vaccine strategies.
Nat Rev Immunol. 2020;20(10):615-632. doi:10.1038/s41577-020-00434-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7472682/

[268] Lu X, Chen Y, Bai B, et al.
Immune responses against severe acute respiratory syndrome coronavirus induced by virus-like particles in mice.
Immunology. 2007;122(4):496-502. doi:10.1111/j.1365-2567.2007.02676.x
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2266036/

Lokugamage KG, Yoshikawa-Iwata N, Ito N, Watts DM, Wyde PR, Wang N, Newman P, Kent Tseng CT, Peters CJ, Makino S.
Chimeric coronavirus-like particles carrying severe acute respiratory syndrome coronavirus (SCoV) S protein protect mice against challenge with SCoV.
Vaccine. 2008 Feb 6;26(6):797-808. doi: 10.1016/j.vaccine.2007.11.092. Epub 2007 Dec 26.

This results in active budding from VLP-producing cells that are structurally identical to the infectious virus but lack the viral genome and therefore are not infectious.
The presence of protein S on the surface of VLPs allows them to bind and enter ACE2 cells. [+]
in the same way as the parent virus [269].
Unlike subunit vaccines, the S protein on the surface of the VLP crosslinks the B-cell receptor and directly activates B cells, but as with subunit and inactivated vaccines, VLPs typically require an adjuvant [270] and repeated administration [271].

Similar to other members of the coronaviridae family, the M protein of SARS-Cov-2 is the most abundant envelope protein that drives other structural components to assemble into VLPs.
Although E protein has also been implicated in viral morphogenesis and release, the mechanistic action remains unclear. The S protein, which is responsible for receptor binding, membrane fusion, and as a target in drug and vaccine development, and the N protein, which encapsulates the viral genome in virions, do not appear to have indispensable roles in the assembly of SARS-CoV-2 VLPs.[272]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7409377/
Schematic of SARS-CoV-2 VLP constructs in the mammalian expression system.

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2267761/

[269] Naskalska A, Dabrowska A, Nowak P, et al.
Novel coronavirus-like particles targeting cells lining the respiratory tract.
PLoS One. 2018;13(9):e0203489. Published 2018 Sep 5. doi:10.1371/journal.pone.0203489
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6124810/

[270] Cimica V, Galarza JM.
Adjuvant formulations for virus-like particle (VLP) based vaccines.
Clin Immunol. 2017;183:99-108. doi:10.1016/j.clim.2017.08.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5673579/

[271] Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK.
Virus-like particle vaccines: immunology and formulation for clinical translation.
Expert Rev Vaccines. 2018;17(9):833-849. doi:10.1080/14760584.2018.1516552
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7103734/

[272] Xu R, Shi M, Li J, Song P, Li N.
Construction of SARS-CoV-2 Virus-Like Particles by Mammalian Expression System
[published correction appears in Front Bioeng Biotechnol. 2020 Sep 09;8:1026]. Front Bioeng Biotechnol. 2020;8:862. Published 2020 Jul 30. doi:10.3389/fbioe.2020.00862.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7409377/

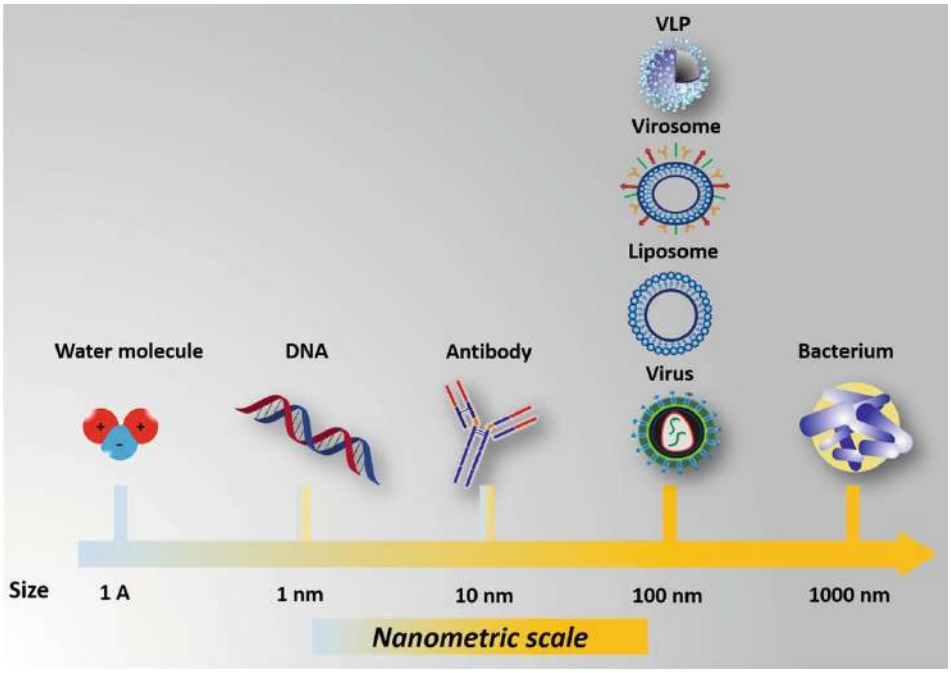
Swann H, Sharma A, Preece B, et al.
Minimal system for assembly of SARS-CoV-2 virus like particles.
Sci Rep. 2020;10(1):21877. Published 2020 Dec 14. doi:10.1038/s41598-020-78656-w
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7736577/

Sharma A, Preece B, Swann H, et al.
Structural stability of SARS-CoV-2 virus like particles degrades with temperature.
Biochem Biophys Res Commun. 2021;534:343-346. doi:10.1016/j.bbrc.2020.11.080
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7699159/

Ghorbani A, Zare F, Sazegari S, Afsharifar A, Eskandari MH, Pormohammad A.
Development of a novel platform of virus-like particle (VLP)-based vaccine against COVID-19 by exposing epitopes: an immunoinformatics approach.
New Microbes New Infect. 2020 Nov;38:100786. doi: 10.1016/j.nmni.2020.100786. Epub 2020 Oct 14.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7556220/

NANOPARTICLE VACCINES

Nanoparticle (NP) vaccines are produced by chemically cross-linking protein antigens and carrier molecules to increase immunogenicity and decrease antigen degradation [273]. These vehicles can be organic (mainly lipid-based) or inorganic (mainly polymer- or metal-based) [274].



https://link.springer.com/chapter/10.1007/978-3-030-31668-6_1

[273] Frietze KM, Peabody DS, Chackerian B.
Engineering virus-like particles as vaccine platforms.
Curr Opin Virol. 2016;18:44-49. doi:10.1016/j.coviro.2016.03.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4983494/

Zhao L, Seth A, Wibowo N, Zhao CX, Mitter N, Yu C, Middelberg AP.
Nanoparticle vaccines.
Vaccine. 2014 Jan 9;32(3):327-37. doi: 10.1016/j.vaccine.2013.11.069. Epub 2013 Dec 2.
https://www.sciencedirect.com/science/article/pii/S0264410X13016319?via%3Dihub

Kheirollahpour M, Mehrabi M, Dounighi NM, Mohammadi M, Masoudi A.
Nanoparticles and Vaccine Development.
Pharm Nanotechnol. 2020;8(1):6-21. doi: 10.2174/2211738507666191024162042.
https://www.researchgate.net/publication/336785181_Nanoparticles_and_Vaccine_Development

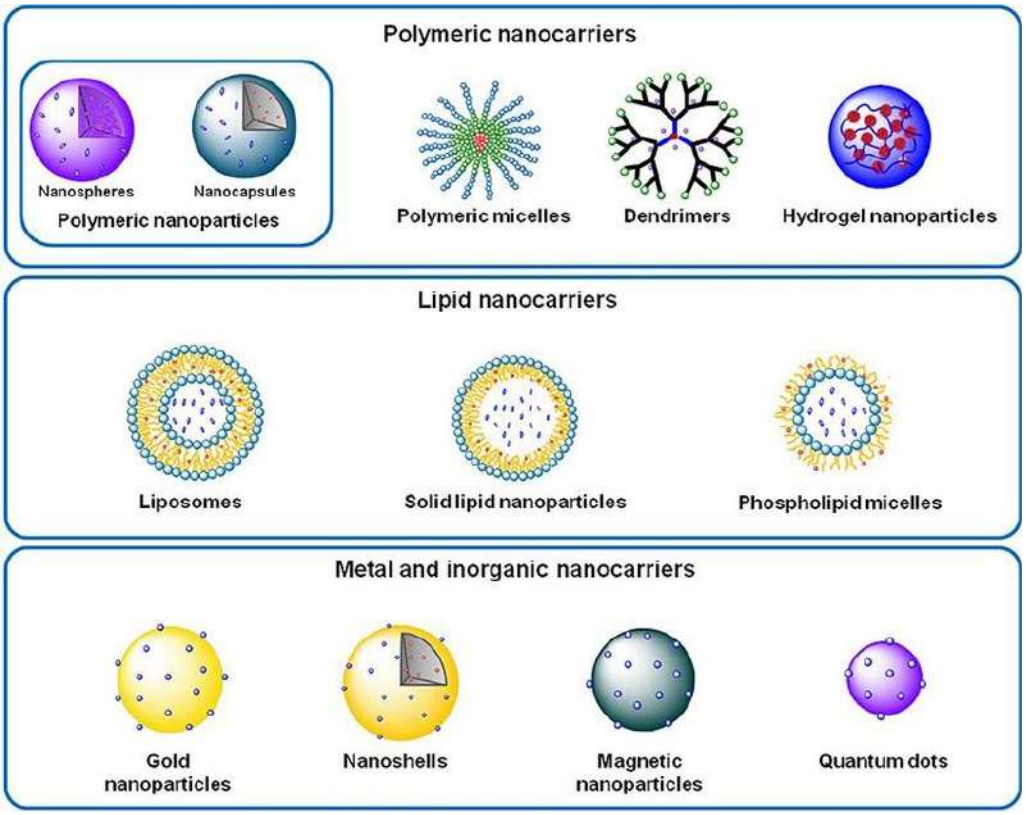
Mamo T, Poland GA.
Nanovaccinology: the next generation of vaccines meets 21st century materials science and engineering.
Vaccine. 2012 Oct 19;30(47):6609-11. doi: 10.1016/j.vaccine.2012.08.023.
https://pubmed.ncbi.nlm.nih.gov/23067445/

Dhande, Rahul & Patel, Arpita & Thakkar, Hetal. (2015).
Nanopharmaceuticals: A Boon or Bane.
https://www.researchgate.net/publication/281456859_Nanopharmaceuticals_A_Boon_or_Bane

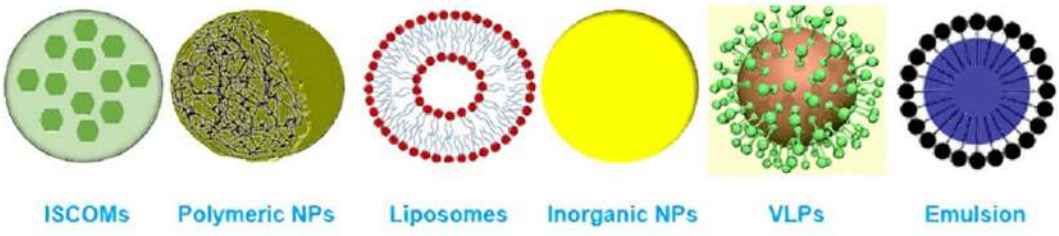
[274] Pati R, Shevtsov M, Sonawane A.
Nanoparticle Vaccines Against Infectious Diseases.
Front Immunol. 2018;9:2224. Published 2018 Oct 4. doi:10.3389/fimmu.2018.02224
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6180194/

Mi-Gyeong Kim, Joo Yeon Park, Yuna Shon, Gunwoo Kim, Gayong Shim, Yu-Kyoung Oh,
Nanotechnology and vaccine development
Asian Journal of Pharmaceutical Sciences, Volume 9, Issue 5, 2014, Pages 227-235, ISSN 1818-0876,
https://doi.org/10.1016/j.ajps.2014.06.002.
http://www.sciencedirect.com/science/article/pii/S181808761400035X.

Rosales-Mendoza S., González-Ortega O. (2019)
Nanovaccines and the History of Vaccinology.
In: Nanovaccines. Springer, Cham. https://doi.org/10.1007/978-3-030-31668-6_1
https://link.springer.com/chapter/10.1007/978-3-030-31668-6_1



https://www.researchgate.net/publication/281456859_Nanopharmaceuticals_A_Boon_or_Bane



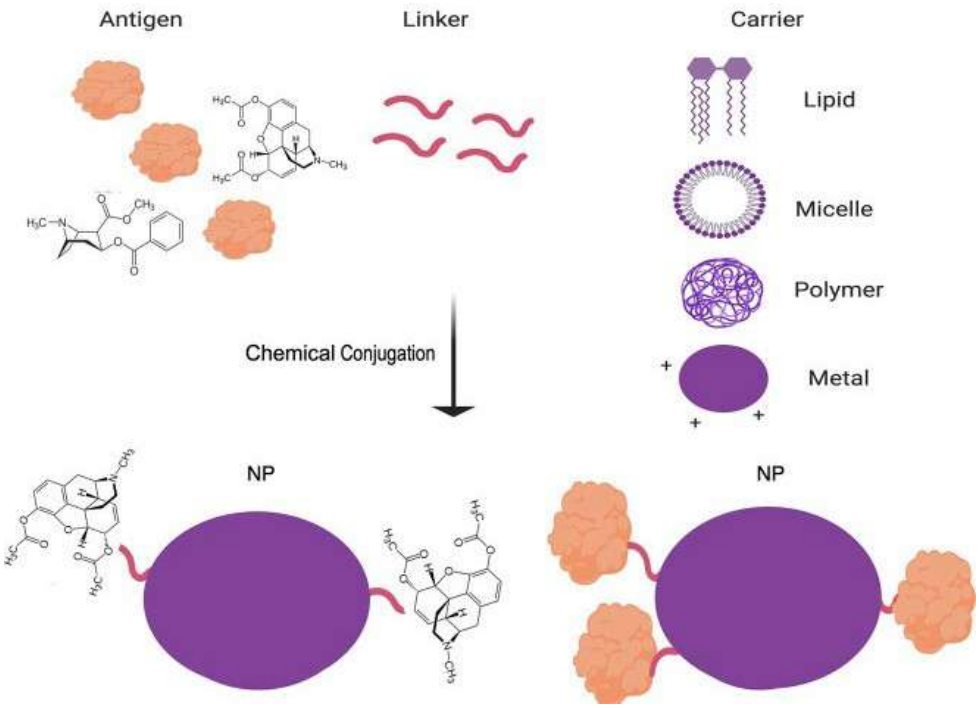
https://www.researchgate.net/publication/336785181_Nanoparticles_and_Vaccine_Development
Structure of nanocarriers for vaccine antigen delivery.

NPs have stability rates as high as those of VLPs, but they do not stimulate the innate immune response to the same extent as VLPs.
However, NPs are simpler in design than VLPs because they lack the multiple protein components of VLPs, which further reduces their production cost and increases their reproducibility and safety.
The challenges associated with the decreased immunogenicity of NP vaccines compared to VLP vaccines can be partly addressed by adapting the vector for the desired antigen based on factors such as size, surface charge, shape, and hydrophobicity.[275]

[275] Al-Halifa S, Gauthier L, Arpin D, Bourgault S, Archambault D.
Nanoparticle-Based Vaccines Against Respiratory Viruses.
Front Immunol. 2019;10:22. Published 2019 Jan 24. doi:10.3389/fimmu.2019.00022
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6353795/

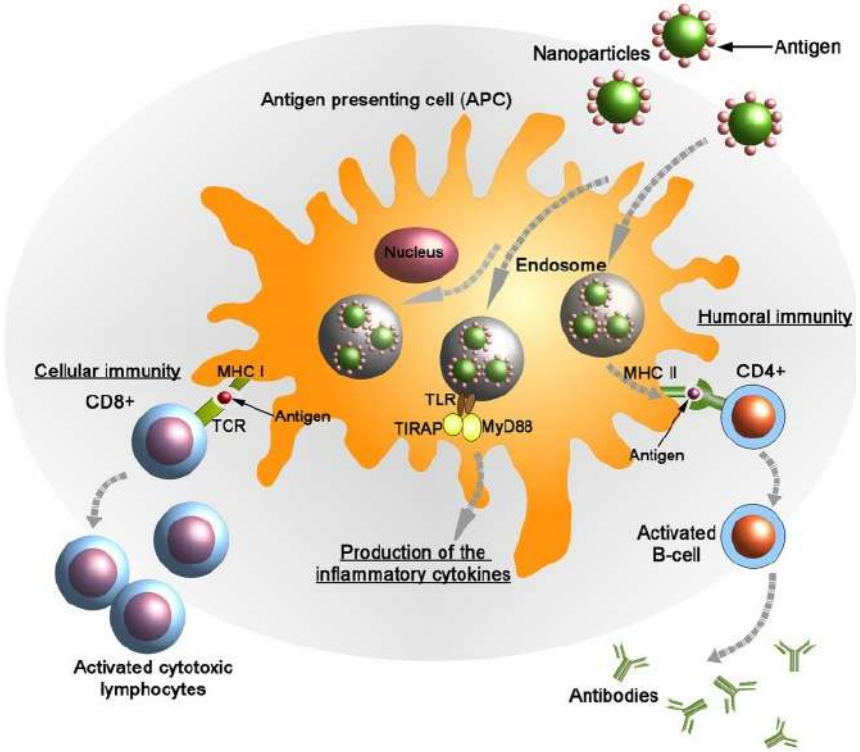
Zhao L, Seth A, Wibowo N, Zhao CX, Mitter N, Yu C, Middelberg AP.
Nanoparticle vaccines.
Vaccine. 2014 Jan 9;32(3):327-37. doi: 10.1016/j.vaccine.2013.11.069. Epub 2013 Dec 2.
https://www.sciencedirect.com/science/article/pii/S0264410X13016319?via%3Dihub

Kheirollahpour M, Mehrabi M, Dounighi NM, Mohammadi M, Masoudi A.
Nanoparticles and Vaccine Development.
Pharm Nanotechnol. 2020;8(1):6-21. doi: 10.2174/2211738507666191024162042.
https://www.researchgate.net/publication/336785181_Nanoparticles_and_Vaccine_Development



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7554600/
Schematic diagram of NP vaccine production. The methodology for producing NP vaccines is summarized in this vignette. Briefly, NP vaccines are produced by assembling an antigen complex, a linker molecule and a carrier molecule by chemical conjugation.

In addition, vectors can be used to directly target NPs to immune cells and to enhance cross-presentation by antigen-presenting cells (APCs).[276]

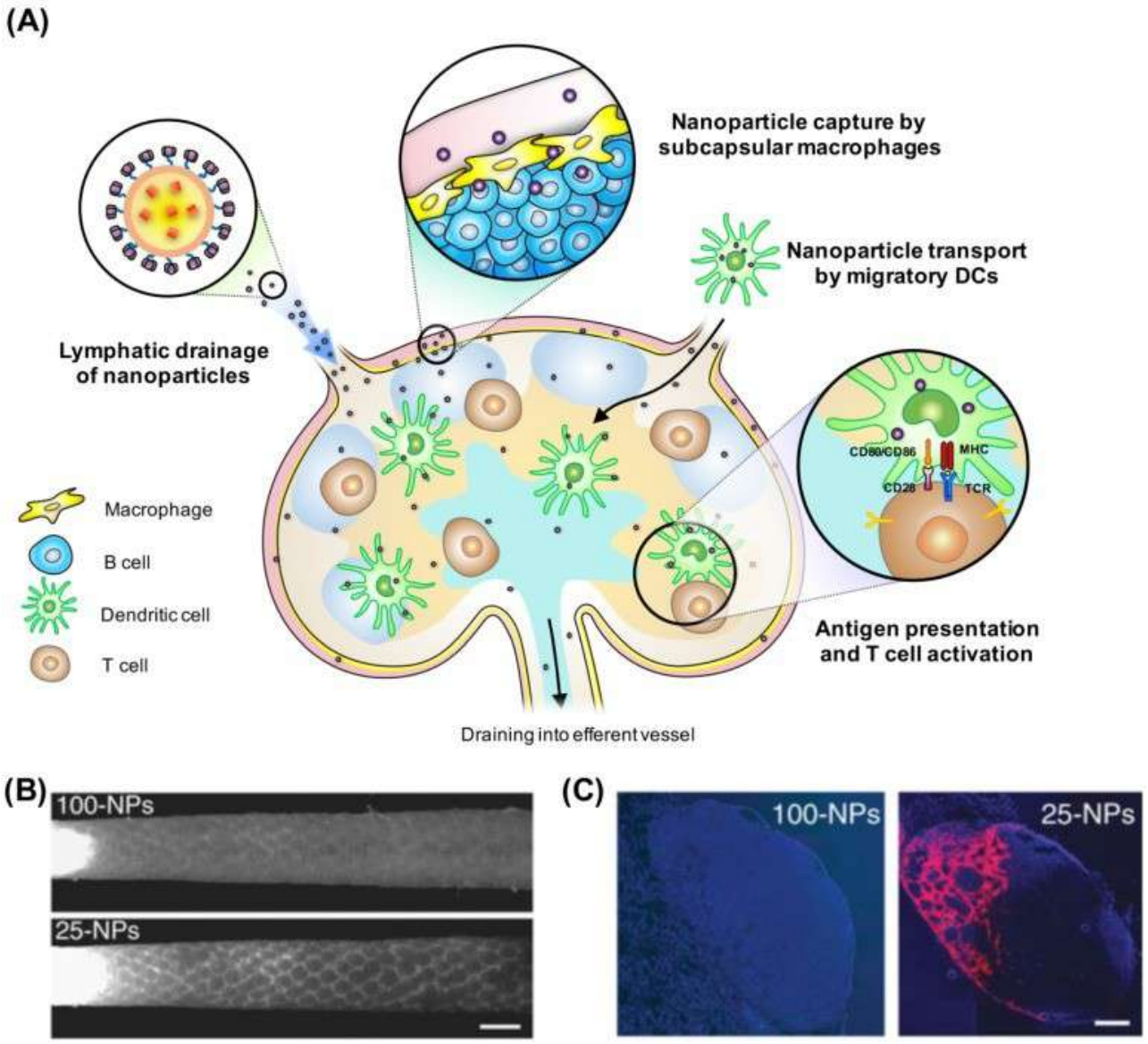


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6180194/
Targeted transport of antigenic molecules using surface-engineered nanoparticles in antigen-presenting cells (APCs). The generated endogenous antigens are presented in a complex with the major histocompatibility complex class I (MHC I) on the membrane of APCs to CD8 T lymphocytes $^{+}$. As a result of the interaction between MHC I and the T cell receptor (TCR) in the presence of co-stimulatory molecules and cytokines, activated $CD8^{+}$ cells kill infected cells by inducing cytotoxicity. Antigens are also presented on the APC surface by MHC class II molecules to helper T lymphocytes ($CD4^{+}$). Subsequently, CD4 cells^{+} activate B cells that produce antimicrobial antibodies. After stimulation, the adaptor proteins MyD88 (marker of myeloid differentiation 88) and TIRAP (TIR domain containing adaptor protein) colocalize with TLR (toll-like receptor) allowing activation of the NF-κB pathway and leading to the production of pro-inflammatory cytokines

[276] Chattopadhyay S, Chen JY, Chen HW, Hu CJ.
Nanoparticle Vaccines Adopting Virus-like Features for Enhanced Immune Potentiation.
Nanotheranostics. 2017;1(3):244-260. Published 2017 Jun 9. doi:10.7150/ntno.19796
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5646730/

Kelly HG, Kent SJ, Wheatley AK.
Immunological basis for enhanced immunity of nanoparticle vaccines.
Expert Rev Vaccines. 2019 Mar;18(3):269-280. doi: 10.1080/14760584.2019.1578216. Epub 2019 Feb 14. PMID: 30707635.
https://pubmed.ncbi.nlm.nih.gov/30707635/

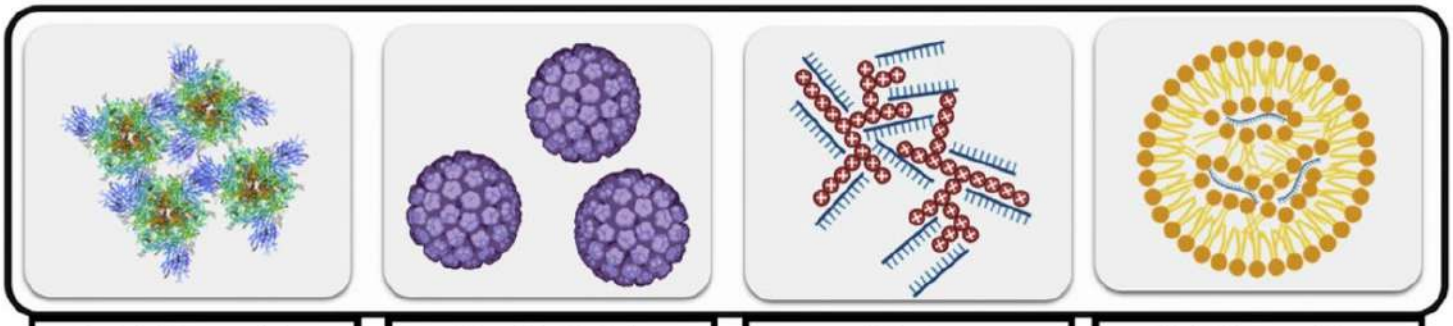
Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F.
Immunological Principles Guiding the Rational Design of Particles for Vaccine Delivery.
ACS Nano. 2017 Jan 24;11(1):54-68. doi: 10.1021/acsnano.6b07343. Epub 2017 Jan 11.
https://pubmed.ncbi.nlm.nih.gov/28075558/



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5646730/

(A) Schematics illustrating the underlying mechanisms and advantages of delivery into the lymph nodes by nanoparticle vaccines. Nanoparticles can exploit both cell-mediated and convective transport for localization in lymph nodes. Particles entering the lymph nodes through interstitial lymphatic drainage are captured by lymph node-resident macrophages. Facilitated antigen release by the nanoparticles facilitates antigen presentation and T-cell activation. (B) Fluorescence microlimangiographic imaging of 100 nm and 25 nm nanoparticles after tailed-based injection. The 25 nm particles cross the lymphatic network more effectively. (C) 25 nm nanoparticles can accumulate more efficiently in lymph nodes than 100 nm nanoparticles, as evidenced by fluorescence microscopy.

The following summarizes the various platforms being tested that employ nanoparticle technologies for SARS-Cov-2 vaccines. Each type of platform is discussed in the dedicated sections.



Protein Nanoparticles
Proteins are made to assemble into aggregate nanoparticles through covalent linkages, ionic interaction, linker molecules (avidin/biotin) or other means.

Advantages: BCR clustering through stimulation with multimeric antigens produces more robust B cell activation, potentially recapitulates quaternary epitopes from the SARS-CoV-2 virion.

Challenges: Must be expressed, stored, and delivered without degradation or denaturation. Proteins delivered without adjuvant may require larger doses.

FDA Approval: None. FluBlok is protein based and approved for influenza but does not form nanoparticles.

Technology: Aggregate spike protein particle co-delivered with Matrix M (Novavax).

Virus-like Particle
Viral protein antigens self assemble into nanoparticle that closely resembles that of a virus.

Advantages: Similar advantages to protein nanoparticles. Antigens recapitulate the surface of the virion surface.

Challenges: Precise ratios of each component which induce self-assembly upon expression must be determined. Expression of particles often competes with expression of free antigens or aggregates. Adjuvant co-delivery is often required.

FDA Approval: VLPs are approved as vaccines (e.g., Cervarix, Gardasil).

Technology: VLPs presenting spike protein. (Medicago, SpyBiotech/Serum Institute of India)

Polyplex
Positively charged polymers ionically interact with nucleotides, forming a nanoparticle complex.

Advantages: Neutralization of charge as well as particle size can facilitate uptake by APCs. Nucleotides maybe partially or fully protected by polymer.

Challenges: Cationic polymers can adduct host DNA and can have toxicity concerns.

FDA Approval: Polyplexes are approved for topical application as wound dressing.

Technology: Branched Poly(β-amino ester) (PBAE) to deliver mRNA encoding spike protein for pulmonary delivery (Translate Bio).

Lipid Nanoparticle
Positively charged liposomes ionically interact with nucleotides to form a nanoparticle or complex.

Advantages: Charge neutralization and size can facilitate APC uptake.

Challenges: Nucleotides potentially available for degradation. Pre-existing or induced immunity against PEG and phosphorylcholine. Cationic lipids can adduct host DNA and have toxicity concerns. Often requires very low storage temperatures.

FDA Approval: Liposomes and lipid complexes have been approved for decades, and internationally inactivated virus in lipids has been approved as a vaccine. siRNA based lipid carriers are FDA approved.

Technology: Lipid complex with mRNA encoding spike protein (Moderna, BioNTech, CureVac).

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7733686/pdf/main.pdf

IN-DEPTH STUDY: NANOTOXICOLOGY

While nanotechnology and nanoparticle production are growing exponentially, the toxicological impact and possible risk of nanoparticles to human health and the environment remain much to be investigated. [277]

Nanomaterials (NM) are natural or engineered materials based on nanoscale particles in a disaggregated state or in the form of an aggregate/agglomerate [278] with a number-size distribution of 50 percent or more of the particles having one or more external dimensions in the size range of 1 to 100 nanometers [279].
Because of small particle sizes and changes in their internal structure, NMs may have different properties that depend on a higher ratio of surface area to volume
[280]. Therefore, the physicochemical properties of NMs may differ from the properties of granular substances or larger particles [281].

The International Organization for Standardization (ISO) has defined nanomaterial as a material with any nanoscale external dimension ("nano-object") or nanoscale internal or surface structure ("nano-structured material")[282].

Specifically, a **nano-object** is defined as a discrete portion of material with one, two or three external dimensions on the nanoscale.

"**Nanoparticles**" are nano-objects with all external dimensions on the nanoscale, where the lengths of the longest and shortest axes do not differ significantly.

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[277] Zielińska A, Costa B, Ferreira MV, et al.
Nanotoxicology and Nanosafety: Safety-By-Design and Testing at a Glance.
Int J Environ Res Public Health. 2020;17(13):4657. Published 2020 Jun 28. doi:10.3390/ijerph17134657
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7369733/

Elsaesser A, Howard CV.
Toxicology of nanoparticles.
Adv Drug Deliv Rev. 2012 Feb;64(2):129-37. doi: 10.1016/j.addr.2011.09.001. Epub 2011 Sep 8.
https://pubmed.ncbi.nlm.nih.gov/21925220/

[278] Boverhof DR, Bramante CM, Butala JH, Clancy SF, Lafranconi M, West J, Gordon SC.
Comparative assessment of nanomaterial definitions and safety evaluation considerations.
Regul Toxicol Pharmacol. 2015 Oct;73(1):137-50. doi: 10.1016/j.yrtph.2015.06.001. Epub 2015 Jun 23.
https://www.sciencedirect.com/science/article/pii/S0273230015001488?via%3Dihub

[279] Rigano, Luigi & Lionetti, Nicola.
Nanobiomaterials in galenic formulations and cosmetics.
(2016). 10.1016/B978-0-323-42868-2.00006-1.
https://www.researchgate.net/publication/303413371_Nanobiomaterials_in_galenic_formulations_and_cosmetics

EFSA Scientific Committee, Hardy A, Benford D, et al.
Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health.
EFSA J. 2018;16(7):e05327. Published 2018 Jul 4. doi:10.2903/j.efsa.2018.5327
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7009542/

[280] Li X, Liu W, Sun L, Aifantis KE, Yu B, Fan Y, Feng Q, Cui F, Watari F.
Effects of physicochemical properties of nanomaterials on their toxicity.
J Biomed Mater Res A. 2015 Jul;103(7):2499-507. doi: 10.1002/jbm.a.35384. Epub 2014 Dec 19.
https://pubmed.ncbi.nlm.nih.gov/25530348/

[281] Shin SW, Song IH, Um SH.
Role of Physicochemical Properties in Nanoparticle Toxicity.
Nanomaterials (Basel). 2015;5(3):1351-1365. Published 2015 Aug 19. doi:10.3390/nano5031351
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5304630/

[282] Nanotechnologies - Plain language explanation of selected terms from the ISO/IEC 80004 series
https://www.iso.org/obp/ui/#iso:std:iso:tr:18401:ed-1:v1:en
https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-2:ed-1:v1:en

If the dimensions differ significantly, typically by more than a factor of three, other terms, such as "**nanofiber**" (two nanoscale outer dimensions) or "**nanoplate**" (one nanoscale outer dimension) may be preferred to the term nanoparticle.

In turn, a "nanostructured material" is defined as a material having an internal or surface nanostructure in which one or more parts are in a nanoscale region. The "**nanoscale**" is between about 1 and 100 nm (ISO, 2017).

According to the ISO vocabulary of nanotechnology, an **'engineered nano-object**' is defined as a nano-object designed for a specific purpose or function; a **'nano-object product'** is defined as a nano-object intentionally produced to have selected properties or composition; and an **'accidental nano-object**' is defined as a nano-object generated as an unintentional by-product of a process (ISO, 2017).

According to ISOs , size is the key parameter for identifying a nanomaterial.
All nanomaterials occur with a size distribution, as the constituent entities do not all have the same size.
Particulate materials often include particles with lengths both shorter and longer than 100 nm.
Due to the reactivity of nanoparticles, mainly related to their high surface free energy, larger clusters ("secondary particles") often result from agglomeration and/or aggregation of the primary constituent particles.
In some cases, the size distribution of manufactured nanomaterials covers a rather wide range of lengths.

These technical definitions, based on size alone, are insufficient from a risk assessment perspective because they do not include other important elements (reviewed below) that should be considered when determining whether a nanomaterial may require further review.

Proper characterization of the nanomaterial, as well as understanding the processes that occur on the surface of nanoparticles in contact with living systems, is critical to understanding possible toxicological effects.

The key parameters for conventional toxicology are concentration and exposure time. These factors can be easily measured for individual chemicals, and after determining the nature of the dose response to a given chemical, threshold levels below which a chemical compound can be considered "safe" or "hazardous" can be determined.

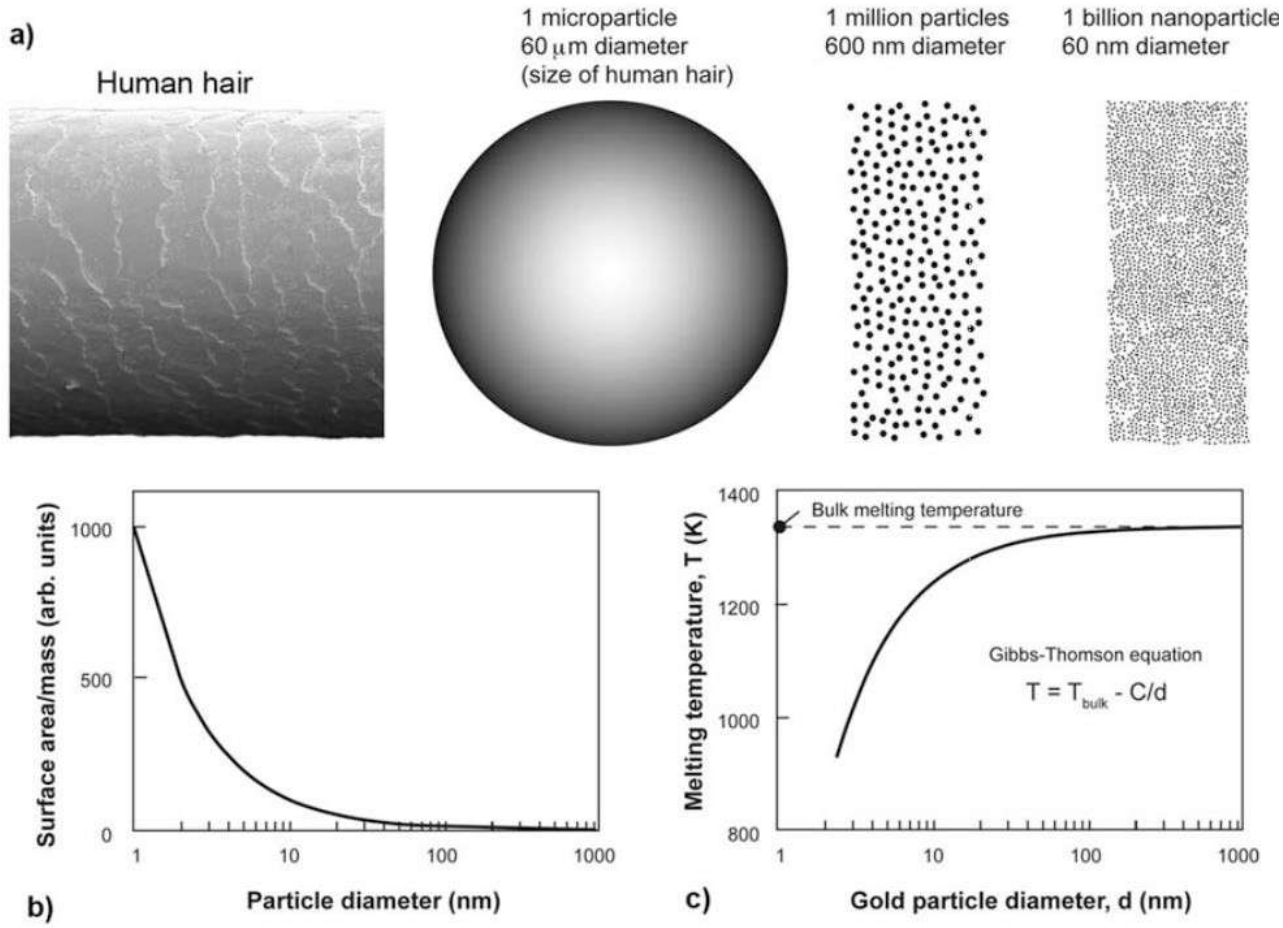
Although we extensively understand the bulk properties of materials (bulk) and/or chemicals at the molecular level, new material properties have been discovered in the area between "molecule" and "bulk," that is, the nanoscale.

As the mass of materials is transformed into smaller and smaller fragments of matter their surface chemistry changes and chemical reactivity increases [283].

[283] Jefferson D. A.
The surface activity of ultrafine particles
Phil. Trans. R. Soc. A. 2000 3582683-2692 http://doi.org/10.1098/rsta.2000.0677
https://royalsocietypublishing.org/doi/pdf/10.1098/rsta.2000.0677

Ju-Nam Y, Lead JR.
Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications.
Sci Total Environ. 2008 Aug 1;400(1-3):396-414. doi: 10.1016/j.scitotenv.2008.06.042. Epub 2008 Aug 19.
https://pubmed.ncbi.nlm.nih.gov/18715626/

Surface reactivity of metal nanoparticles - importance of surface-active agents and biomolecules from a transformation, mobility and toxicity perspective: Sara Skoglund Doctoral thesis
https://www.diva-portal.org/smash/get/diva2:1063551/FULLTEXT01.pdf



https://arxiv.org/pdf/0801.3280.pdf
(a) Schematics illustrating a 60-µm-diameter microparticle-about the size of a human hair-shown at left in scale, and the number of 600-nm-diameter and 60-nm-diameter nanoparticles having the same mass as a 60-µm-diameter microparticle. (b) Surface area normalized to mass versus particle diameter. (c) Melting temperature of gold as a function of particle diameter, according to t h e Gibbs-Thomson equation, shown in the inset; melting temperature of gold mass is 1336 K.

According to "REACH" (Registration, Evaluation, Authorization and Restriction of Chemicals), the safety assessment of nanomaterials should follow the risk assessment methodology adopted for conventional chemicals, which is based on the following requirements:

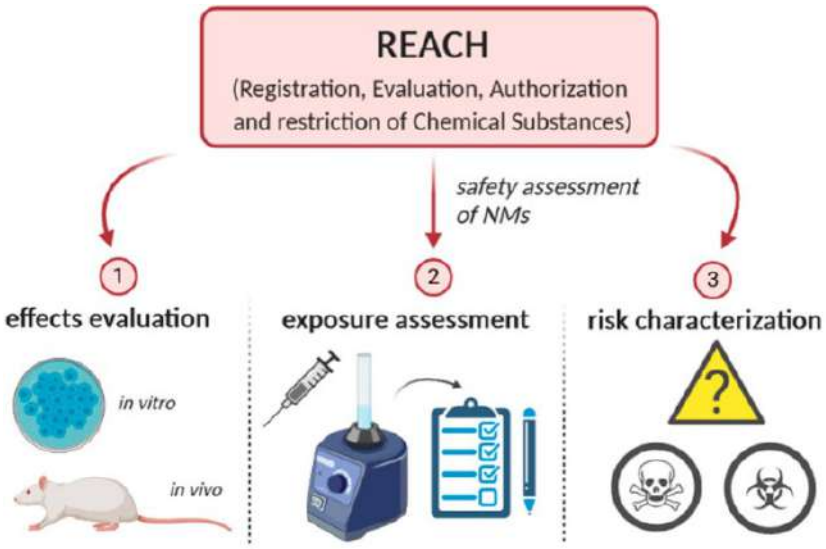
(1) evaluation of effects ,
(2) exposure assessment and
(3) Risk characterization.

Step 1 includes the evaluation of effects. The hazard quotient is considered acceptable (greater than 1) when the estimated exposure value is less than the concentration of the agent at which no adverse effect was observed in the experimental study performed to evaluate the point under consideration, e.g., inhalation toxicity or genotoxicity.
If it is necessary to conduct in vitro and/or in vivo experiments to evaluate effects, other procedures may also be recommended to characterize the NMs under study.

Molleman B , Hiemstra T .
Size and shape dependency of the surface energy of metallic nanoparticles: unifying the atomic and thermodynamic approaches. Phys Chem Phys. 2018 Aug 8;20(31):20575-20587. doi: 10.1039/c8cp02346h.
https://pubmed.ncbi.nlm.nih.gov/30059091/

Buzea C, Pacheco II, Robbie K.
Nanomaterials and nanoparticles: sources and toxicity.
Biointerphases. 2007 Dec;2(4):MR17-71. doi: 10.1116/1.2815690.
https://arxiv.org/pdf/0801.3280.pdf

This includes gathering information on the most relevant physicochemical parameters that may influence toxicity, i.e., size distribution, aggregation/agglomeration state, shape, surface area, reactivity, water solubility, surface property, and long-term stability.
Step 2 includes identifying all potential sources of exposure. Therefore, it is important to understand the entire production process and the most likely routes of exposure.
This is also important for choosing the appropriate testing strategy and making recommendations on risk prevention measures (**Phase 3**). [284]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7369733/
The risk assessment methodology for nanomaterials (NM) based on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) requirements

However, NMs have different properties that may affect their toxicological profile, such as: [285]

(1) **particle size**: toxicity and uptake by cells will increase with smaller NM sizes;
(2) **particle charge**: positively charged NMs have higher toxicity due to greater interactions with negatively charged biological surfaces;

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[284] Schwirn K, Voelker D, Galert W, Quik J, Tietjen L.
Environmental Risk Assessment of Nanomaterials in the Light of New Obligations Under the REACH Regulation: Which Challenges Remain and How to Approach Them?
Integr Environ Assess Manag. 2020;16(5):706-717. doi:10.1002/ieam.4267
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7497025/

Boros BV, Ostafe V.
Evaluation of Ecotoxicology Assessment Methods of Nanomaterials and Their Effects.
Nanomaterials (Basel). 2020;10(4):610. Published 2020 Mar 26. doi:10.3390/nano10040610
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7221575/

Liu X, Tang K, Harper S, Harper B, Steevens JA, Xu R. Predictive modeling of nanomaterial exposure effects in biological systems. Int J Nanomedicine. 2013;8 Suppl 1(Suppl 1):31-43. doi:10.2147/IJN.S40742
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3790277/

Hofmann-Amtenbrink M, Grainger DW, Hofmann H.
Nanoparticles in medicine: Current challenges facing inorganic nanoparticle toxicity assessments and standardizations.
Nanomedicine. 2015 Oct;11(7):1689-94. doi: 10.1016/j.nano.2015.05.005. Epub 2015 Jun 4.
https://pubmed.ncbi.nlm.nih.gov/26051651/

[285] Singh AV, Laux P, Luch A, Sudrik C, Wiehr S, Wild AM, Santomauro G, Bill J, Sitti M.
Review of emerging concepts in nanotoxicology: opportunities and challenges for safer nanomaterial design.
Toxicol Mech Methods. 2019 Jun;29(5):378-387. doi: 10.1080/15376516.2019.1566425. Epub 2019 Feb 12.
https://pubmed.ncbi.nlm.nih.gov/30636497/

Albanese A, Tang PS, Chan WC.
The effect of nanoparticle size, shape, and surface chemistry on biological systems.
Annu Rev Biomed Eng. 2012;14:1-16. doi: 10.1146/annurev-bioeng-071811-150124. Epub 2012 Apr 18.
https://pubmed.ncbi.nlm.nih.gov/22524388/

(3) The **ionic dissolution**: higher ionic dissolution leads to higher toxicity, and
(4) the **shape**: rod-shaped anisotropic NMs are less efficient and can cause significant near-infrared damage, resulting in destruction of target cells .

The cytotoxicity of nanoparticles is induced by several factors.
Some cases of nanomaterials inducing cytotoxicity are due to the substance itself, and some nanoparticles show toxicity without a clear mechanism. [286]
Nanoparticles of a particular substance are considered to pose greater toxicity risks than larger particles of the same substance [287].
Above all, the distribution within the body and the accumulation of a specific type of particle in a particular district or organ, which depends on the size of the particle and the characteristics of its surface area, are considered critical factors [288]. In addition, when nanoparticles accumulate in the body without adequate excretion, they can cause continued toxicity.

The major distribution sites and target organs of nanoparticles are unknown; however, it appears that the liver and spleen are target organs [289].
If nanoparticles are ingested, inhaled or absorbed through the skin, they can induce the formation of reactive oxygen species (ROS) including free radicals [290].
ROS produce oxidative stress, inflammation and subsequent damage to various biological components such as proteins, DNA, etc.

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[286] Lewinski N, Colvin V, Drezek R.
Cytotoxicity of nanoparticles.
Small. 2008 Jan;4(1):26-49. doi: 10.1002/smll.200700595.
https://pubmed.ncbi.nlm.nih.gov/18165959/

Favi PM, Gao M, Johana Sepúlveda Arango L, Ospina SP, Morales M, Pavon JJ, Webster TJ.
Shape and surface effects on the cytotoxicity of nanoparticles: gold nanospheres versus gold nanostars.
J Biomed Mater Res A. 2015 Nov;103(11):3449-62. doi: 10.1002/jbm.a.35491. Epub 2015 May 6.
https://pubmed.ncbi.nlm.nih.gov/25904210/

[287] Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, Schmid G, Brandau W, Jahnen-Dechent W.
Size-dependent cytotoxicity of gold nanoparticles.
Small. 2007 Nov;3(11):1941-9. doi: 10.1002/smll.200700378.
https://pubmed.ncbi.nlm.nih.gov/17963284/

Napierska D, Thomassen LC, Rabolli V, Lison D, Gonzalez L, Kirsch-Volders M, Martens JA, Hoet PH.
Size-dependent cytotoxicity of monodisperse silica nanoparticles in human endothelial cells.
Small. 2009 Apr;5(7):846-53. doi: 10.1002/smll.200800461.
https://pubmed.ncbi.nlm.nih.gov/19288475/

Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, Jones RL, Schlager JJ.
Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species.
J Phys Chem B. 2008 Oct 30;112(43):13608-19. doi: 10.1021/jp712087m. Epub 2008 Oct 3.
https://pubmed.ncbi.nlm.nih.gov/18831567/

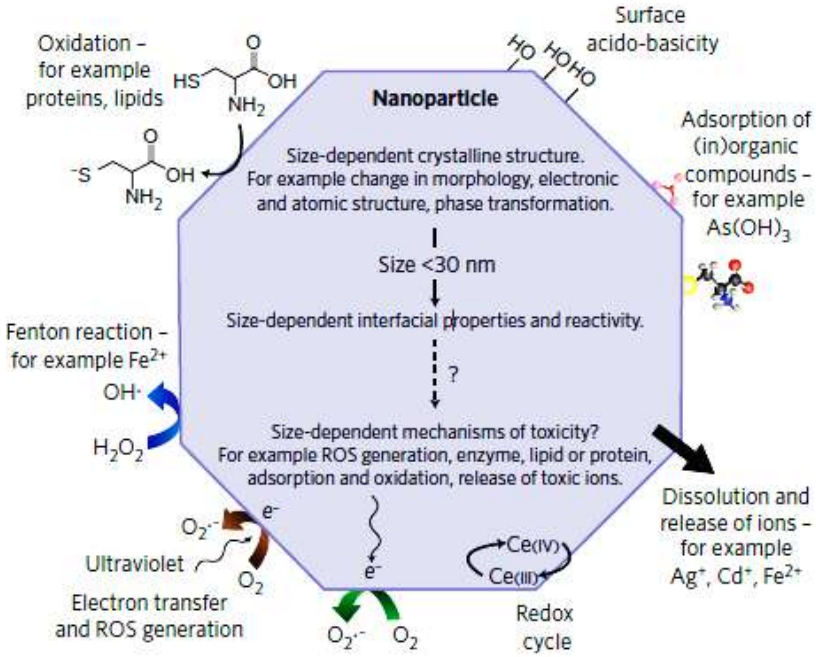
[288] Oberdörster G, Oberdörster E, Oberdörster J.
Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles
[published correction appears in Environ Health Perspect. 2010 Sep;118(9):A380]. Environ Health Perspect. 2005;113(7):823-839. doi:10.1289/ehp.7339
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1257642/

[289] Handy RD, Owen R, Valsami-Jones E.
The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs.
Ecotoxicology. 2008 Jul;17(5):315-25. doi: 10.1007/s10646-008-0206-0. Epub 2008 Apr 12
https://pubmed.ncbi.nlm.nih.gov/18408994/

Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ.
In vitro toxicity of nanoparticles in BRL 3A rat liver cells.
Toxicol In Vitro. 2005 Oct;19(7):975-83. doi: 10.1016/j.tiv.2005.06.034. Epub 2005 Aug 25.
https://pubmed.ncbi.nlm.nih.gov/16125895/

[290] Brown JS, Zeman KL, Bennett WD.
Ultrafine particle deposition and clearance in the healthy and obstructed lung.
Am J Respir Crit Care Med. 2002 Nov 1;166(9):1240-7. doi: 10.1164/rccm.200205-399OC.
https://pubmed.ncbi.nlm.nih.gov/12403694/

In addition to ROS production, other factors affecting toxicity include size, morphology, state of agglomeration, shape, chemical composition, structure and surface charge, state of aggregation, and solubility [291].



https://www.researchgate.net/publication/258559761_Surface_Reactivity_of_Manufactured_Nanoparticles
On the surface of an inorganic nanoparticle, numerous chemical and physical mechanisms can occur. The potential relationship between the size dependence of the structure crystalline of the nanoparticles (typically <30 nm), their interfacial properties (e.g., dissolution, oxidation, adsorption/desorption, electron transfer, redox cycling, Fenton reactions, and surface acid-base), and potential toxicity mechanisms (e.g., ROS generation, toxic ion release, protein oxidation, and pollutant adsorption).
OH -, hydroxyl radical; O2 ¨ , superoxide anion

Because of their small size, nanoparticles can cross tissue junctions and even cell membranes where they induce structural damage to mitochondria, [292] or invade the nucleus where they cause severe DNA mutations [293] capable of causing cell death, as will be discussed in more detail below. [294].

The above factors can be classified into the five nanoparticle characteristics, which are:

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[291] Holsapple MP, Farland WH, Landry TD, Monteiro-Riviere NA, Carter JM, Walker NJ, Thomas KV.
Research strategies for safety evaluation of nanomaterials, part II: toxicological and safety evaluation of nanomaterials, current challenges and data needs.
Toxicol Sci. 2005 Nov;88(1):12-7. doi: 10.1093/toxsci/kfi293. Epub 2005 Aug 24.
https://pubmed.ncbi.nlm.nih.gov/16120754/

Auffan, Melanie & Rose, Jerome & Chanéac, Corinne & Jolivet, Jean-Pierre & Masion, Armand & Wiesner, Mark & Bottero, Jean-Yves.
Surface Reactivity of Manufactured Nanoparticles.
Nanoethics and Nanotoxicology. (2011). 269-. 10.1007/978-3-642-20177-6_12.
https://www.researchgate.net/publication/258559761_Surface_Reactivity_of_Manufactured_Nanoparticles

[292] Hoshino A, Fujioka K, Oku T, Nakamura S, Suga M, Yamaguchi Y, Suzuki K, Yasuhara M, Yamamoto K.
Quantum dots targeted to the assigned organelle in living cells.
Microbiol Immunol. 2004;48(12):985-94. doi: 10.1111/j.1348-0421.2004.tb03621.x.
https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1348-0421.2004.tb03621.x

Salnikov V, Lukyánenko YO, Frederick CA, Lederer WJ, Lukyánenko V.
Probing the outer mitochondrial membrane in cardiac mitochondria with nanoparticles.
Biophys J. 2007;92(3):1058-1071. doi:10.1529/biophysj.106.094318
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1779971/

[293] Donaldson K, Stone V.
Current hypotheses on the mechanisms of toxicity of ultrafine particles.
Ann Ist Super Sanita. 2003;39(3):405-10.
https://pubmed.ncbi.nlm.nih.gov/15098562/

[294] Wilson RF.
Nanotechnology: the challenge of regulating known unknowns.
J Law Med Ethics. 2006 Winter;34(4):704-13. doi: 10.1111/j.1748-720X.2006.00090.x.
https://pubmed.ncbi.nlm.nih.gov/17199812/

Albanese A, Tang PS, Chan WC.
The effect of nanoparticle size, shape, and surface chemistry on biological systems.
Annu Rev Biomed Eng. 2012;14:1-16. doi: 10.1146/annurev-bioeng-071811-150124. Epub 2012 Apr 18.
https://pubmed.ncbi.nlm.nih.gov/22524388/

size, surface area, surface electrostatic state, morphology and agglomeration state.
[295]

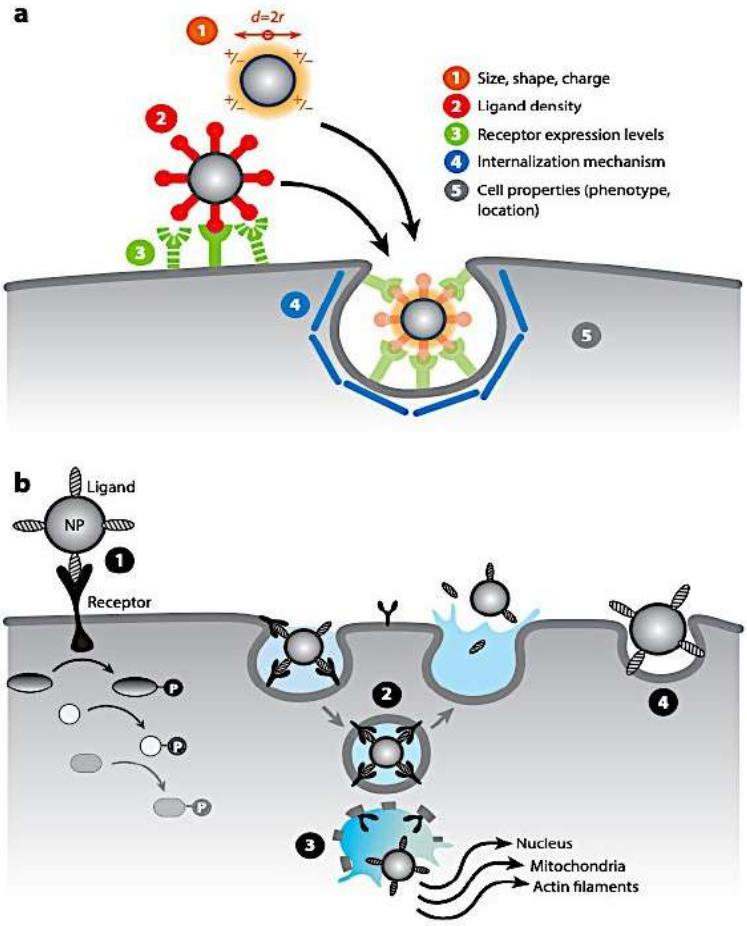
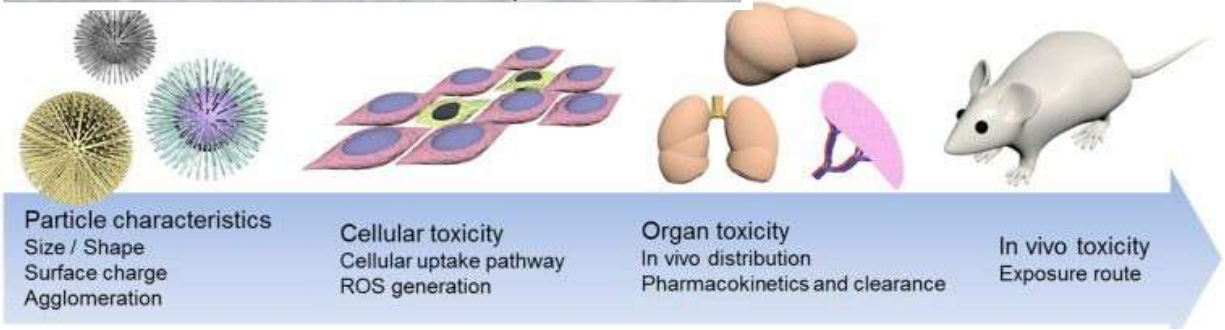


Fig.1 https://pubmed.ncbi.nlm.nih.gov/22524388/ Nanoparticle-cell interactions**. (a)** List of factors that may influence nanoparticle-cell interactions at the nano-bio interface. **(b)** Ligand-coated nanoparticles interacting with cells. Ligand-coated nanoparticles bind to receptors on the membrane and induce a signaling cascade without entering the cell**. 1) Ligand-coated** nanoparticles can also be internalized and exocytosed from the cell, without ever leaving the vesicle**. 2) They** bind to the membrane receptor, enter the cells and then leave the cell.
3) Internalized nanoparticles can escape the vesicle and interact with various organelles. They bind to membrane receptors, enter the cell and target subcellular structures.
4) Nanoparticles can interact nonspecifically with the cell surface membrane. They are subsequently internalized.
(c) Multiple 15 nm transferrin-coated gold nanoparticles are internalized by HeLa cells into intracellular vesicles. Abbreviation: NP, nanoparticle.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5304630/
Schematic drawing of nanoparticle-induced cytotoxicity. The intrinsic characteristics of nanoparticles, such as size, surface charge, and agglomeration, can significantly influence cytotoxicity. Such cytotoxicity can be affected at the level of the cell, organ, and even in vivo systems.

Fig.2

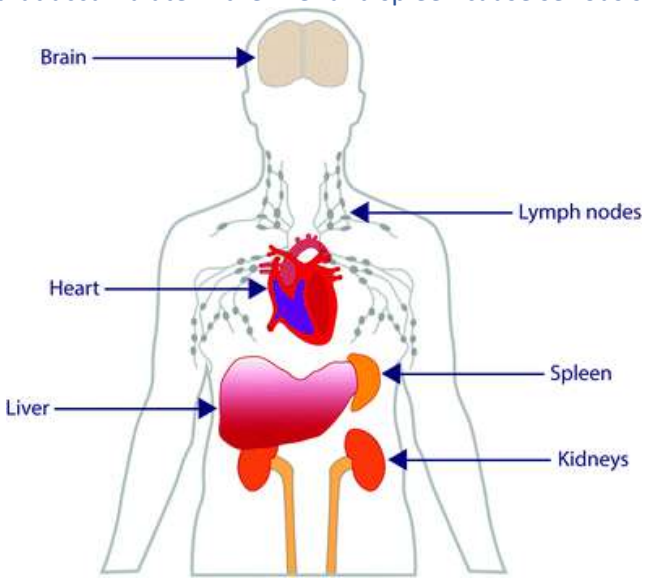


[295] Shin SW, Song IH, Um SH.
Role of Physicochemical Properties in Nanoparticle Toxicity.
Nanomaterials (Basel). 2015;5(3):1351-1365. Published 2015 Aug 19. doi:10.3390/nano5031351
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5304630/

Ahmet Aydın, Hande Sipahi and Mohammad Charehsaz (October 31, 2012).
Nanoparticles Toxicity and Their Routes of Exposures, Recent Advances in Novel Drug Carrier Systems,
Ali Demir Sezer, IntechOpen, DOI: 10.5772/51230. Available from: https://www.intechopen.com/books/recent-advances-in-novel-drug-carrier-systems/nanoparticles-toxicity-and-their-routes-of-exposures
https://www.intechopen.com/books/recent-advances-in-novel-drug-carrier-systems/nanoparticles-toxicity-and-their-routes-of-exposures

Dimensions

Cytotoxicity is induced by the interaction between the nanomaterial surface and cellular components.
As the diameter decreases, the surface area of the particle increases exponentially, so even when the particles have the same composition, they can have significantly different levels of cytotoxicity depending on particle size and surface reactivity.
In addition, particle size induces significant differences in cell release mechanism and distribution in vivo.
Many studies have examined the in vivo distribution of nanomaterials [296].
Nanoparticles with a diameter greater than 6 nm cannot be excreted by the kidneys and accumulate in specific organs, such as the liver and spleen, until clearance by the mononuclear phagocyte system occurs [297] .
Most of the nanoparticles that accumulate in the liver and spleen cause serious side effects.



https://pubs.rsc.org/en/content/articlehtml/2020/bm/d0bm00558d
Schematic representation of the main areas of translocation and accumulation of nanoparticles after administration.

For example, quantum dots [298] of cadmium selenide (CdSe) remain in the tissue for up to eight months and cause hepatotoxicity [299] .

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[296] Varna, Mariana & Ratajczak, Philippe & Ferreira, Irmine & Leboeuf, Christophe & Bousquet, Guilhem & Janin, Anne.
In vivo Distribution of Inorganic Nanoparticles in Preclinical Models.
Journal of Biomaterials and Nanobiotechnology. (2012). 03. 269-279. 10.4236/jbnb.2012.322033.
https://www.researchgate.net/publication/276489705_In_vivo_Distribution_of_Inorganic_Nanoparticles_in_Preclinical_Models

El-Ansary A, Al-Daihan S.
On the toxicity of therapeutically used nanoparticles: an overview.
J Toxicol. 2009;2009:754810. doi:10.1155/2009/754810
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2809332/

[297] Albanese A, Tang PS, Chan WC.
The effect of nanoparticle size, shape, and surface chemistry on biological systems.
Annu Rev Biomed Eng. 2012;14:1-16. doi: 10.1146/annurev-bioeng-071811-150124. Epub 2012 Apr 18.
https://pubmed.ncbi.nlm.nih.gov/22524388/

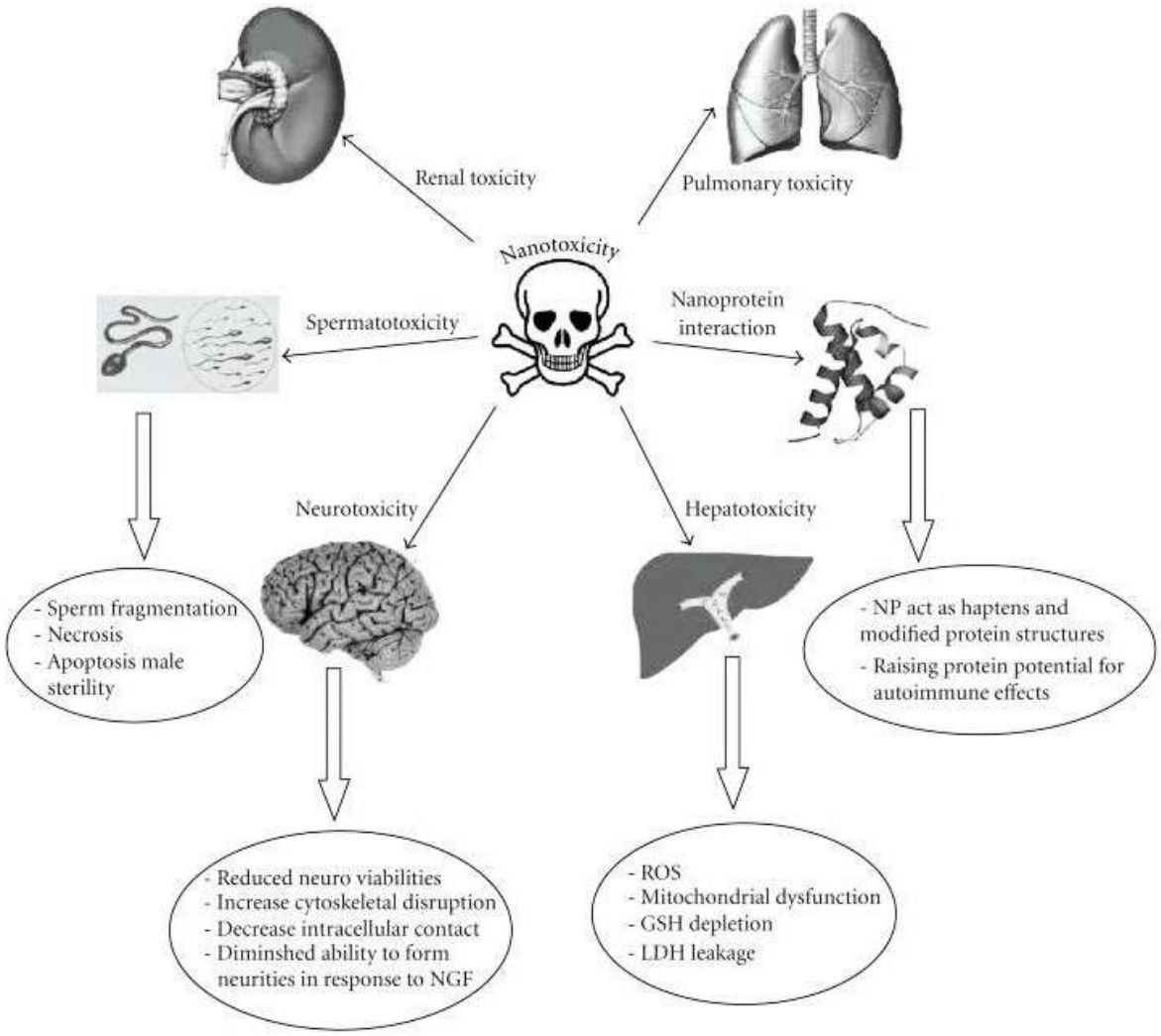
Foulkes R , Man E , Thind J , Yeung S , Joy A , Hoskins C .
The regulation of nanomaterials and nanomedicines for clinical application: current and future perspectives.
Biomater Sci. 2020 Sep 7;8(17):4653-4664. doi: 10.1039/d0bm00558d. Epub 2020 Jul 16.
https://pubs.rsc.org/en/content/articlehtml/2020/bm/d0bm00558d

[298] Tyler Maxwell, Maria Gabriela Nogueira Campos, Stephen Smith, Mitsushita Doomra, Zon Thwin, Swadeshmukul Santra,
Chapter 15 - Quantum Dots, Editor(s): Eun Ji Chung, Lorraine Leon, Carlos Rinaldi, In Micro and Nano Technologies,
Nanoparticles for Biomedical Applications, Elsevier, 2020, Pages 243-265, ISBN 9780128166628,
https://doi.org/10.1016/B978-0-12-816662-8.00015-1.
http://www.sciencedirect.com/science/article/pii/B9780128166628000151

Quantum dots: history, fabrication methods and applications
http://tesi.cab.unipd.it/33121/1/tesi_577834_copia.pdf

[299] Ballou B, Lagerholm BC, Ernst LA, Bruchez MP, Waggoner AS.

These characteristic pharmacokinetics of nanoparticles depend on particle size and surface chemistry.



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2809332/
A summary of the most important recorded toxic effects of therapeutically used nanoparticles

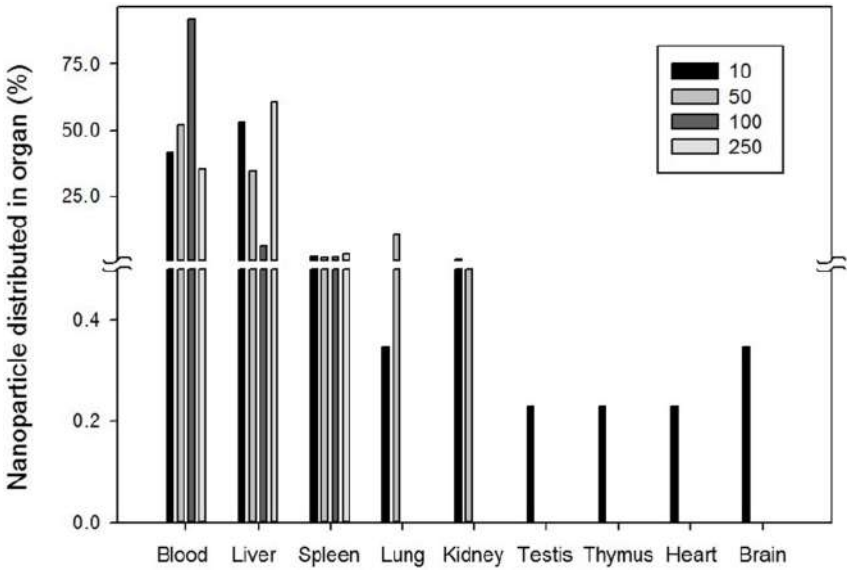
The in vivo size distribution of gold nanoparticles was evaluated by De Jong et al. [300], using particle sizes ranging from 10 to 250 nm after intravenous injection in a rat model.

The authors found that the 10-nm nanoparticles were distributed differently than their larger counterparts and were found in almost every organ, including blood, liver, spleen, kidney, testes, thymus, heart, lungs and brain.

Meanwhile, most nanoparticles larger than 50 nm have been detected only in blood, liver and spleen.

Noninvasive imaging of quantum dots in mice.
Bioconjug Chem. 2004 Jan-Feb;15(1):79-86. doi: 10.1021/bc034153y.
https://pubmed.ncbi.nlm.nih.gov/22524388/

[300] De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJ, Geertsma RE.
Particle size-dependent organ distribution of gold nanoparticles after intravenous administration.
Biomaterials. 2008 Apr;29(12):1912-9. doi: 10.1016/j.biomaterials.2007.12.037. Epub 2008 Feb 1.
https://pubmed.ncbi.nlm.nih.gov/18242692/

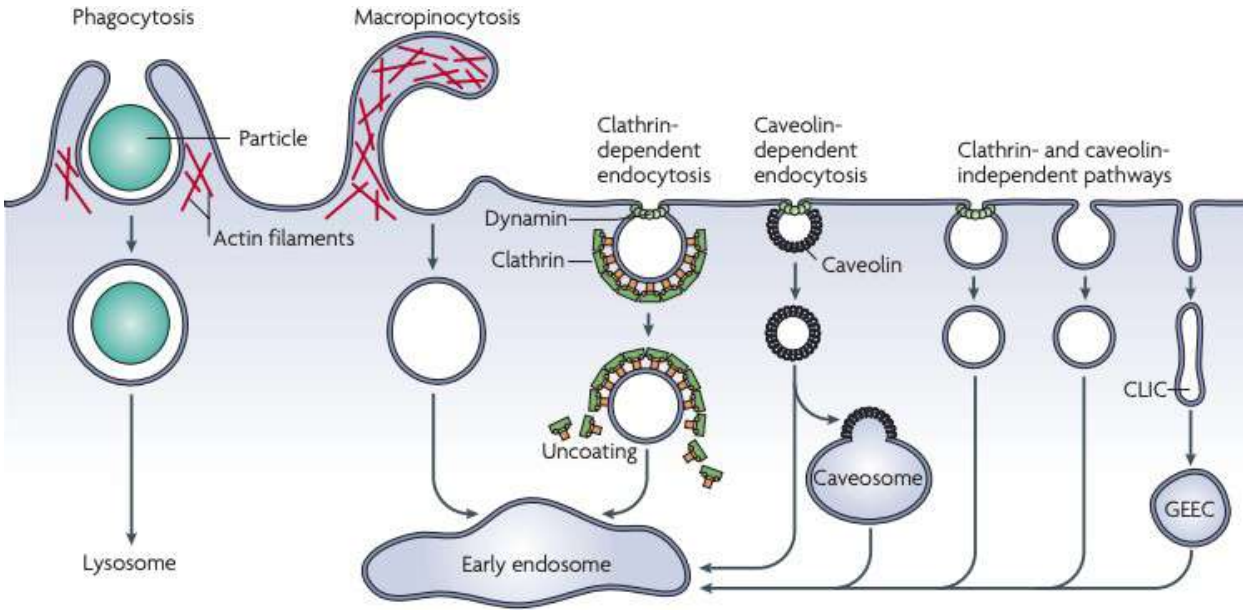


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5304630/
Distribution of gold nanoparticles in different organs in the rat according to particle size (nm).

Size-dependent cellular uptake and cytotoxicity

In terms of cellular interaction, nanoparticle uptake mechanism and efficiency are key factors influencing cytotoxicity.
One of the main factors determining the efficiency and mechanism of cellular uptake is the size of the nanoparticles. Suitable sizes for adsorption range from 10 to 500 nm.

Large particles (> 1 µm) are most likely to be swallowed by macropinocytosis. The size of a vesicle involved in clathrin-mediated endocytosis is about 100 nm, while the size involved in caveolae-mediated endocytosis is usually about 60-80 nm.



https://www.nature.com/articles/nrm2216
Routes of entry into cells. Routes of entry into cells. Larger particles can be taken up by phagocytosis, while fluid uptake occurs by macropinocytosis. Both processes appear to be triggered by and depend on actin-mediated remodeling of the plasma membrane on a large scale. Compared with the other endocytic pathways, the size of vesicles formed by phagocytosis and macropinocytosis is much larger. Numerous cargoes can be endocytosed by mechanisms independent of the clathrin-coating protein and the fission GTPase, dynamin. This review focuses on clathrin-independent pathways, some of which are also dynamin-independent (FIGS 2,3). Most internalized cargoes are delivered to the early endosome via vesicles (clathrin- or caveolin-coated vesicles) or tubular intermediates (known as clathrin- and dynamin-independent carriers (CLICs)) that arise from the plasma membrane. Some pathways may first switch to intermediate compartments, such as caveosome or glycosyl posphatidylinositol-enriched early endosomal compartment (GEEC) protein, en route to the early endosome.

Surface area of the plot:

A larger surface area can cause greater reactivity with nearby particles, resulting in potentially harmful effects when used in fillers, cosmetics, and as drug carriers [301].
It can be concluded that by decreasing the particle size, its biological activity increases substantially.
Smaller particles occupy less volume, so that more particles can occupy a unit area, resulting in increased mechanisms of pathophysiological toxicity, e.g., oxidative stress, ROS generation, mitochondrial perturbation, etc. [302].

Changes in surface charge result in significant differences in the in vivo biodistribution of nanoparticles [303]and varying degrees of toxicity [304]. Nanoparticles with a positively charged surface tend to have much higher toxicity.
It has been widely recognized in the scientific community that nanoparticles in a biological or environmental context never consist of "naked" particles [305].

As soon as particles come into contact with heterogeneous, liquid or gaseous environments, smaller structures such as atoms, clusters of atoms, single molecules and/or macromolecules attach themselves to the surface of the particle, binding strongly or weakly.

In a biological environment where biomolecules such as proteins and polymers are present, this surface layer has been called the "corona" [306].

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[301] Nel A, Xia T, Mädler L, Li N.
Toxic potential of materials at the nanolevel.
Science. 2006 Feb 3;311(5761):622-7. doi: 10.1126/science.1114397.
https://pubmed.ncbi.nlm.nih.gov/16456071/

[302] Oberdörster G et al; ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group.
Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy.
Part Fibre Toxicol. 2005 Oct 6;2:8. doi: 10.1186/1743-8977-2-8.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1260029/

[303] Hirn S, Semmler-Behnke M, Schleh C, et al.
Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration.
Eur J Pharm Biopharm. 2011;77(3):407-416. doi:10.1016/j.ejpb.2010.12.029
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3051057/

Asati A, Santra S, Kaittanis C, Perez JM. Surface-charge-dependent cell localization and cytotoxicity of cerium oxide nanoparticles. ACS Nano. 2010;4(9):5321-5331. doi:10.1021/nn100816s
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2947560/

[304] Jiang, J., Oberdörster, G. & Biswas, P.
Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies.
J Nanopart Res 11, 77-89 (2009). https://doi.org/10.1007/s11051-008-9446-4
https://link.springer.com/article/10.1007%2Fs11051-008-9446-4

Fröhlich E.
The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles.
Int J Nanomedicine. 2012;7:5577-5591. doi:10.2147/IJN.S36111
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3493258/

Ruenraroengsak P, Tetley TD.
Differential bioreactivity of neutral, cationic and anionic polystyrene nanoparticles with cells from the human alveolar compartment: robust response of alveolar type 1 epithelial cells.
Part Fibre Toxicol. 2015 Jul 2;12:19. doi: 10.1186/s12989-015-0091-7.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4489088/

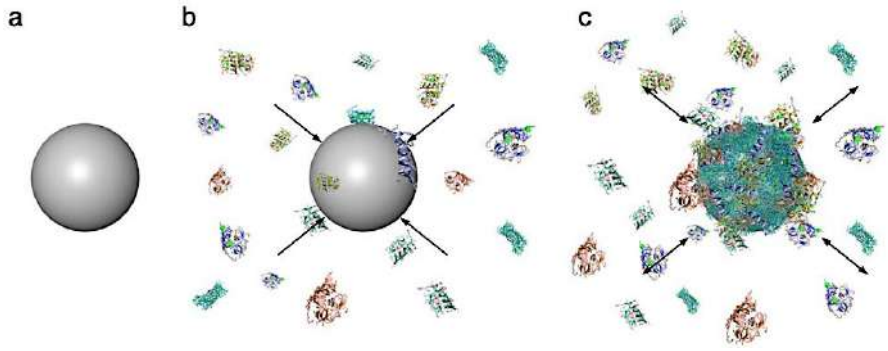
[305] Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, Quigg A, Santschi PH, Sigg L. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi.
Ecotoxicology. 2008 Jul;17(5):372-86. doi: 10.1007/s10646-008-0214-0. Epub 2008 May 7.
https://pubmed.ncbi.nlm.nih.gov/18461442/

[306] Lynch I, Cedervall T, Lundqvist M, Cabaleiro-Lago C, Linse S, Dawson KA.
The nanoparticle-protein complex as a biological entity; a complex fluids and surface science challenge for the 21st century.
Adv Colloid Interface Sci. 2007 Oct 31;134-135:167-74. doi: 10.1016/j.cis.2007.04.021. Epub 2007 May 5.
https://pubmed.ncbi.nlm.nih.gov/17574200/

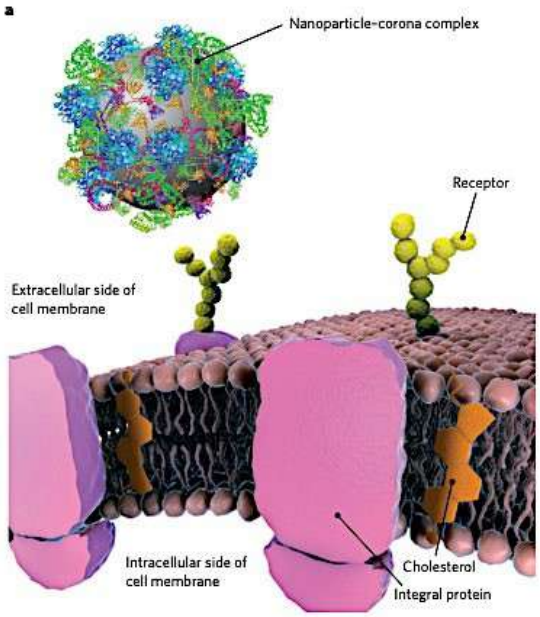
Hartland, A., Lead, J. R., Slaveykova, V. I., O'Carroll, D. & Valsami-Jones, E.

Research has shown that it is not the nanomaterial itself but the "corona" that mainly defines the properties of the "particle + corona" compound.
It follows that it is necessary to study not only the properties of the nanomaterial but also the environment in which the nanoparticles are found during nanotoxicity testing [307]

Nanoparticle + corona formation: a) "naked" particle, b) nanoparticles in contact with proteins, c) corona formation. The crown can consist of a "hard crown," proteins firmly attached to the surface, or a "soft crown" of proteins that are only weakly bound to the nanoparticles and form a layer in equilibrium with the surrounding matrix.



https://pubmed.ncbi.nlm.nih.gov/21925220/ https://pubmed.ncbi.nlm.nih.gov/21925220/



https://pubmed.ncbi.nlm.nih.gov/23212421/

Morphology

Like other well-established inhalable fibers (e.g., asbestos), nanoscale fibers (e.g., carbon nanotubes) have a serious risk of lung inflammation.
In addition, prolonged exposure can cause several types of cancer. It is difficult to determine whether there is a certain toxic effect of individual nanotubes or a set of such tubes. Some studies have shown that carbon nanotubes are more toxic than other ultrafine carbon black or silica powders. [308]

The Environmental Significance of Natural Nanoparticles.
Nature Education Knowledge (2013) 4(8):7
https://www.nature.com/scitable/knowledge/library/the-environmental-significance-of-natural-nanoparticles-105737311/

Cedervall T, Lynch I, Lindman S, et al.
Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci U S A. 2007;104(7):2050-2055. doi:10.1073/pnas.0608582104
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1892985/

Monopoli MP, Aberg C, Salvati A, Dawson KA.
Biomolecular coronas provide the biological identity of nanosized materials.
Nat Nanotechnol. 2012 Dec;7(12):779-86. doi: 10.1038/nnano.2012.207.
https://pubmed.ncbi.nlm.nih.gov/23212421/

Murr LE, Garza KM, Soto KF, et al.
Cytotoxicity assessment of some carbon nanotubes and related carbon nanoparticle aggregates and the implications for anthropogenic carbon nanotube aggregates in the environment.
Int J Environ Res Public Health. 2005;2(1):31-42. doi:10.3390/ijerph2005010031
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3814694/

[307] Lynch I, Salvati A, Dawson KA.
Protein-nanoparticle interactions: What does the cell see?
Nat Nanotechnol. 2009 Sep;4(9):546-7. doi: 10.1038/nnano.2009.248.
https://www.nature.com/articles/nnano.2009.248

Elsaesser A, Howard CV.
Toxicology of nanoparticles.
Adv Drug Deliv Rev. 2012 Feb;64(2):129-37. doi: 10.1016/j.addr.2011.09.001. Epub 2011 Sep 8.
https://pubmed.ncbi.nlm.nih.gov/21925220/

[308] Cha KE, Myung H.
Cytotoxic effects of nanoparticles assessed in vitro and in vivo.
J Microbiol Biotechnol. 2007 Sep;17(9):1573-8.
https://pubmed.ncbi.nlm.nih.gov/18062241/

NANOPARTICLES AND CELLS

Membranes

In a cellular context, **membranes** are phospho-lipid bilayers that subdivide different intracellular compartments with each having specific functions and also encapsulate the entire cell.

To facilitate exchanges between compartments and/or cells, membranes must be permeable.
The outer cell membrane is the cell's interface with its external environment and enables the selective transport of ions, molecules, and even nanoparticles.
Intracellular membranes separate distinct compartments (mitochondria, vesicles, nucleus, etc.) from the cytosol. Membrane stability can be affected by nanoparticles directly (e.g., physical damage) or indirectly (e.g., oxidation), which can lead to cell death.

It is the ability of membranes to control intracellular homeostasis, through selective permeability and transport mechanisms, that makes them a vulnerable target for the possible damaging effects of nanoparticles.
The interactions of nanoparticles with membranes depend largely on the surface properties of the nanoparticles.
This is why surface modifications are crucial in the design of drug delivery systems in order to improve nanoparticle uptake into cells.
Nanoparticle size also plays an important role as it affects surface pressure and adhesion forces. [309]

Mitochondria, as the power plants of the cell, appear to be the main target of fullerenes and carbon nanotubes [310].
However, other nanoparticles (e.g., titanium dioxide, carbon nanotubes, polystyrene, and silver) also appear to be able to affect mitochondrial function, leading to apoptosis [311].

Signatures CP 3rd, Bandaru PR.
Toxicity issues in the application of carbon nanotubes to biological systems.
Nanomedicine. 2010 Apr;6(2):245-56. doi: 10.1016/j.nano.2009.07.003. Epub 2009 Aug 20. https://pubmed.ncbi.nlm.nih.gov/19699321/

[309] Vasir JK, Labhasetwar V.
Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles.
Biomaterials. 2008;29(31):4244-4252. doi:10.1016/j.biomaterials.2008.07.020
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2570224/

Peetla C, Labhasetwar V. Biophysical characterization of nanoparticle-endothelial model cell membrane interactions.
Mol Pharm. 2008 May-Jun;5(3):418-29. doi: 10.1021/mp700140a. Epub 2008 Feb 14.
https://pubmed.ncbi.nlm.nih.gov/18271547/

[310] Foley S, Crowley C, Smaihi M, Bonfils C, Erlanger BF, Seta P, Larroque C.
Cellular localization of a water-soluble fullerene derivative.
Biochem Biophys Res Commun. 2002 May 31;294(1):116-9. doi: 10.1016/S0006-291X(02)00445-X.
https://pubmed.ncbi.nlm.nih.gov/12054749/

Zhu Y, Zhao Q, Li Y, Cai X, Li W.
The interaction and toxicity of multi-walled carbon nanotubes with Stylonychia mytilus.
J Nanosci Nanotechnol. 2006 May;6(5):1357-64. doi: 10.1166/jnn.2006.194.
https://pubmed.ncbi.nlm.nih.gov/16792365/

[311] Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ.
In vitro toxicity of nanoparticles in BRL 3A rat liver cells.
Toxicol In Vitro. 2005 Oct;19(7):975-83. doi: 10.1016/j.tiv.2005.06.034. Epub 2005 Aug 25.
https://pubmed.ncbi.nlm.nih.gov/16125895/

Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, Sioutas C, Yeh JI, Wiesner MR, Nel AE.
Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm.
Nano Lett. 2006 Aug;6(8):1794-807. doi: 10.1021/nl061025k.
https://pubmed.ncbi.nlm.nih.gov/16895376/

Jia G, Wang H, Yan L, Wang X, Pei R, Yan T, Zhao Y, Guo X.
Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene.
Environ Sci Technol. 2005 Mar 1;39(5):1378-83. doi: 10.1021/es048729l.

Another preferred intracellular compartment is the **lysosomes**, the digestive system of the cell. Research has shown that nanoparticles generally end up in lysosomes where the cell tries to digest or excrete them [312].

Finally, the nuclear **membrane** uses nuclear pores to transport substances in and out of the nucleus. Some nanoparticles, generally smaller, appear to be able to diffuse through these pores [313] or be transported via receptor-mediated transport mechanisms to gain access to the intra-nuclear area. [314]

Macromolecules and proteins

The cellular apparatus is largely based on proteins and other macromolecules.
These exist in the form of enzymes (e.g., gastrin), cell signaling molecules (e.g., hormones) or structural proteins (e.g., tubulin).

Their normal functioning is therefore essential for all vital cellular activities.
Proper molecular conformation is essential for proteins to function as intended, and slight conformational changes can alter or destroy protein function.

During their assembly process, chaperones play an important role in controlling the way proteins fold [315], in order to achieve a certain conformation.

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https://pubmed.ncbi.nlm.nih.gov/15787380/

312Mohammadinejad R, Moosavi MA, Tavakol S, et al.
Necrotic, apoptotic and autophagic cell fates triggered by nanoparticles.
Autophagy. 2019;15(1):4-33. doi:10.1080/15548627.2018.1509171
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6287681/

Al-Rawi M, Diabaté S, Weiss C.
Uptake and intracellular localization of submicron and nano-sized SiO_2 particles in HeLa cells.
Arch Toxicol. 2011 Jul;85(7):813-26. doi: 10.1007/s00204-010-0642-5. Epub 2011 Jan 15.
https://pubmed.ncbi.nlm.nih.gov/21240478/

[313] Parfenov AS, Salnikov V, Lederer WJ, Lukyánenko V.
Aqueous diffusion pathways as a part of the ventricular cell ultrastructure.
Biophys J. 2006;90(3):1107-1119. doi:10.1529/biophysj.105.071787
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1367097/

Godbey WT, Wu KK, Mikos AG.
Tracking the intracellular pathway of poly(ethylenimine)/DNA complexes for gene
delivery. Proc Natl Acad Sci U S A. 1999;96(9):5177-5181. doi:10.1073/pnas.96.9.5177
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC21837/

[314] Panté N, Kann M.
Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm.
Mol Biol Cell. 2002;13(2):425-434. doi:10.1091/mbc.01-06-0308
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC65638/

Williams Y, Sukhanova A, Nowostawska M, Davies AM, Mitchell S, Oleinikov V, Gun'ko Y, Nabiev I, Kelleher D, Volkov Y.
Probing cell-type-specific intracellular nanoscale barriers using size-tuned quantum dots.
Small. 2009 Nov;5(22):2581-8. doi: 10.1002/smll.200900744.
https://pubmed.ncbi.nlm.nih.gov/19685445/

Hoshino A, Fujioka K, Oku T, Nakamura S, Suga M, Yamaguchi Y, Suzuki K, Yasuhara M, Yamamoto K.
Quantum dots targeted to the assigned organelle in living cells.
Microbiol Immunol. 2004;48(12):985-94. doi: 10.1111/j.1348-0421.2004.tb03621.x.
https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1348-0421.2004.tb03621.x

[315] Englander SW, Mayne L, Krishna MM.
Protein folding and misfolding: mechanism and principles.
Q Rev Biophys. 2007;40(4):287-326. doi:10.1017/S0033583508004654
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3433742/

Chandel TI, Zaman M, Khan MV, Ali M, Rabbani G, Ishtikhar M, Khan RH.
A mechanistic insight into protein-ligand interaction, folding, misfolding, aggregation and inhibition of protein aggregates: An overview.
Int J Biol Macromol. 2018 Jan;106:1115-1129. doi: 10.1016/j.ijbiomac.2017.07.185. Epub 2017 Sep 8.
https://pubmed.ncbi.nlm.nih.gov/28890370/

Molecular chaperones and protein folding Cellular function and intracellular trafficking

Nanoparticles the same size as protein molecules, are able to interfere with cell signaling processes [316] or interact with proteins [317], either with chaperone-like activity [318] or by changing the configuration of peptides into aggregation and fibrillation forms [319].

Protein misfolding and peptide fibrillation leading to amyloid-like structures are associated with neurodegenerative diseases.[320]
The study of possible misformation and overproduction of proteins and macromolecules at the cellular level is important in nanotoxicological considerations . [321]

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[316] F. Marano F, Hussain S, Rodrigues-Lima F, Baeza-Squiban A, Boland S.
Nanoparticles: molecular targets and cell signalling.
Arch Toxicol. 2011 Jul;85(7):733-41. doi: 10.1007/s00204-010-0546-4. Epub 2010 May 26. PMID: 20502881.
https://pubmed.ncbi.nlm.nih.gov/20502881/

[317] Dawson KA, Salvati A, Lynch I.
Nanotoxicology: nanoparticles reconstruct lipids.
Nat Nanotechnol. 2009 Feb;4(2):84-5. doi: 10.1038/nnano.2008.426. PMID: 19197306.
https://www.nature.com/articles/nnano.2008.426

Shang W, Nuffer JH, Dordick JS, Siegel RW.
Unfolding of ribonuclease A on silica nanoparticle surfaces.
Nano Lett. 2007 Jul;7(7):1991-5. doi: 10.1021/nl070777r. Epub 2007 Jun 9. PMID: 17559285.
https://pubmed.ncbi.nlm.nih.gov/17559285/

[318] Takahashi H, Sawada S, Akiyoshi K. A
mphiphilic polysaccharide nanoballs: a new building block for biomedical engineering nanogels and artificial chaperones.
ACS Nano. 2011 Jan 25;5(1):337-45. doi: 10.1021/nn101447m. Epub 2010 Dec 7. PMID: 21138322.
https://pubmed.ncbi.nlm.nih.gov/21138322/

K. Akiyoshi K, Sasaki Y, Sunamoto J.
Molecular chaperone-like activity of hydrogel nanoparticles of hydrophobized pullulan: thermal stabilization with refolding of carbonic anhydrase B.
Bioconjug Chem. 1999 May-Jun;10(3):321-4. doi: 10.1021/bc9801272. PMID: 10346859.
https://pubmed.ncbi.nlm.nih.gov/10346859/

[319] Wu WH, Sun X, Yu YP, Hu J, Zhao L, Liu Q, Zhao YF, Li YM.
TiO2 nanoparticles promote beta-amyloid fibrillation in vitro.
Biochem Biophys Res Commun. 2008 Aug 22;373(2):315-8. doi: 10.1016/j.bbrc.2008.06.035. Epub 2008 Jun 20. PMID: 18571499.
https://pubmed.ncbi.nlm.nih.gov/18571499/

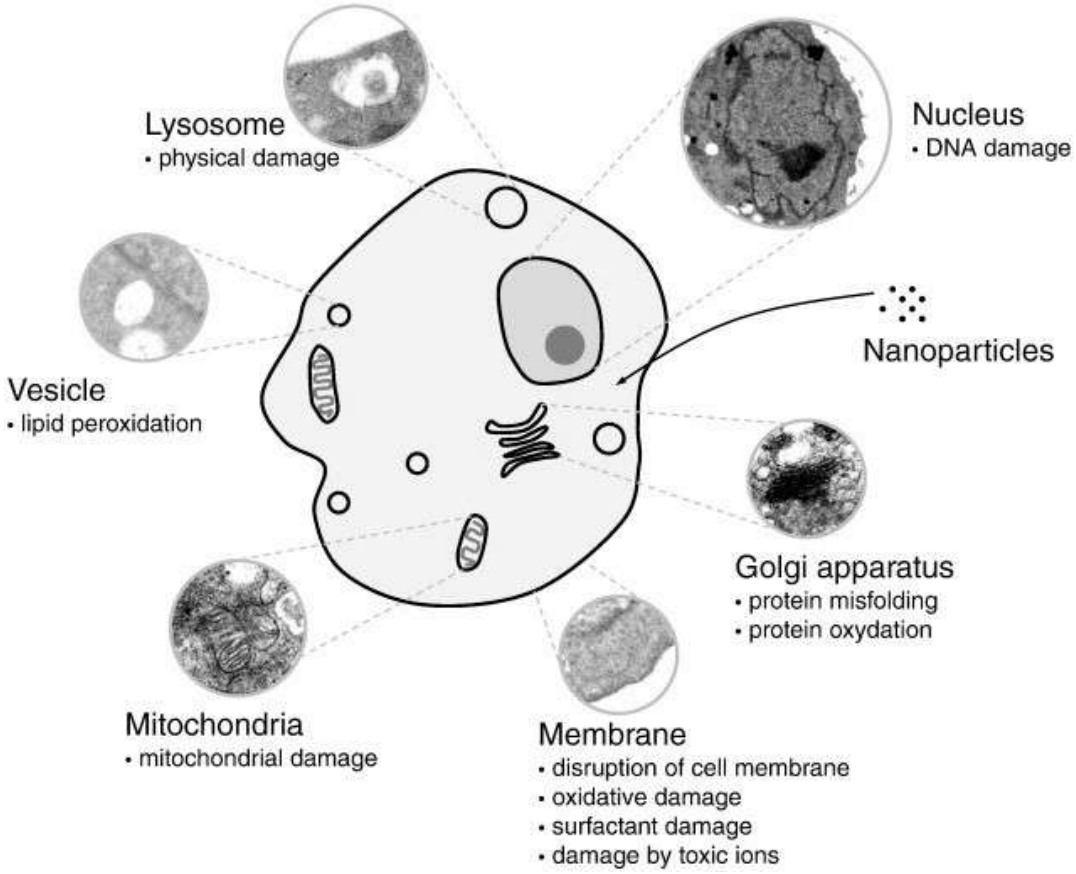
Wagner SC, Roskamp M, Pallerla M, Araghi RR, Schlecht S, Koksch B.
Nanoparticle-induced folding and fibril formation of coiled-coil-based model peptides.
Small. 2010 Jun 21;6(12):1321-8. doi: 10.1002/smll.200902067. PMID: 20517875.
https://pubmed.ncbi.nlm.nih.gov/20517875/

Aili D, Enander K, Rydberg J, Nesterenko I, Björefors F, Baltzer L, Liedberg B.
Folding induced assembly of polypeptide decorated gold nanoparticles.
J Am Chem Soc. 2008 Apr 30;130(17):5780-8. doi: 10.1021/ja711330f. Epub 2008 Apr 2. PMID: 18380430.
https://pubmed.ncbi.nlm.nih.gov/18380430/

[320] Proteins that "fibrillate": amyloidosis. New hope for a disease that cardiologists need to know about Eloisa
Arbustini, Antonello Gavazzi*, Giampaolo Merlini
(Ital Heart J Suppl 2002; 3(6): 590-597)
https://www.giornaledicardiologia.it/allegati/00771_2002_06/fulltext/06-02_02%20590-597.pdf

[321] Dawson KA, Salvati A, Lynch I.
Nanotoxicology: nanoparticles reconstruct lipids.
Nat Nanotechnol. 2009 Feb;4(2):84-5. doi: 10.1038/nnano.2008.426. PMID: 19197306.
https://www.nature.com/articles/nnano.2008.426

Bucciantini M, Giannoni E, Chiti F, Baroni F, Formigli L, Zurdo J, Taddei N, Ramponi G, Dobson CM, Stefani M.
Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature. 2002 Apr 4;416(6880):507-11. doi: 10.1038/416507a. PMID: 11932737.
https://pubmed.ncbi.nlm.nih.gov/11932737/



https://www.sciencedirect.com/science/article/abs/pii/S0169409X11002328
Interaction of nanoparticles with cells: intracellular targets and nanotoxicological mechanisms.

DNA

Since the earliest studies in nanotoxicology, DNA has attracted special attention in the evaluation of potential toxicological risks caused by nanomaterials. As researchers have reported that nanoparticles are able to enter the nuclear envelope, interest in the possible genotoxic effects of nanoparticles has shifted to center stage. [322]

Several nanoparticles have been tested for genotoxicity [323]. However, these studies have not been able to clearly identify which nanoparticle parameter is responsible for the positive or negative outcomes.

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[322] Bhabra G, et al.
Nanoparticles can cause DNA damage across a cellular barrier.
Nat Nanotechnol. 2009 Dec;4(12):876-83. doi: 10.1038/nnano.2009.313. PMID: 19893513.
https://pubmed.ncbi.nlm.nih.gov/19893513/

Myllynen P.
Nanotoxicology: damaging DNA from a distance.
Nat Nanotechnol. 2009 Dec;4(12):795-6. doi: 10.1038/nnano.2009.365. PMID: 19966824.
https://www.nature.com/articles/nnano.2009.365

[323] Zhanataev AK, Anisina EA, Kulakova AV, Shilovskiy IP, Lisitsyn AA, Koloskova OO, Khaitov MR, Durnev AD.
Genotoxicity of cationic lipopeptide nanoparticles.
Toxicol Lett. 2020 Aug 1;328:1-6. doi: 10.1016/j.toxlet.2020.04.011. Epub 2020 Apr 19.
https://pubmed.ncbi.nlm.nih.gov/32315709/

Charles S, Jomini S, Fessard V, Bigorgne-Vizade E, Rousselle C, Michel C.
Assessment of the in vitro genotoxicity of TiO2 nanoparticles in a regulatory context.
Nanotoxicology. 2018 May;12(4):357-374. doi: 10.1080/17435390.2018.1451567. Epub 2018 Mar 19. PMID: 29553842.
https://pubmed.ncbi.nlm.nih.gov/29553842/

Rodriguez-Garraus A, Azqueta A, Vettorazzi A, López de Cerain A.
Genotoxicity of Silver Nanoparticles.
Nanomaterials (Basel). 2020;10(2):251. Published 2020 Jan 31. doi:10.3390/nano10020251

Moreover, the mechanism of potential DNA damage is not completely understood. Apart from direct intercalation or physical and/or electrochemical interaction with nanoparticles [324], it is again believed that ROS play a key role in DNA damage.

This means that the particles do not necessarily have to reach the nucleus, but could, for example, induce genotoxicity through oxidative stress.

THE NANOTOXICOLOGY OF ADJUVANT ALUMINUM.

Adjuvant aluminum has been used in vaccine practice for more than 50 years to induce effective immunization.
Glenny and his collaborators were the first researchers to demonstrate the adjuvanting effect of aluminum compounds in 1926 by injecting diphtheria toxoid precipitated with **potassium alum** ($KAl(_{SO4})_{2\cdot 12H2O}$).
Vaccines prepared according to this principle are referred to in vaccine practice as alum-precipitated vaccines.
Because such preparations turn out to be particularly heterogeneous, **hydrated aluminum hydroxide gels** with the ability to adsorb antigen proteins from an aqueous solution according to well-standardized procedures have been developed; such preparations are called adsorbed aluminum vaccines.
Aluminum phosphate was introduced in 1946 following Ericson's research in which diphtheria toxoid was co-precipitated in an aluminum phosphate matrix.

Adjuvant aluminum elicits a **strong Th2 response**, but is rather ineffective against pathogens that require Th1-cell-mediated immunity.
Induces the immune response through a **depot effect and activation of APCs** (antigen-presenting cells).

Recently, the **NLRP3 inflammasome** has been linked to its immunostimulatory properties (and also its toxicity). [325]
A mechanism of inflammasome activation has been explained by lysosomal damage induced by aluminum hydroxide, which triggers the release of free cathepsin B that can initiate inflammasome assembly, activate caspase-1, and release active cytokines.
Initial activation of nuclear transcription factor (NF-κB) by lipopolysaccharide (LPS) seems to be required for activation of NLRP3 by aluminum hydroxide [62]. In addition, aluminum hydroxide may increase uric acid levels at the vaccination site, which could promote the conversion of pro-IL-1b and pro-IL-18 into their active forms through activation of NLRP3. [326]

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7075128/

Karlsson HL. The comet assay in nanotoxicology research.
Anal Bioanal Chem. 2010 Sep;398(2):651-66. doi: 10.1007/s00216-010-3977-0. Epub 2010 Jul 18. PMID: 20640410.
https://pubmed.ncbi.nlm.nih.gov/20640410/

[324] Xie W, Wang L, Zhang Y, Su L, Shen A, Tan J, Hu J.
Nuclear targeted nanoprobe for single living cell detection by surface-enhanced Raman scattering.
Bioconjug Chem. 2009 Apr;20(4):768-73. doi: 10.1021/bc800469g. PMID: 19267459.
https://pubmed.ncbi.nlm.nih.gov/19267459/

Mehrabi M, Wilson R.
Intercalating gold nanoparticles as universal labels for DNA detection.
Small. 2007 Sep;3(9):1491-5. doi: 10.1002/smll.200700230. PMID: 17661307.
https://pubmed.ncbi.nlm.nih.gov/17661307/

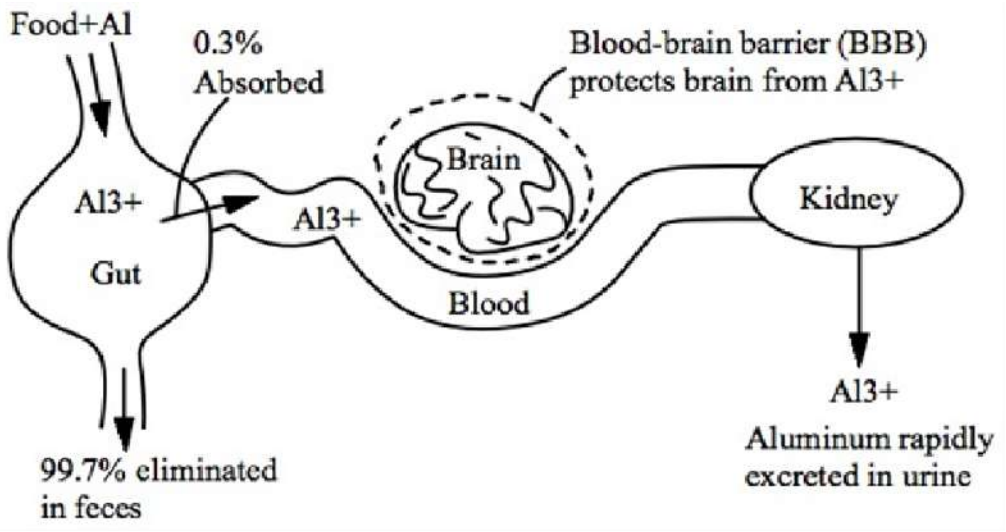
[325] Ghimire TR.
The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm.
Springerplus. 2015;4:181. Published 2015 Apr 16. doi:10.1186/s40064-015-0972-0
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4406982/

[326] Ivanov K, Garanina E, Rizvanov A, Khaiboullina S.
Inflammasomes as Targets for Adjuvants.
Pathogens. 2020;9(4):252. Published 2020 Mar 30. doi:10.3390/pathogens9040252
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7238254/

It is important to keep in mind that the aluminum used as an adjuvant is in the form of **Al hydroxide and/or Al phosphate (henceforth abbreviated as NA) nanoparticles,** and that the safety of aluminum as an adjuvant has been based on studies related to its ingestion, for which there is little absorption (about 0.3%) [327] , compared with intramuscular administration, which achieves an absorption of about 100%. [328]

Kinetics of ingested aluminum

Ingested aluminum enters the blood through the intestine. In the blood, orally assimilated aluminum is in water-soluble ionic form, typically Al^{+3} or as an aluminum complex (e.g., with amino acids, ferritin ect).
Ionic aluminum is toxic, but its penetration into the brain is blocked by the blood-brain barrier (BBB), and it is then rapidly filtered from the blood into the kidneys without causing excessive toxicity, if in small amounts. These natural defenses are adequate to protect the brain from the normal naturally occurring levels of ingested aluminum.



http://vaccinepapers.org/vaccine-aluminum-travels-to-the-brain/

Kinetics of intramuscularly administered aluminum.

In contrast, when aluminum is injected intramuscularly, NAs are phagocytosed by macrophages before they can go into solution.
The problem with NAs is that they are not digested by macrophage enzymes, and when they are in them they go into solution much more slowly.

NAs then persist for a long time and macrophages slowly let aluminum escape.
Macrophages that phagocytize NA then become highly contaminating aluminum vehicles, as they spread it to every district of the body, including the brain because they are able to cross the blood-brain barrier.
Once inside the brain, the aluminum damages brain cells, and the toxic substances produced in turn trigger inflammation that attracts more macrophages, which can carry more aluminum, and thus set up a vicious cycle that leads to more and more brain damage.

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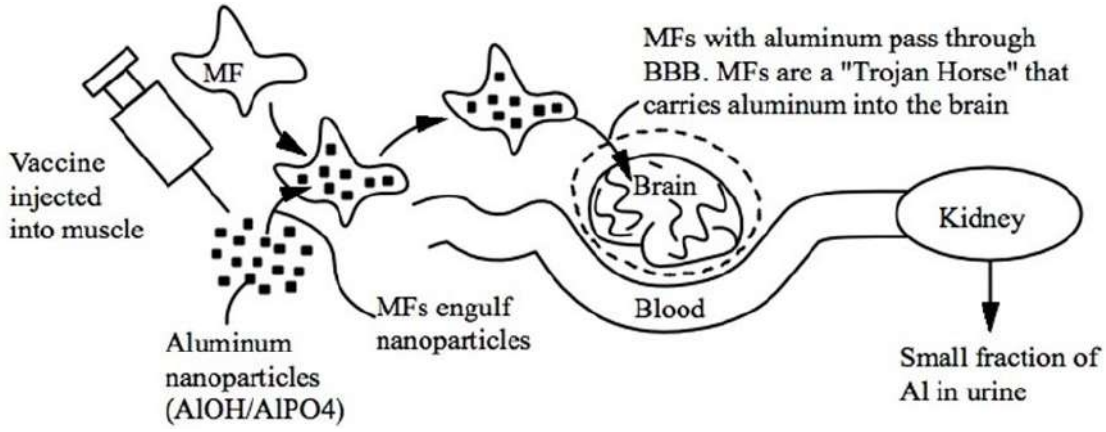
[327] Yokel RA, Hicks CL, Florence RL.
Aluminum bioavailability from basic sodium aluminum phosphate, an approved food additive emulsifying agent, incorporated into cheese.
Food Chem Toxicol. 2008;46(6):2261-2266. doi:10.1016/j.fct.2008.03.004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2449821/

[328] Yokel RA, McNamara PJ.
Aluminium toxicokinetics: an updated minireview.
Pharmacol Toxicol. 2001 Apr;88(4):159-67. doi: 10.1034/j.1600-0773.2001.d01-98.x. PMID: 11322172.
https://pubmed.ncbi.nlm.nih.gov/11322172/



http://vaccinepapers.org/vaccine-aluminum-travels-to-the-brain/
Before the aluminum nanoparticles can dissolve, they are ingested by macrophages (MFs). The MFs then transport the aluminum nanoparticles throughout the body, including the brain. MFs through the BBB. Inside the brain, aluminum is slowly released by the MFs and causes brain damage.

The scientific evidence supporting this mechanism of aluminum delivery/translocation and toxicity

Keep in mind that the brain is an organ that is extremely sensitive to aluminum; aluminum concentrations of 10-100 nanoM (equal to 270 nanograms/L of aluminum) are capable of causing inflammation of blood vessel cells respectively [329] and human neurons, respectively.[330]

Scientific evidence for this delivery mechanism is documented by multiple studies conducted by universities and government-funded laboratories to demonstrate the following steps:

-Macrophage incorporation of NA, translocation of macrophages into the brain
-The observation that macrophages transport nanoparticles into the brain.

In addition, the whole process has also been demonstrated: NA injected into experimental animals has been detected and photographed in the brain.

The results of some of the many studies are given below:

Mold et al (2014 [331] e 2016 [332]) demonstrated for the first time the unequivocal identification of adjuvant aluminum nanoparticles within THP-1 (monocytic T helper 1 cell line) cells and that a

[329] Alexandrov PN, Kruck TP, Lukiw WJ.
Nanomolar aluminum induces expression of the inflammatory systemic biomarker C-reactive protein (CRP) in human brain microvessel endothelial cells (hBMECs).
J Inorg Biochem. 2015 Nov;152:210-3. doi: 10.1016/j.jinorgbio.2015.07.013. Epub 2015 Aug 1. PMID: 26265215.
https://pubmed.ncbi.nlm.nih.gov/26265215/.

[330] Lukiw WJ, Percy ME, Kruck TP.
Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture.
J Inorg Biochem. 2005 Sep;99(9):1895-8. doi: 10.1016/j.jinorgbio.2005.04.021. PMID: 15961160.
https://pubmed.ncbi.nlm.nih.gov/15961160/

[331] Mold M, Eriksson H, Siesjö P, Darabi A, Shardlow E, Exley C.
Unequivocal identification of intracellular aluminium adjuvant in a monocytic THP-1 cell line.
Sci Rep. 2014;4:6287. Published 2014 Sep 5. doi:10.1038/srep06287
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4155332/

Mold M, Shardlow E, Exley C.
Insight into the cellular fate and toxicity of aluminum adjuvants used in clinically approved human vaccinations.
Sci Rep. 2016;6:31578. Published 2016 Aug 12. doi:10.1038/srep31578
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4981857/

[332] Mold M, Shardlow E, Exley C.
Insight into the cellular fate and toxicity of aluminum adjuvants used in clinically approved human vaccinations.
Sci Rep. 2016;6:31578. Published 2016 Aug 12. doi:10.1038/srep31578
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4981857/

overload of aluminum hydroxide in the cytoplasm of THP-1 cells, while not inducing immediate cytotoxicity may promote its subsequent transport through the body including access to the brain.

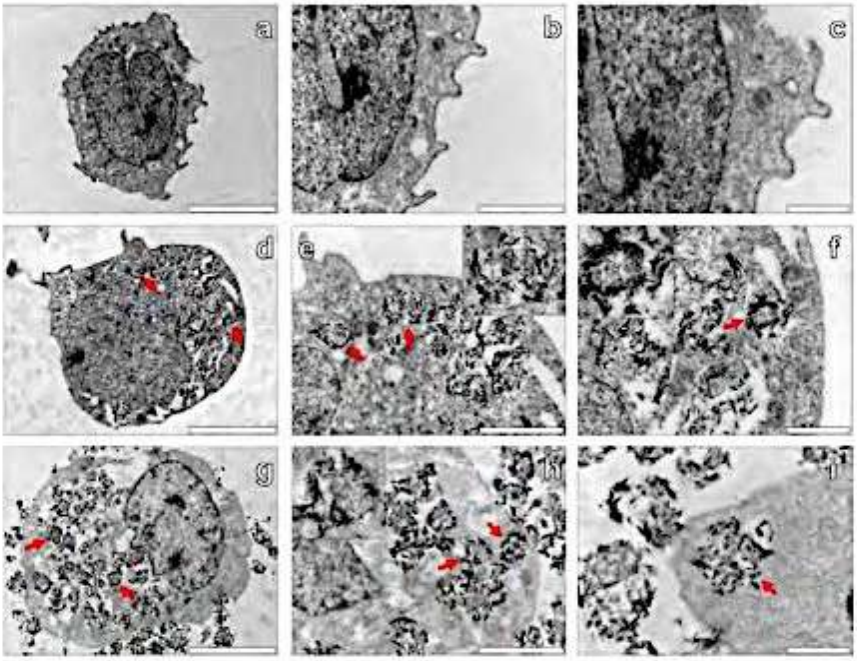
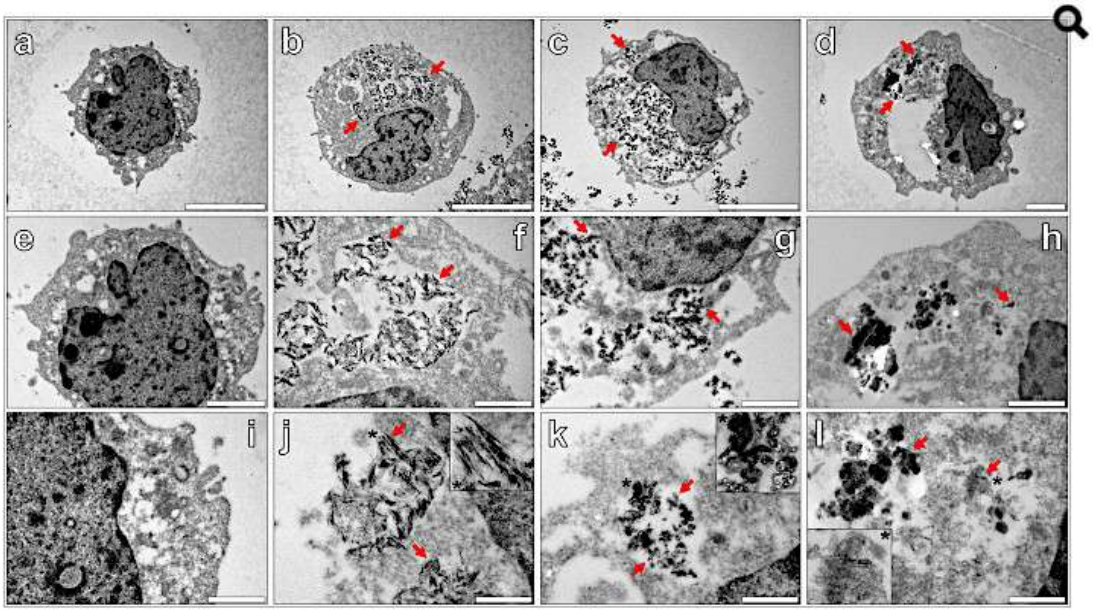


Figure 5 | Representative electron micrographs from TEM of Spurr resin-sectioned (100 nm sections) native THP-1 cells (a–c), THP-1 cells co-cultured with 50 µg/mL AlO(OH)^{Sigma} adjuvant (24 h) (d–f) & THP-1 cells co-cultured with 200 µg/mL AlO(OH)^{Sigma} adjuvant (24 h) (g–i). Cell resin-sections were stained for 20 min with 2% ethanolic uranyl acetate, rinsed with 30% ethanol followed by ultrapure water and finally allowed 24 h drying time prior to analysis via TEM. Inserts show close-ups of intracellular adjuvant particles contained within vesicle-like structures and the red arrows highlight their presence within the respective cell images. Magnification & scale bars: (a)., (d). & (g). × 8 K, 5 µm, (b)., (e). & (h). × 20 K, 2 µm, (c)., (f). & (i). × 30 K, 1 µm, respectively.

Figure 8



Representative electron micrographs from TEM of Spurr resin-sectioned (100 nm sections) native THP-1 cells (**a**,**e**,**i**). THP-1 cells co-cultured with 50 µg/mL Alhydrogel® (24 h) (**b**,**f**,**j**) and 50 µg/mL Adju-Phos® (Brenntag Biosector, Denmark) adjuvant (24 h) (**c**,**g**,**k**) and 50 µg/mL Imject™ Alum (Pierce, Thermo Scientific) adjuvant (24 h) (**d**,**h**,**l**). Cell resin-sections were stained for 20 min with 2% ethanolic uranyl acetate, rinsed with 30% ethanol followed by ultrapure water and finally allowed 24 h drying time prior to analysis via TEM. Inserts show close-ups of intracellular adjuvant particles contained within vesicle-like structures and the red arrows highlight their presence within the respective cell images. Magnification and scale bars: (**a–c**) X 8 K, 5 µm, (**d**) X 10 K, 2 µm, (**e**) X 15 K, 2 µm, (**f–h**) X 30 K, 1 µm, (**i**) X 30 K, 1 µm and (**j–l**) X 60 K, 0.5 µm, respectively.

Electron microscopy images of adjuvant aluminum nanoparticles inside macrophages, which ingest (phagocytose) the nanoparticles
Fig. 5 https://pubmed.ncbi.nlm.nih.gov/25498314/
Fig. 8 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4981857/

These studies by the group of Mold et al, [333] have made it possible to demonstrate that phagocytosis is one of the most recognized mechanisms governing the cellular internalization and subsequent degradation of adjuvant aluminum nanoparticles, through the molecular process of autophagy.

In the latter stages of autophagy, maturation of autophagosomes into autolysosomes acidifies the resulting vesicular compartments formed at about pH 4.0 to 4.5.

This causes degradation of the internalized adjuvant aluminum particulate in lysosomes, thereby releasing Al^{3+} (aq) into the cell cytosol.

The release of the enzyme cathepsin B acts as an endogenous danger signal, which together with adjuvant aluminum degradation products that induce the production of immunostimulatory molecules (damage associated molecular patterns: DAMPs), transform antigen-presenting cells into inflammatory cells; this effect increases cell death in vitro and could be responsible for inflammation at the injection site.

Studies by Gherardi et a l [334]

[333] Exley C, Mold MJ.
The binding, transport and fate of aluminium in biological cells.
J Trace Elem Med Biol. 2015 Apr;30:90-5. doi: 10.1016/j.jtemb.2014.11.002. Epub 2014 Nov 20. PMID: 25498314.
https://pubmed.ncbi.nlm.nih.gov/25498314/

Ohlsson L, Exley C, Darabi A, Sandén E, Siesjö P, Eriksson H.
Aluminium based adjuvants and their effects on mitochondria and lysosomes of phagocytosing cells.
J Inorg Biochem. 2013 Nov;128:229-36. doi: 10.1016/j.jinorgbio.2013.08.003. Epub 2013 Aug 9. PMID: 23992993.
https://pubmed.ncbi.nlm.nih.gov/23992993/.

Mile I, Svensson A, Darabi A, Mold M, Siesjö P, Eriksson H.
Al adjuvants can be tracked in viable cells by lumogallion staining.
J Immunol Methods. 2015 Jul;422:87-94. doi: 10.1016/j.jim.2015.04.008. epub 2015 Apr 17. PMID: 25896212.
https://pubmed.ncbi.nlm.nih.gov/25896212/

[334] Crépeaux G, Eidi H, David MO, Baba-Amer Y, Tzavara E, Giros B, Authier FJ, Exley C, Shaw CA, Cadusseau J, Gherardi RK.
Non-linear dose-response of aluminum hydroxide adjuvant particles: Selective low dose neurotoxicity.
Toxicology. 2017 Jan 15;375:48-57. doi: 10.1016/j.tox.2016.11.018. Epub 2016 Nov 28. PMID: 27908630.
https://pubmed.ncbi.nlm.nih.gov/27908630/

Gherardi RK, Aouizerate J, Cadusseau J, Yara S, Authier FJ.
Aluminum adjuvants of vaccines injected into the muscle: Normal fate, pathology and associated disease.
Morphologie. 2016 Jun;100(329):85-94. doi: 10.1016/j.morpho.2016.01.002. Epub 2016 Apr 6. PMID: 26948677.
https://pubmed.ncbi.nlm.nih.gov/26948677/

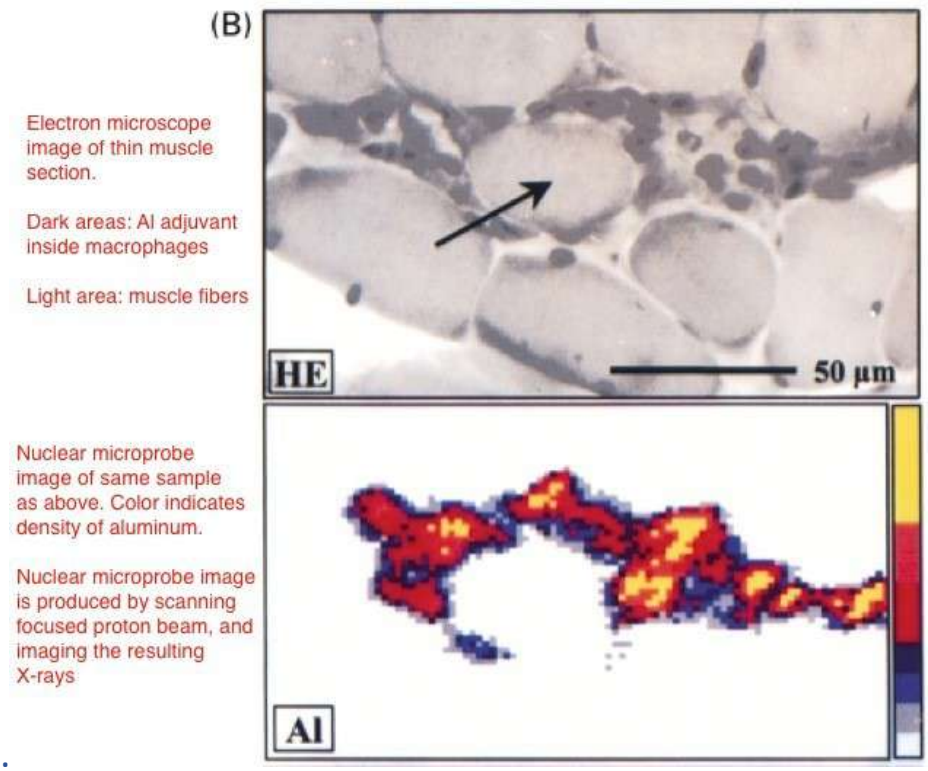
Gherardi RK, Eidi H, Crépeaux G, Authier FJ, Cadusseau J.
Biopersistence and brain translocation of aluminum adjuvants of vaccines.
Front Neurol. 2015;6:4. Published 2015 Feb 5. doi:10.3389/fneur.2015.00004
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4318414/

Eidi H, David MO, Crépeaux G, et al.
Fluorescent nanodiamonds as a relevant tag for the assessment of alum adjuvant particle biodisposition.
BMC Med. 2015;13:144. Published 2015 Jun 17. doi:10.1186/s12916-015-0388-2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4482291/

Gherardi RK, Cadusseau J, Authier FJ.
Bio-persistance et distribution systémique des particules injectées par voie intra-musculaire: quelle incidence sur la tolérance à long terme des adjuvants aluminiques? [Biopersistence and systemic distribution of intramuscularly injected particles: what impact on long-term tolerability of alum adjuvants?].
Bull Acad Natl Med. 2014 Jan;198(1):37-48; discussion 49-53. French. PMID: 26259285.
https://pubmed.ncbi.nlm.nih.gov/26259285/

Khan Z, Combadière C, Authier FJ, et al.
Slow CCL2-dependent translocation of biopersistent particles from muscle to brain.
BMC Med. 2013;11:99. Published 2013 Apr 4. doi:10.1186/1741-7015-11-99
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3616851/

A first study [335] in patients with macrophage myofasciitis (MMF) detected NA within macrophages at the site of intramuscular injections of the vaccine.
Samples were obtained from the biopsy of muscle tissue from 3 months to 8 years after vaccine injection (mean: 36 months). The presence of aluminum within macrophages was confirmed by 3 different methods. Aluminum was present only in macrophages and not in muscle cells.



https://pubmed.ncbi.nlm.nih.gov/11522584/
Electron microscope (top) and nuclear microprobe (or proton-induced X-ray emission (PIXE); bottom) images of thin sections of muscle taken from patients with macrophage myofasciitis (MMF). This study showed that NA remains within macrophages (MFS) in muscle tissue for 8 years after intramuscular injection.

Inflammation caused by the movement of macrophages:

A 2009 study by **D'Mello et al.** [336] showed that liver-specific inflammation (liver damage due to bile duct blockage) caused peripheral macrophages to enter the CNS.
Specifically, in the presence of hepatic inflammation, the mice had elevated levels of MCP-1 (monocyte chemoattractant protein or CCL2) and increased numbers of circulating monocytes expressing CCR2 (CCR2: receptor for CCL2; monocytes: are the precursors of macrophages).

Recall that MCP-1 is also a predictive marker of the fatal outcome of sepsis, and its increase is associated with the degree of systemic inflammation. [337]

[335] Gherardi RK, Coquet M, Cherin P, Belec L, Moretto P, Dreyfus PA, Pellissier JF, Chariot P, Authier FJ.
Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle.
Brain. 2001 Sep;124(Pt 9):1821-31. doi: 10.1093/brain/124.9.1821. PMID: 11522584.
https://pubmed.ncbi.nlm.nih.gov/11522584/

[336] D'Mello C, Le T, Swain MG.
Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation.
J Neurosci. 2009;29(7):2089-2102. doi:10.1523/JNEUROSCI.3567-08.2009
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6666330/.

[337] Zhu T, Liao X, Feng T, Wu Q, Zhang J, Cao X, Li H.
Plasma Monocyte Chemoattractant Protein 1 as a Predictive Marker for Sepsis Prognosis: A Prospective Cohort Study.
Tohoku J Exp Med. 2017 Feb;241(2):139-147. doi: 10.1620/tjem.241.139. PMID: 28202856.
https://www.jstage.jst.go.jp/article/tjem/241/2/241_139/_pdf/-char/en

In this inflammation model, microglia (the macrophage component residing in the CNS) were activated and produced MCP-1/CCL2 prior to monocyte infiltration, and it was seen that this stimulation was mediated by the peripheral TNF-alpha (mediator of inflammation) signal pathway.

In other words, microglia in the CNS can detect hepatic inflammation and activate by peripherally produced TNF-alpha; activated microglia release MCP-1, which attracts macrophages into the brain from the periphery.
This is one of the ways in which NAs are transported by macrophages into the CNS.
The results of that study identify the existence of a new immune system-SNC communication pathway that occurs during inflammation localized in peripheral organs and may have specific implications in the development of alterations in brain neurotransmission that are encountered in various inflammatory diseases that occur outside the CNS; thus, inflammation anywhere in the body can cause macrophages carrying NA to enter the brain.

Adjuvant aluminum nanoparticles photographed in mouse brain tissue:

Important work by **Khan et al.** [338] documented that NA and other nanoparticles (e.g., Latex) injected intramuscularly penetrated into the brain and kidneys and could be detected up to a year after injection.
These results contradict an assumption regarding the absence of toxicity of IM-inoculated adjuvant aluminum, namely that the aluminum remains at the injection site without causing harm.

Below is the photo showing the NAs in the brain and kidneys:

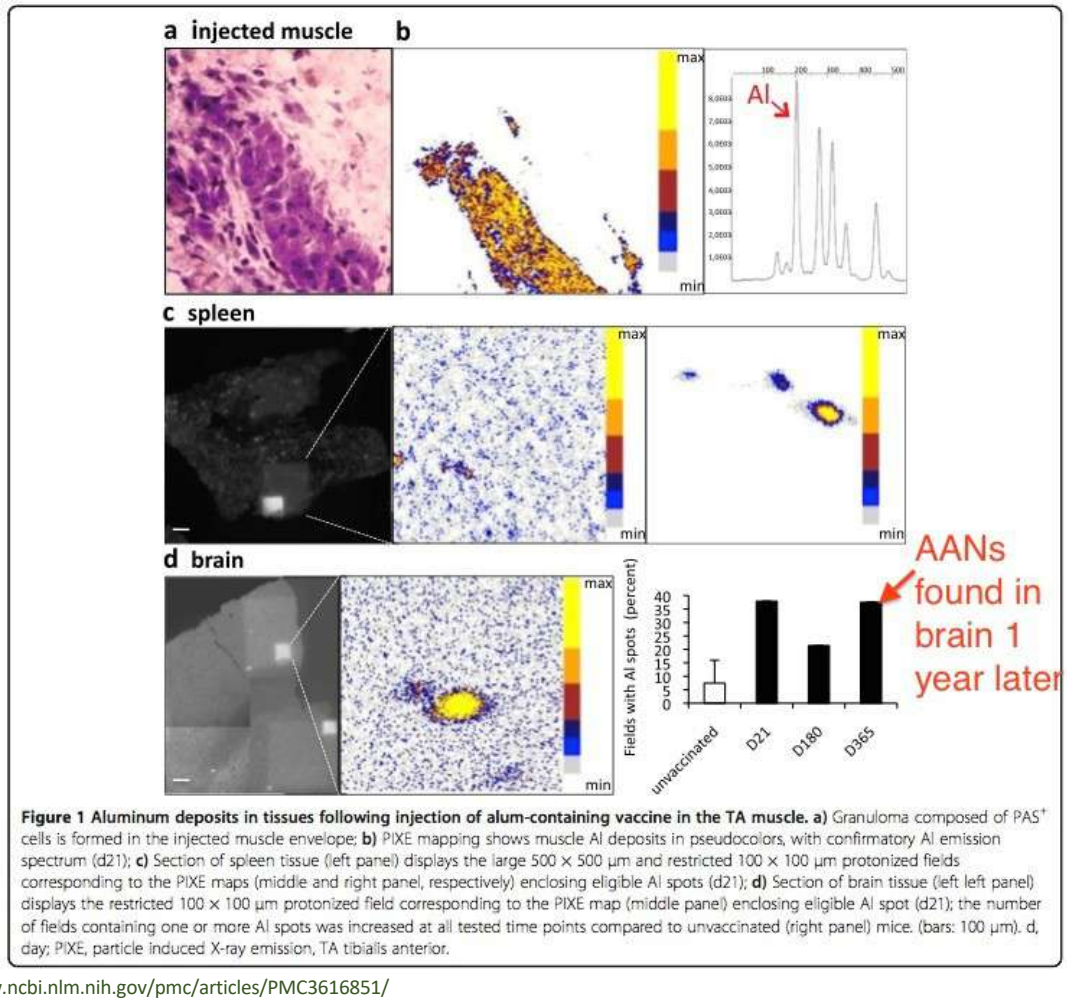


Figure 1 Aluminum deposits in tissues following injection of alum-containing vaccine in the TA muscle. a) Granuloma composed of PAS$^+$ cells is formed in the injected muscle envelope; **b)** PIXE mapping shows muscle Al deposits in pseudocolors, with confirmatory Al emission spectrum (d21); **c)** Section of spleen tissue (left panel) displays the large 500 × 500 µm and restricted 100 × 100 µm protonized fields corresponding to the PIXE maps (middle and right panel, respectively) enclosing eligible Al spots (d21); **d)** Section of brain tissue (left left panel) displays the restricted 100 × 100 µm protonized field corresponding to the PIXE map (middle panel) enclosing eligible Al spot (d21); the number of fields containing one or more Al spots was increased at all tested time points compared to unvaccinated (right panel) mice. (bars: 100 µm). d, day; PIXE, particle induced X-ray emission, TA tibialis anterior.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3616851/

[338] Khan Z, Combadière C, Authier FJ, et al.
Slow CCL2-dependent translocation of biopersistent particles from muscle to brain.
BMC Med. 2013;11:99. Published 2013 Apr 4. doi:10.1186/1741-7015-11-99
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3616851/

Khan also noted that NA transport in the CNS and tissues is MPC-1- dependent, and this fact is further evidence that macrophages are responsible for nanoparticle transport.
The mechanism by which nanoparticles are delivered through macrophages to all districts of the body (a mechanism known as the "Trojan horse") is being studied for pharmaceutical application to bring drugs into the brain through the BBB.

Studies have shown that nanoparticles (e.g., containing serotonin, cancer drugs, or anti-HIV drugs) can be transported across the BBB using macrophages as carriers [339]; another important application of nanoparticles is in vivo diagnostics of brain inflammation.
[340]

It follows that the transport of nanoparticles carried by macrophages into the brain is a widely accepted and verified phenomenon.
Macrophages, however, do the same thing with NA from vaccines. Since aluminum is a potent neurotoxin and strongly stimulates inflammation in the brain, [341] this is a serious concern for the safety of vaccines.

Some recent studies on the administration of adjuvant Al in mice revealed an increased complexity of adjuvant Al transport. [342]

Such studies have shown that:
1) **transport depends on the injection site** (translocation is greater following subcutaneous injection than intramuscular injection). In addition, the dose range that causes transport into the brain may vary depending on the anatomical site of injection.

2) **transport depends inversely on dosage**. A dosage of 200 mcg/kg caused transport in the brain (and behavioral changes) while the dosage of 400 mcg/kg had no effect

[339] Reynolds JL, Mahato RI.
Nanomedicines for the Treatment of CNS Diseases.
J Neuroimmune Pharmacol. 2017 Mar;12(1):1-5. doi: 10.1007/s11481-017-9725-x. Epub 2017 Feb 1. PMID: 28150132.
https://pubmed.ncbi.nlm.nih.gov/28150132/.

Choi MR, Bardhan R, Stanton-Maxey KJ, et al.
Delivery of nanoparticles to brain metastases of breast cancer using a cellular Trojan horse.
Cancer Nanotechnol. 2012;3(1-6):47-54. doi:10.1007/s12645-012-0029-9
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3505533/

[340] Kirschbaum K, Sonner JK, Zeller MW, et al.
In vivo nanoparticle imaging of innate immune cells can serve as a marker of disease severity in a model of multiple sclerosis.
Proc Natl Acad Sci U S A. 2016;113(46):13227-13232. doi:10.1073/pnas.1609397113
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5135308/

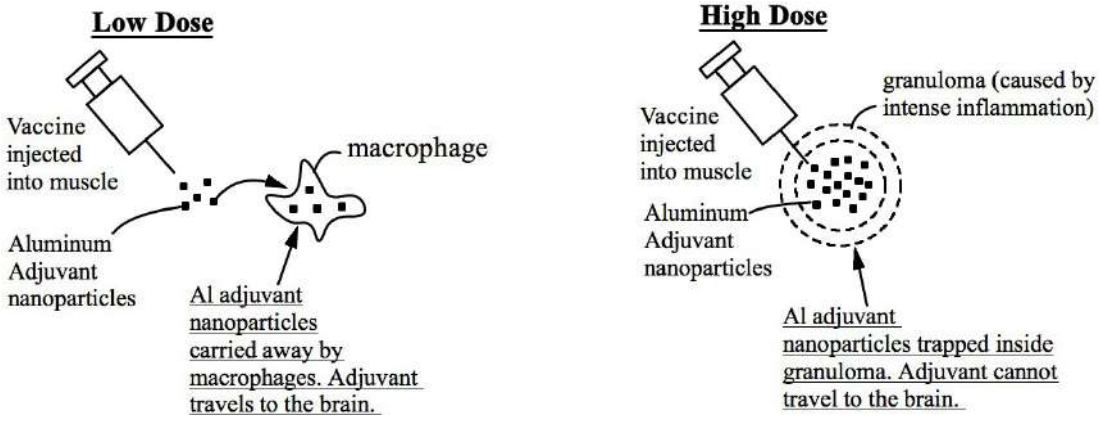
[341] Alexandrov PN, Kruck TP, Lukiw WJ.
Nanomolar aluminum induces expression of the inflammatory systemic biomarker C-reactive protein (CRP) in human brain microvessel endothelial cells (hBMECs).
J Inorg Biochem. 2015 Nov;152:210-3. doi: 10.1016/j.jinorgbio.2015.07.013. Epub 2015 Aug 1. PMID: 26265215.
https://pubmed.ncbi.nlm.nih.gov/26265215/.

Jovanova-Nesic K, Shoenfeld Y, Spector NH.
Aluminum excytotoxicity and neuroautotoimmunity: the role of the brain expression of CD32+ (FcγRIIa), ICAM-1+ and CD3ξ in aging.
Curr Aging Sci. 2012 Dec;5(3):209-17. doi: 10.2174/1874609811205030007. PMID: 23387884.
https://pubmed.ncbi.nlm.nih.gov/23387884/

[342] Crépeaux G, Eidi H, David MO, Tzavara E, Giros B, Exley C, Curmi PA, Shaw CA, Gherardi RK, Cadusseau J.
Highly delayed systemic translocation of aluminum-based adjuvant in CD1 mice following intramuscular injections.
J Inorg Biochem. 2015 Nov;152:199-205. doi: 10.1016/j.jinorgbio.2015.07.004. Epub 2015 Jul 22. PMID: 26384437.
https://pubmed.ncbi.nlm.nih.gov/26384437/

Eidi H, David MO, Crépeaux G, et al.
Fluorescent nanodiamonds as a relevant tag for the assessment of alum adjuvant particle biodisposition.
BMC Med. 2015;13:144. Published 2015 Jun 17. doi:10.1186/s12916-015-0388-2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4482291/

analogues. This may be because macrophage mobility is impaired at high doses.
Increased local inflammation at the injection site may cause reduced mobility of macrophages.[343]



http://vaccinepapers.org/al-adjuvant-causes-brain-inflammation-behavioral-disorders/

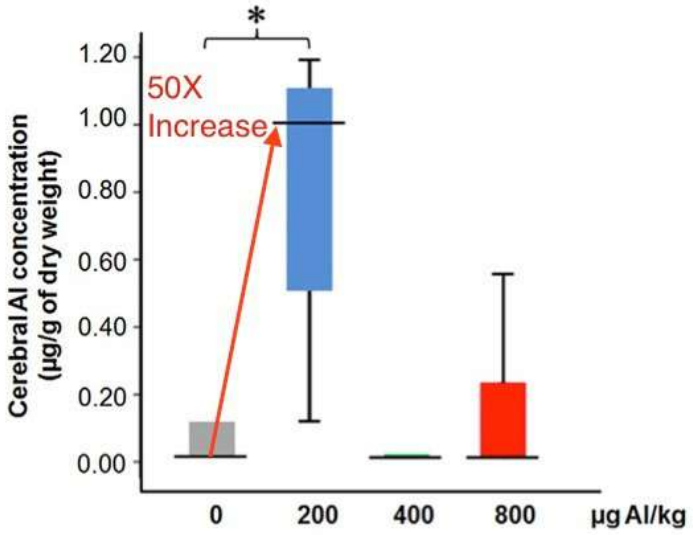


Fig. 3. Aluminium level determination in brain (µg/g of dry weight). Increased cerebral concentrations of aluminium were selectively observed with 200 µg/kg low Alhydrogel® dose. 5 mice/group; results expressed as median and range values, with quartiles boxes; non parametric Kruskal-Wallis test followed by Mann-Whitney test. * $p < 0.05$.

https://pubmed.ncbi.nlm.nih.gov/27908630/

3) **The extreme biological persistence of the adjuvant Al particles**. The particles have been observed in peripheral tissues and organs for up to 270 days after administration, including the brain, spleen, and lymph nodes. [344] It follows from what has been discussed that adjuvant nanoparticles are biologically active, which is why they are used in vaccines. [345]

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[343] Crépeaux G, Eidi H, David MO, Baba-Amer Y, Tzavara E, Giros B, Authier FJ, Exley C, Shaw CA, Cadusseau J, Gherardi RK.
Non-linear dose-response of aluminum hydroxide adjuvant particles: Selective low dose neurotoxicity.
Toxicology. 2017 Jan 15;375:48-57. doi: 10.1016/j.tox.2016.11.018. Epub 2016 Nov 28.
https://pubmed.ncbi.nlm.nih.gov/27908630/

[344] Vaccines and Autoimmunity June 2015, Wiley-Blackwell
Yehuda Shoenfeld , Nancy Agmon-Levin , Lucija Tomljenovic
Pg 261-270

[345] Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, Lee LK, Swartz MA, Hubbell JA.
Exploiting lymphatic transport and complement activation in nanoparticle vaccines.
Nat Biotechnol. 2007 Oct;25(10):1159-64. doi: 10.1038/nbt1332. Epub 2007 Sep 16. PMID: 17873867.
https://www.nature.com/articles/nbt1332

Swartz MA, Hubbell JA, Reddy ST.
Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design.
Semin Immunol. 2008 Apr;20(2):147-56. doi: 10.1016/j.smim.2007.11.007. Epub 2008 Jan 16. PMID: 18201895.

NAs cause inflammation and activation of the immune system (Th2 polarization of the immune system, production of inflammatory cytokines, activation of complement), and as already seen they travel to all parts of the body (including the brain, which is protected by the blood-brain barrier from entry of Al ions $^{3+}$).

The following figure summarizes the various levels at which aluminum toxicity acts.

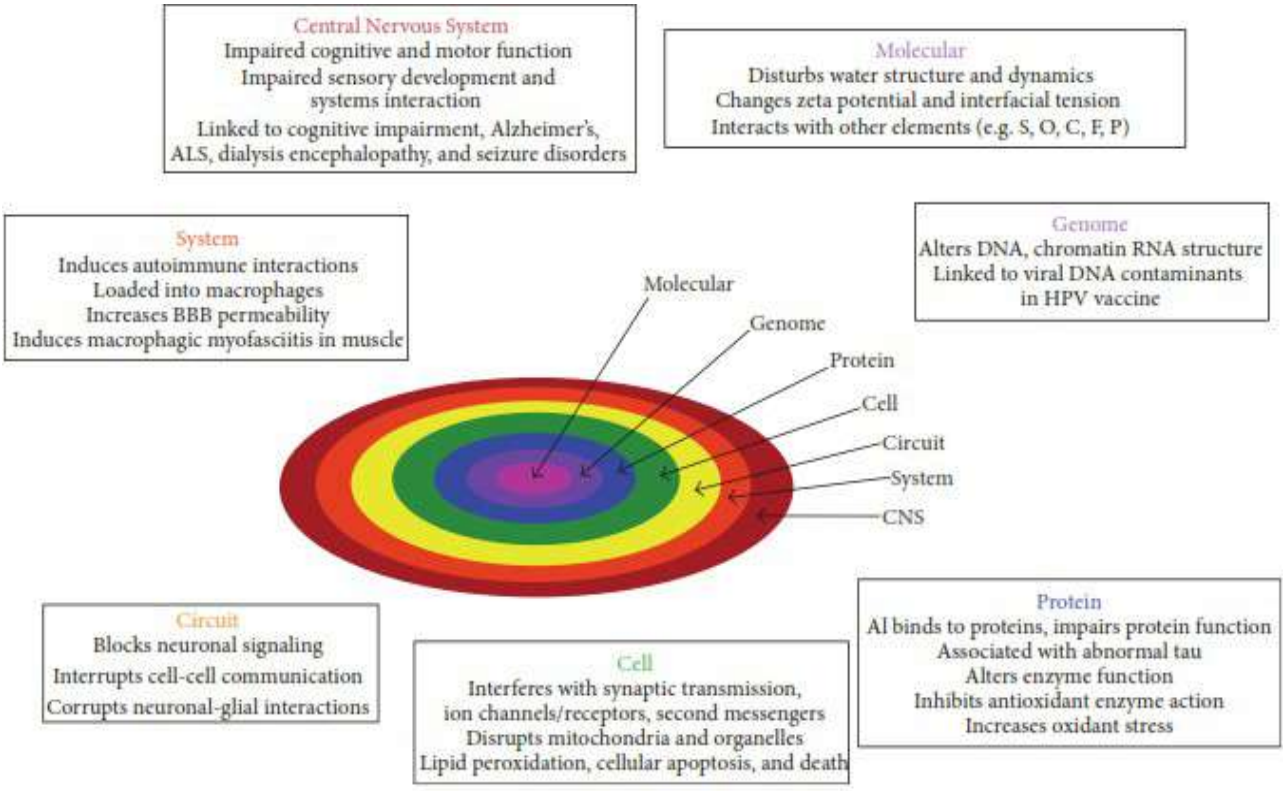


FIGURE 4: Schematic of the biosemiotic levels at which Al can impact the body and CNS.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4202242/

The following is the EMA's response on the toxicity of aluminum adjuvant to Mr. Ivan Catalano, former parliamentarian and vice chair of the last Depleted Uranium Commission:

EMA/133566/201816 March 2018
1.7. Aluminum as an adjuvant
For your information, keep in mind that **the use of aluminum hydroxide and aluminum phosphate in vaccines as adjuvants (used to enhance immune response) has been well established for many years.** These substances are defined in the European Pharmacopoeia. [9] Specifically, for human vaccines, **the pharmacopeia specifies a maximum of 1.25 mg of aluminum per single human dose when aluminum is used in this way**, unless otherwise justified and approved for a specific product. Similar standards apply in other parts of the world.
There are currently 17 vaccines for human use that have been approved through the EMA (through the so-called centralized procedure), as well as many other nationally (member state) licensed vaccines that use aluminum hydroxide, aluminum phosphate, or amorphous aluminum hydroxyphosphate sulfate as an adjuvant.
The benefits of an adjuvant in a vaccine must be weighed against the risk of any inherent adverse reaction to it. **Current attitudes toward the risk-benefit of vaccination favor safety over efficacy when a vaccine is administered to a healthy population. A final safety evaluation of the new vaccine formulation can only be conducted on the basis of clinical trials.**[18]
More information on adjuvants can be found at the following Web page:
http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000904.jsp&mid=WC0b01ac058002956b

[9] Vaccines for human use monograph (Ph.Eur. 07/2017 0153)

https://pubmed.ncbi.nlm.nih.gov/18201895/

Aluminium (2.5.13): maximum 1.25 mg of aluminium (Al) per single human dose where an aluminium adsorbent has been used in the vaccine, unless otherwise stated.
Calcium (2.5.14): maximum 1.3 mg of calcium (Ca) per single human dose where a calcium adsorbent has been used in the vaccine, unless otherwise stated.
Free formaldehyde (2.4.18): maximum 0.2 g/L of free formaldehyde in the final product where formaldehyde has been used in the preparation of the vaccine, unless otherwise stated.
Phenol (2.5.15): maximum 2.5 g/L in the final product where phenol has been used in the preparation of the vaccine, unless otherwise stated.
[18] Adjuvants in vaccines for human use
http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003809.pdf

Also added is the question to the European Parliament E-001327/2018 March 2, 2018 by Prof. Romain Gherardi and the subsequent answer:

Subject: Scientific data on aluminum in vaccine adjuvants
In its February 11, 2016 response, the Commission said that "available scientific evidence supports the safe and effective use of aluminum adjuvants in vaccines." A report published in September 2017 by the French National Agency for the Safety of Medicines and Health Products reignited the debate in the scientific community. The French National Institute for Health and Medical Research (INSERM) found that aluminum in vaccine adjuvants injected into mice remains in their bodies for several months and eventually reaches the brain. At the same time, a representative of the French National Academy of Pharmacy argued that animal testing is a poor predictor of risk to humans.
According to Professor Romain Gherardi, a researcher at INSERM, it is likely that only some people with genetic variations experience aluminum-related side effects, such as chronic fatigue. More than 600 cases of macrophage myofasciitis (MFM), a complex disease that causes joint pain, extreme fatigue and cognitive impairment, have been diagnosed in France. An association for people living with MFM said the disease is caused by aluminum adjuvants.
1. What does the Commission make of these results?
2. Will you call for further research on the risks presented by aluminum in adjuvants?

E-001327/2018 (2018/06/05)
Answer given by Mr. Moedas on behalf of the Commission.
1. Vaccines may contain small amounts of aluminum that serve as adjuvants. They are nationally or centrally authorized. The European Medicines Agency (EMA)[1] is in contact with France's National Agency for the Safety of Medicines and Health Products (ANMS), which funded the research referred to by the MEP. In September 2017, ANMS reported that the results of the new studies, as well as the available publication, do not change the positive benefit/risk ratio of the vaccines[2] . The EMA, in collaboration with EU member states, continuously monitors the safety of aluminum-containing vaccines, including studies reported in the global scientific literature, and will take all necessary measures if a safety issue is identified.
2. Through the Innovative Medicines Initiative (IMI)[3] , the Commission funds research on vaccine safety. The IMI ADVANCE[4] and BioVacSafe projects[5] work to provide reliable benefit/risk data on marketed vaccines and to improve vaccine safety testing and monitoring, respectively.

(1) http://www.ema.europa.eu/ema/
(2) http://ansm.sante.fr/S-informer/Communiques-Communiques-Points-presse/Les-vaccins-contenant-de-l-aluminium-sont-surs-Communique
(3) http://www.imi.europa.eu/
(4) ADVANCE, http://www.advance-vaccines.eu/
(5) BioVacSafe, http://www.biovacsafe.eu/

The statement that "*the use of aluminum hydroxide and aluminum phosphate in vaccines as adjuvants (used to enhance immune response) has been well-established for many years,*" being unsupported by data from injection toxicology studies (the agency provided only oral and continuous-use studies) is not sufficient assurance of safety.

BioVacSafe's large investment of funds for vaccine risk assessment (€30.2 million) has not resulted in any publication of aluminum-specific safety studies in clinical settings. (http://www.biovacsafe.eu/scientific-publications).

It follows that the lack of data that should provide unequivocal assurance, and not on the basis of established use, of the safety of adjuvanted aluminum administered by injection is in blatant contrast to the concluding statement, *"The current attitude toward the risk-benefit ratio of vaccination favors safety over efficacy when a vaccine is administered to a healthy population,"* as well as the following: "*A final safety evaluation of the new vaccine formulation can only be conducted on the basis of clinical studies,"* since no clinical studies aimed at evaluating medium- to long-term safety have ever been conducted with control groups that have never been vaccinated.

Finally, we point out in confirmation of the data presented, additional very recent bibliographic references to which we refer for further study. [346]

THE ENVIRONMENTAL SIGNIFICANCE OF NATURAL NANOPARTICLES [347]

Natural water-borne nanoparticles are ubiquitous and critical in buffering environmental systems, as they play the dual role of limiting concentrations of potentially toxic metals, while at the same time providing metals at levels that allow biochemical reactions to take place.

Environmental colloids and nanoparticles can be found in a multitude of compositions and conformations.

The environmental significance of colloids and nanoparticles lies in their interactions **with trace metals** (**TM**), of which they regulate the circulation and bioavailability in natural waters.

Colloids are mixtures whose particles are intermediate in size between those of a solution and those of a suspension, that is, **between 1nm and 100 nm** (they are therefore visible only under an ultramicroscope). Two phases can be distinguished in colloidal systems: the particles constitute the dispersed phase and are uniformly distributed in the water, which is the dispersing phase.
Colloids are responsible for the turbidity or color of surface water [348]

[346] Asín J, Pascual-Alonso M, Pinczowski P, et al.
Cognition and behavior in sheep repetitively inoculated with aluminum adjuvant-containing vaccines or aluminum adjuvant only.
J Inorg Biochem. 2020;203:110934. doi:10.1016/j.jinorgbio.2019.110934
https://pubmed.ncbi.nlm.nih.gov/31783216/

Exley C.
An aluminium adjuvant in a vaccine is an acute exposure to aluminium.
J Trace Elem Med Biol. 2020;57:57-59. doi:10.1016/j.jtemb.2019.09.010
https://pubmed.ncbi.nlm.nih.gov/31561170/

Shardlow E, Mold M, Exley C.
The interaction of aluminum-based adjuvants with THP-1 macrophages in vitro: Implications for cellular survival and systemic translocation.
J Inorg Biochem. 2020;203:110915. doi:10.1016/j.jinorgbio.2019.110915
https://pubmed.ncbi.nlm.nih.gov/31751817/

de Miguel R, Asín J, Rodríguez-Largo A, et al.
Detection of aluminum in lumbar spinal cord of sheep subcutaneously inoculated with aluminum-hydroxide containing products.
J Inorg Biochem. 2020;204:110871. doi:10.1016/j.jinorgbio.2019.110871
https://pubmed.ncbi.nlm.nih.gov/31901536/

[347] Hartland, A., Lead, J. R., Slaveykova, V. I., O'Carroll, D. & Valsami-Jones, E.
The Environmental Significance of Natural Nanoparticles. Nature Education Knowledge (2013) 4(8):7
https://www.nature.com/scitable/knowledge/library/the-environmental-significance-of-natural-nanoparticles-105737311/

[348] https://www.lenntech.it/colloidi.htm

http://www.galenotech.org/colloidi.htm

The **surface area/volume ratio** provides colloids with very good free ion adsorption properties.
This ion adsorption phenomenon involves the presence of electronic charge on the surface, which determines some repulsion forces and stability in solution.

There are **different origins**: dissolution of minerals, erosion, decomposition of organic matter, crop wastes and wastewater.

Hydrophobic colloids are responsible for water coloration and have at their base an organic origin with R-NH2 or R-OH functional groups. These electronegative parts generate hydrogen bonds with water molecules. This layer opposes the aggregation of colloids and is a stabilizing factor.

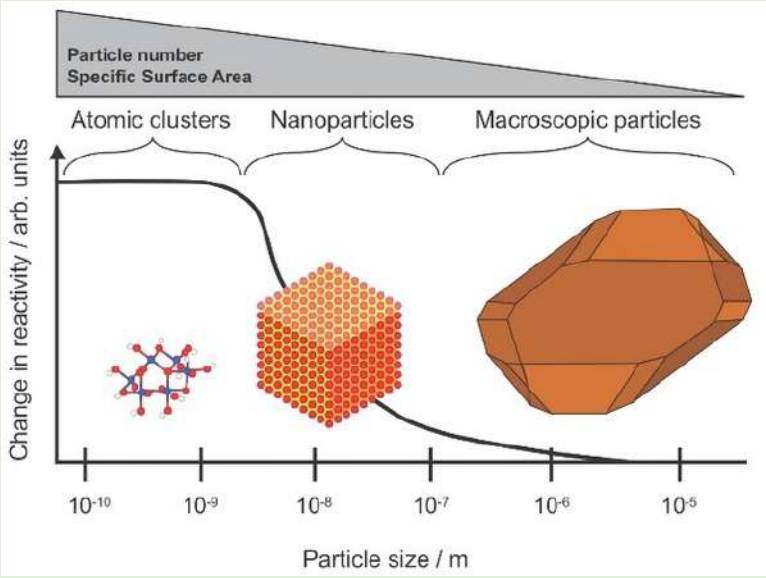
Hydrophilic colloids have mineral origins. There are concentrated negative charges on their surface that make agglomeration impossible. Colloids are never 100 percent hydrophilic nor 100 percent hydrophobic; the actual percentages depend on their molecular constitution.

Nanoparticle

A particle of any shape with an equivalent diameter of approximately 1 to 100 nm

Colloid

Molecules or particles dispersed in a medium with at least in one direction a dimension roughly between 1 nm and 1 µm



https://www.nature.com/scitable/knowledge/library/the-environmental-significance-of-natural-nanoparticles-105737311/

Much of the environmental pool of colloids consists of low-molecular-weight degradation products of biological decay (humic matter), various fibrillar and mesh-like organic compounds, and minerals generated during chemical erosion of rocks, mainly oxides and oxyhydroxides of iron (Fe), manganese (Mn) and aluminum (Al) and aluminosilicates.

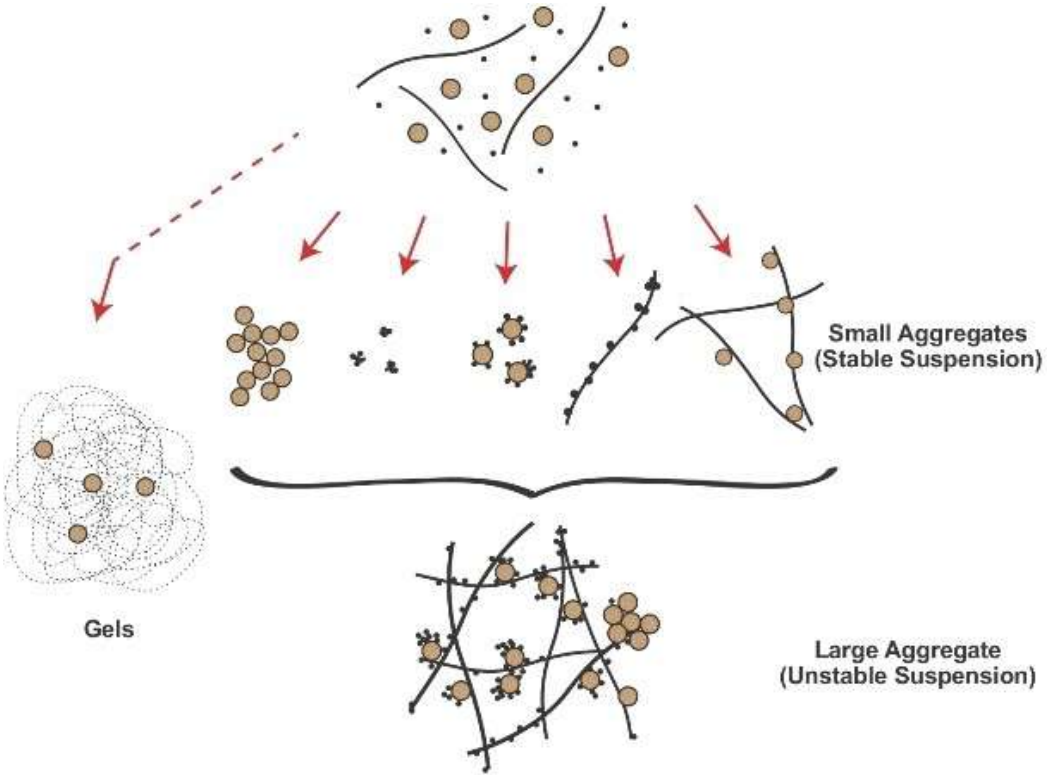
The most studied subset of environmental organic matter, **humic substances (HS)**, are an operationally defined and chemically extracted fraction of the total natural organic carbon pool.

https://www.istitutomedici.edu.it/servizi-online/materiali-scaricabili/materiale-didattico/dispense-on-line/materiale-prof-giovanni-nalin/chemistry/323-colloids-and-colloid-systems/files

Although not fully representative of **natural organic matter (NOM)** [349], in terms of binding to trace metals, extracted HS behave similarly, although not identically, to their natural counterparts and may represent the most functionally significant component of NOM [350].

HS exists as a dispersed material at the lower end of the nanometer range (<5 nm), but it can aggregate to form larger structures (often as composites with mineral colloids (e.g., Fe and Mn oxides and aluminosilicates), reaching sizes potentially outside the nano-range [351].

The most abundant organic material found in natural waters are peptides, proteins, peptidoglycans, polysaccharides and similar biomolecules.
Polysaccharides have fibrillar or mesh-like configurations, while proteins are often globular and differ from HS in that they are "fresher," that is, less degraded.
These types of compounds have the effect of generally increasing the size of nanoparticles and colloids through aggregation, while HS often, but not always, reduces aggregation through charge and/or steric stabilization [352].



https://www.nature.com/scitable/knowledge/library/the-environmental-significance-of-natural-nanoparticles-105737311/

[349] Filella, Montserrat.
NOM site binding heterogeneity in natural waters: Discrete approaches.
Journal of Molecular Liquids - J MOL LIQ. (2008). 143. 42-51. 10.1016/j.molliq.2008.04.018.
https://www.researchgate.net/publication/238117397_NOM_site_binding_heterogeneity_in_natural_waters_Discrete_approaches

[350] E. Tipping, D.C. Higgins,
The effect of adsorbed humic substances on the colloid stability of haematite particles,
Colloids and Surfaces, Volume 5, Issue 2, 1982, Pages 85-92, ISSN 0166-6622, https://doi.org/10.1016/0166-6622(82)80064-4.
http://www.sciencedirect.com/science/article/pii/0166662282800644

[351] Björn, Stolpe & Hassellöv, Martin.
Changes in size distribution of fresh water nanoscale colloidal matter and associated elements on mixing with seawater.
Geochemistry et Cosmochemistry Acta. (2007). 71. 3292-3301. 10.1016/j.gca.2007.04.025.
https://www.researchgate.net/publication/222198993_Changes_in_size_distribution_of_fresh_water_nanoscale_colloidal_matter_and_associated_elements_on_mixing_with_seawater

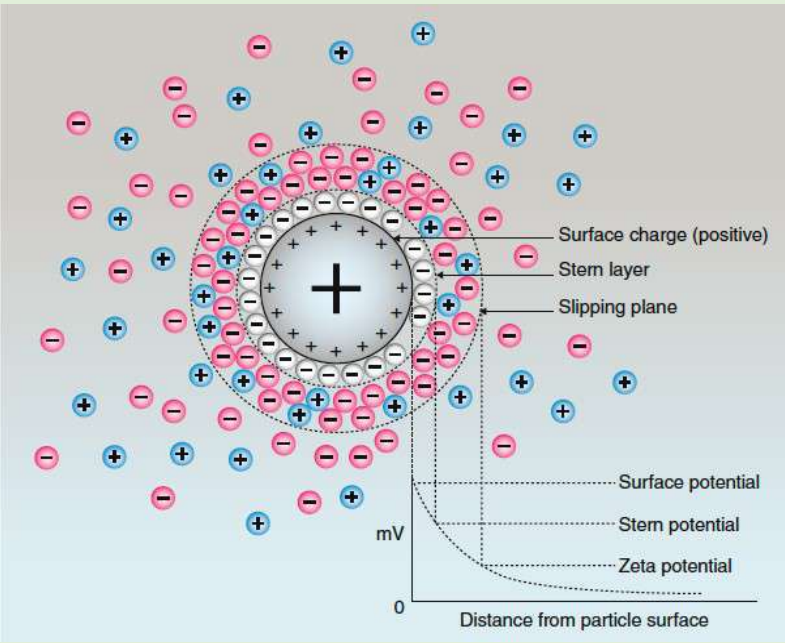
[352] Buffle J, Leppard GG. Characterization of aquatic colloids and macromolecules. Structure and behavior of colloidal material.
Environ Sci Technol. 1995 Sep 1;29(9):2169-75. doi: 10.1021/es00009a004. PMID: 22280252.
https://pubs.acs.org/doi/10.1021/es00009a004

Main types of aggregates formed in the three-component colloidal system: fulvic compounds (FCs) (or aromatic refractory organic matter) small dots; inorganic colloids (ICs)-circles; rigid biopolymers -lines. Both FCs and polysaccharides can also form gels, which are depicted here as gray areas in which IC can be incorporated.

In all environmental systems, metal oxides, especially iron and manganese, are also important nanoscale phases because of their ability to bind trace elements.
At the approximately neutral pH of natural waters (~ pH 7), nanoparticles and colloids typically possess a negative electrostatic surface charge due to surface coatings of humic substances [353].
Charge differences between particle surfaces confer stability through charge repulsion, which means that nanoparticles and colloids tend to remain dispersed in water, forming a suspension.
The surface charge of all particles can be equalized by counter-ions, which form a double layer around the particle.

Charged particles in an aqueous suspension attract ions of opposite charges (counter ions), as illustrated by the Gouy-Chapman bilayer model.
This model describes the existence of an inner layer, the Stern layer, and a more diffuse outer layer surrounding the particles. The electric potential decreases with distance from the surface of the particles, and that at the outer edge of the diffuse layer is known as the **zeta potential**.

This should also be kept in mind for colloidal compounds used in vaccines, such as the aluminum adjuvant. Electrostatic attraction between the aluminum adjuvant and a protein antigen is possible when the adjuvant and antigen have opposite electrical charges. Aluminum hydroxide has an alkaline point of zero charge (PZC). At pH values below the PZC, the aluminum hydroxide is positively charged and at pH values above the PZC, the aluminum hydroxide is negatively charged. This is reflected in the zeta potential of the particle. A similar situation exists for adjuvant aluminum phosphate, only in this case the PZC is acidic. The actual pH value for PZC for both aluminum hydroxide and adjuvant aluminum phosphate depends on the type of production.



Subunit Vaccine - Camilla Foged p. 39

[353] A. W. P. Vermeer, W. H. van Riemsdijk, and L. K. Koopal
Adsorption of Humic Acid to Mineral Particles. Specific and Electrostatic Interactions
Langmuir 1998, 14, 10, 2810-2819 https://doi.org/10.1021/la970624r
https://pubs.acs.org/doi/10.1021/la970624r

E. Tipping, D. C. Higgins,
The effect of adsorbed humic substances on the colloid stability of haematite particles,
Colloids and Surfaces, Volume 5, Issue 2, 1982, Pages 85-92, ISSN 0166-6622, https://doi.org/10.1016/0166-6622(82)80064-4.
http://www.sciencedirect.com/science/article/pii/0166662282800644

The importance of nanoparticles (compared to colloids in general) lies in their ability to bind large amounts of trace metals, which affects the bioavailability of vital and toxic metals in natural waters.

The principles governing these bonding reactions are believed to be similar, regardless of the size and chemistry of the colloids, and typically occur through the inner sphere, e.g., with covalent bonding, and complexation reactions with surface functional groups.

Complexation within the sphere between metal ions and common metal oxides, which also occurs for humic substances and other colloids, has been shown to be the predominant mechanism of adsorption.
By definition, metals in inner sphere complexes are more bound and therefore are less labile than metals in outer sphere complexes (ion pairs), with implications for bioavailability and ecotoxicity of metals.

Biological availability is defined as "*the degree to which a substance is absorbed by a living organism.*" [354].
Because of the interaction of trace metals with other constituents in the aquatic system (such as nanoparticles), only a fraction of the total mass of trace metals present interacts with organisms, and thus the remainder is not available either as a nutrient or as a toxin unless it is absorbed directly before dissociation. [355]

Nanoparticles and colloids regulate the bioavailability of trace metals by influencing their speciation and other processes at the organism-environment interface.

Determining **speciation** involves distributing a metal, present in a given sample, among different forms or species.
This is an extremely important process for being able to assess the bioavailability and toxicity of an element.

It is useful to distinguish between the physical one, that is, its distribution among soluble, colloidal or particulate forms, and the chemical one.
The latter refers to the distribution among various distinct chemical species in solution, including both the distinction between complexed and free metal, but also between different oxidation states.

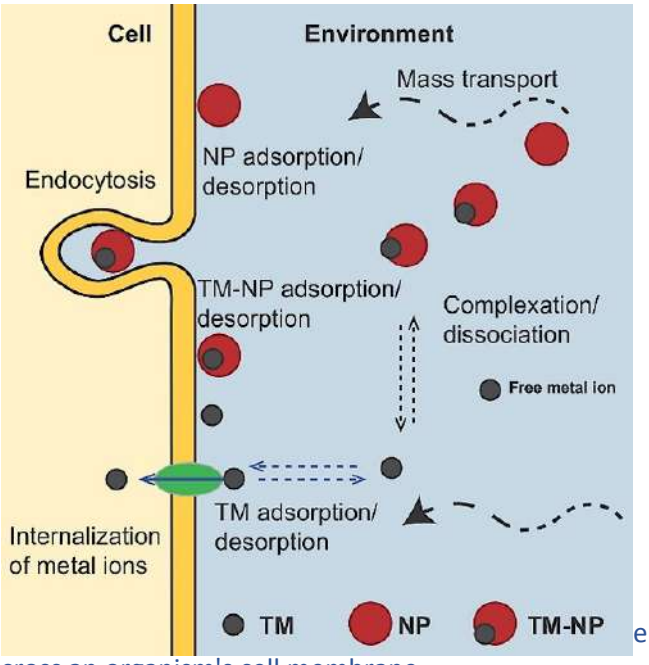
The sum of the concentrations of the various forms gives the total concentration. This work is done because it is known that different chemical and physical forms of an element can manifest different environmental impact and different toxicity.

In general, labile species are defined as those present at equilibrium and inert species as those in a non-equilibrium state.
It has been shown that for most metals, the most toxic forms are free metals or water-ions; strong complexes and colloidal associations, on the other hand, are the least toxic.
Toxicity is related to the ability to cross biological membranes. It should be pointed out that it is impossible to determine all chemical forms of the element in the sample.
What is normally done is to determine some of the fractions present; in particular, we try to evaluate the labile metal fraction, which is the most interesting one for bioavailability studies. [356]

[354] Nordberg, Monica & Duffus, John & Templeton, Douglas.
Explanatory dictionary of key terms in toxicology: Part II (IUPAC Recommendations).
Pure Appl. Chem. (2010).82. 679-751. 10.1351/pac200779091583.
https://www.researchgate.net/publication/228530064_Explanatory_dictionary_of_key_terms_in_toxicology_Part_II_IUPAC_Recommendations

[355] Zhao, Chun-Mei & Campbell, Peter & Wilkinson, Kevin.
When are metal complexes bioavailable?
Environmental Chemistry. (2016).13. 425-433. 10.1071/EN15205.
https://www.publish.csiro.au/en/pdf/EN15205

[356] https://www.tesionline.it/tesi/brano/speciazione-dei-metalli/13980



https://www.nature.com/scitable/knowledge/library/the-environmental-significance-of-natural-nanoparticles-105737311/
Processes at the organism-environment interface that determine the bioavailability of TM. In this case, TM represents trace free metal and NP a nanoparticle, TM-NP is the TM bound to the nanoparticle. Different chemical forms are transported from the soil into the vicinity of organisms; during transport, TM complexes (e.g., with nanoparticles) can dissociate/associate; chemical species can adsorb to the protective layer of organisms (e.g., mucus or cell walls) and cell membranes, then cross cell membranes by different mechanisms. Depending on the nature of the MT, the organism and its environment, any of the above processes could limit the rate. If MT transport across the cell membrane limits the overall metal transport process in the cell, bio-responses to both essential and toxic elements can be related to any metal species in an equilibrium state, including metal bound to receptor sites on organisms-the biotic ligand model; or free metal ions in solution-the free ion activity model [1] . Under nutrient-limited conditions or in biofilms where mass transport is the limiting factor[1] , TM complexes should contribute to bioavailability depending on their mobility and lability.

e metals by decreasing their free fraction available to cross an organism's cell membrane.
In fact, colloids of different compositions have been shown to limit the bioavailability (and harmful effects) of toxic metals (e.g., Ag, Cd, Cu, Ni, and Pb) for various organisms, including bacteria, fungi, phytoplankton, and daphnia, in a manner directly proportional to the concentrations of free metal ions [357].

Given the large proportion of trace metals bound to nanoparticles and colloids in surface waters, they are expected to mitigate the harmful effects of toxic trace elements by generally decreasing their bioavailability.
Furthermore, by regulating the bioavailability of micronutrients (Cu, Co, Fe, Mn, Mo, Ni, and Zn) used in enzymatic processes [358] , nanoparticles could affect phytoplankton biomass and biodiversity in the ocean and lakes [359].
Such an alteration could have profound consequences for the biogeochemical cycles of C, N, Fe and for food web interactions, as phytoplankton are responsible for more than 40 percent of primary productivity on Earth and form the basis of the aquatic food chain.

[357] Slaveykova, V.I. & Wilkinson, Kevin.
Predicting the Bioavailability of Metals and Metal Complexes: Critical Review of the Biotic Ligand Model. Environmental Chemistry. (2005) 2. 10.1071/EN04076.
https://www.researchgate.net/publication/37421640_Predicting_the_Bioavailability_of_Metals_and_Metal_Complexes_Critical_Review_of_the_Biotic_Ligand_Model

[358] Morel FM, Price NM.
The biogeochemical cycles of trace metals in the oceans.
Science. 2003 May 9;300(5621):944-7. doi: 10.1126/science.1083545. PMID: 12738853.
https://pubmed.ncbi.nlm.nih.gov/12738853/

[359] Sterner, R. W. et al.
Phosphorus and trace metal limitation of algae and bacteria in Lake Superior
Limnol. Oceanogr. 49, 495-507 (2004) https://doi.org/10.4319/lo.2004.49.2.0495
https://aslopubs.onlinelibrary.wiley.com/doi/pdf/10.4319/lo.2004.49.2.0495

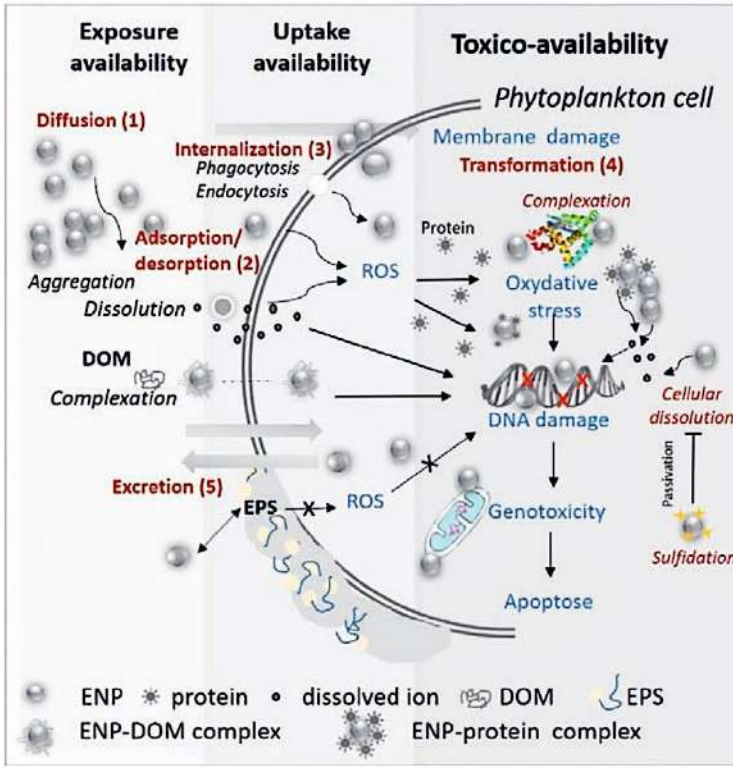
Boyd, P., Ellwood, M.
The biogeochemical cycle of iron in the ocean.
Nature Geosci 3, 675-682 (2010). https://doi.org/10.1038/ngeo964
https://www.nature.com/articles/ngeo964

Tosca NJ, Jiang CZ, Rasmussen B, Muhling J.
Products of the iron cycle on the early Earth.
Free Radic Biol Med. 2019 Aug 20;140:138-153. doi: 10.1016/j.freeradbiomed.2019.05.005. Epub 2019 May 6. PMID: 31071438.
https://www.sciencedirect.com/science/article/pii/S0891584918324936?via%3Dihub.

The influence of nanoparticles on the bioavailability of both nutrient and toxic elements has been a factor in the evolution and development of higher organisms, potentially acting with a buffering effect on environmental systems against change [360].

The reason that industrially produced nanoparticles (for pharmaceutical purposes or as waste from industrial or urban processes) are the subject of toxicological study, despite being present in much smaller quantities than their natural counterparts, is because man-made nanoparticles consist of specific structures and chemicals, distinct from those found in nature, for which organisms may not have appropriate defense mechanisms. [361]
In the environment, particularly the aquatic environment, engineered nanoparticles (ENPs) are present as complex mixtures with other pollutants, such as trace metals, with which they may act in synergism, additivity or antagonism of their combined effects.
Despite the fact that the toxicity and environmental risk of ENPs have received a great deal of attention in recent years, the interactions of ENPs with other pollutants and the resulting effects on aquatic organisms represent a major challenge in (nano)ecotoxicology.

Below are two figures representing the various mechanisms of damage induction at the cellular level.



When Environmental Chemistry Meets Ecotoxicology: Bioavailability of Inorganic Nanoparticles to Phytoplankton ENP: engineered nanoparticles; DOM: dissolved organic matter; EPS: extracellular polymeric substances
It is an important paradigm in ecotoxicology that a contaminant must be biologically available (bioavailable) to have a significant effect on living organisms such as phytoplankton. In the case of ENPs, bioavailability to phytoplankton is the result of several interconnected processes including (1) transport of ENPs from the exposure medium to the cell surface, e.g., by diffusion. This process is size-dependent and differs for single dissolved ENPs and aggregates formed in the medium of the environment; (2) reversible adsorption of different forms of ENPs on cell walls and membrane;
(3) internalization (or not) through different mechanisms, e.g., by endocytosis and phagocytosis and/or altering cell membranes, (4) distribution and cellular transformation, and (5) excretion. In addition, bioavailability can be considered to consist of three main components: "exposure availability," "actual or potential uptake availability," and "toxic availability." Phytoplankton species can influence the presence and fate of ENPs in the aquatic environment directly by producing metal nanoparticles from dissolved metal ions, by inducing the transformation of ENPs within cells or on the cell surface, and indirectly by secreting various small molecules and extracellular polymeric substances (EPSs).

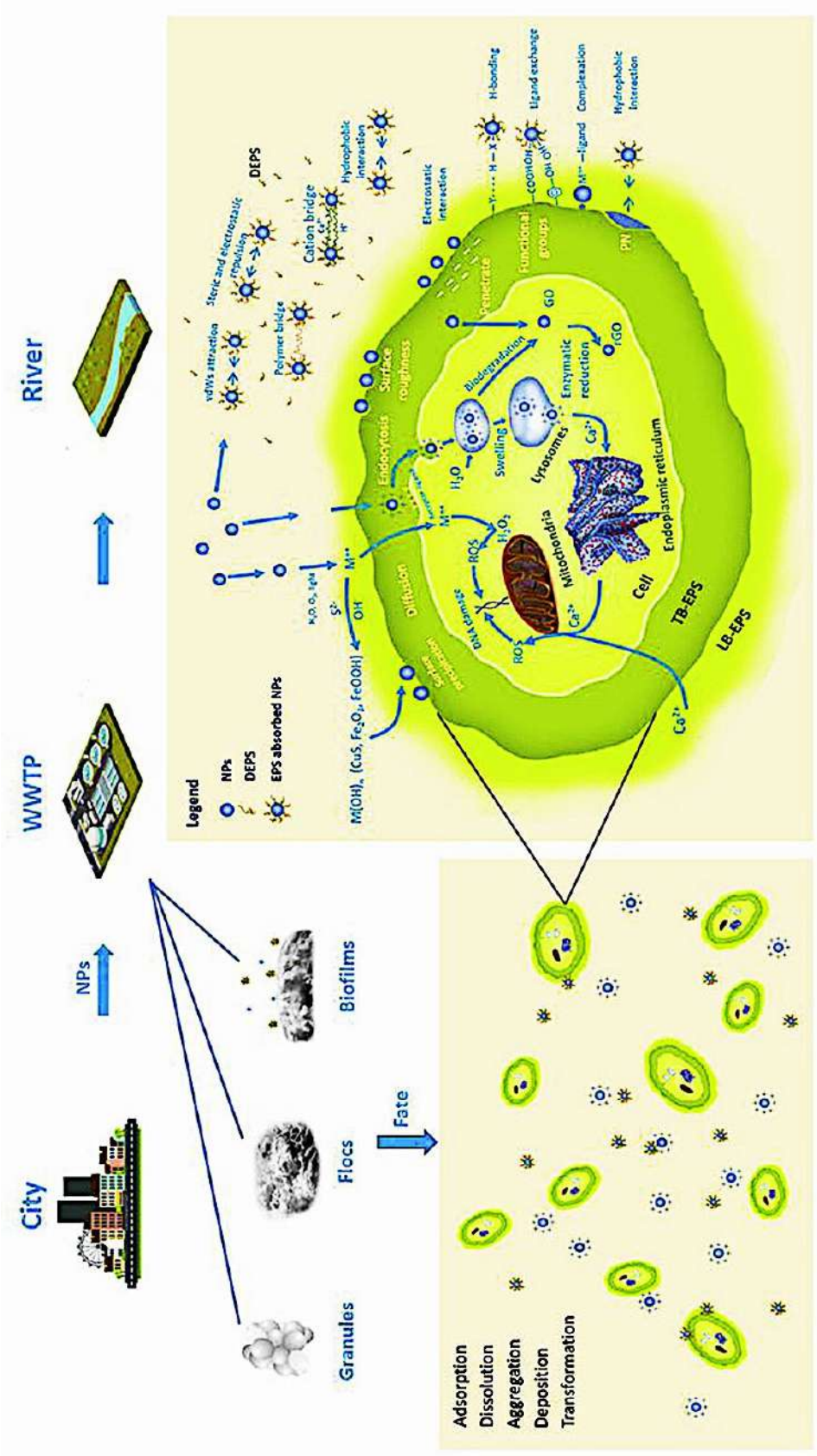
[360] Tipping, E. Cation binding by humic substances. Cambridge: University Press (2001).

[361] Xiaoliu Huangfu, Yanghui Xu, Caihong Liu, Qiang He, Jun Ma, Chengxue Ma, Ruixing Huang,
A review on the interactions between engineered nanoparticles with extracellular and intracellular polymeric substances from wastewater treatment aggregates,
Chemosphere, Volume 219, 2019, Pages 766-783, ISSN 0045-6535,
https://doi.org/10.1016/j.chemosphere.2018.12.044.
https://www.sciencedirect.com/science/article/abs/pii/S004565351832366X.

Li, M.; Liu, W.; Slaveykova, V.I.
Effects of Mixtures of Engineered Nanoparticles and Metallic Pollutants on Aquatic Organisms.
Environments 2020, 7, 27. https://doi.org/10.3390/environments7040027
https://www.mdpi.com/2076-3298/7/4/27

Slaveykova VI, Li M, Worms IA, Liu W.
When Environmental Chemistry Meets Ecotoxicology: Bioavailability of Inorganic Nanoparticles to Phytoplankton.
Chimia (Aarau). 2020 Mar 25;74(3):115-121. doi: 10.2533/chimia.2020.115. PMID: 32197668.
http://docserver.ingentaconnect.com/deliver/connect/scs/00094293/v74n3/s3.pdf?expires=1611089689&id=0000&titleid=10984&checksum=9FD24A62BB69CE1582A7B3D2CAD08065

https://www.sciencedirect.com/science/article/abs/pii/S004565351832366X
Engineered nanoparticles (ENPs) will inevitably enter wastewater treatment plants (WWTPs) because of their widespread application; therefore, it is necessary to study the migration and transformation of nanoparticles in wastewater treatment systems. Extracellular polymeric substances (EPS) such as polysaccharides, proteins, nucleic acids, humic acids and other polymers are polymers released by microorganisms under certain conditions. Intracellular polymeric substances (IPS) are microbial substances contained in the body with compositions similar to those of extracellular polymers. This figure summarizes the characteristics of EPS and IPS from microbial wastewater collection aggregates containing pure bacteria, activated sludge, granular sludge, and biofilms. Also shown are the dissolution, adsorption, aggregation, deposition, oxidation and other chemical transformation processes of nanoparticles, such as metals, metal oxides and nonmetal oxides, in particular, the mechanisms of migration and transformation of nanoparticles in EPS and IPS matrices, including physical, chemical and biological interaction mechanisms

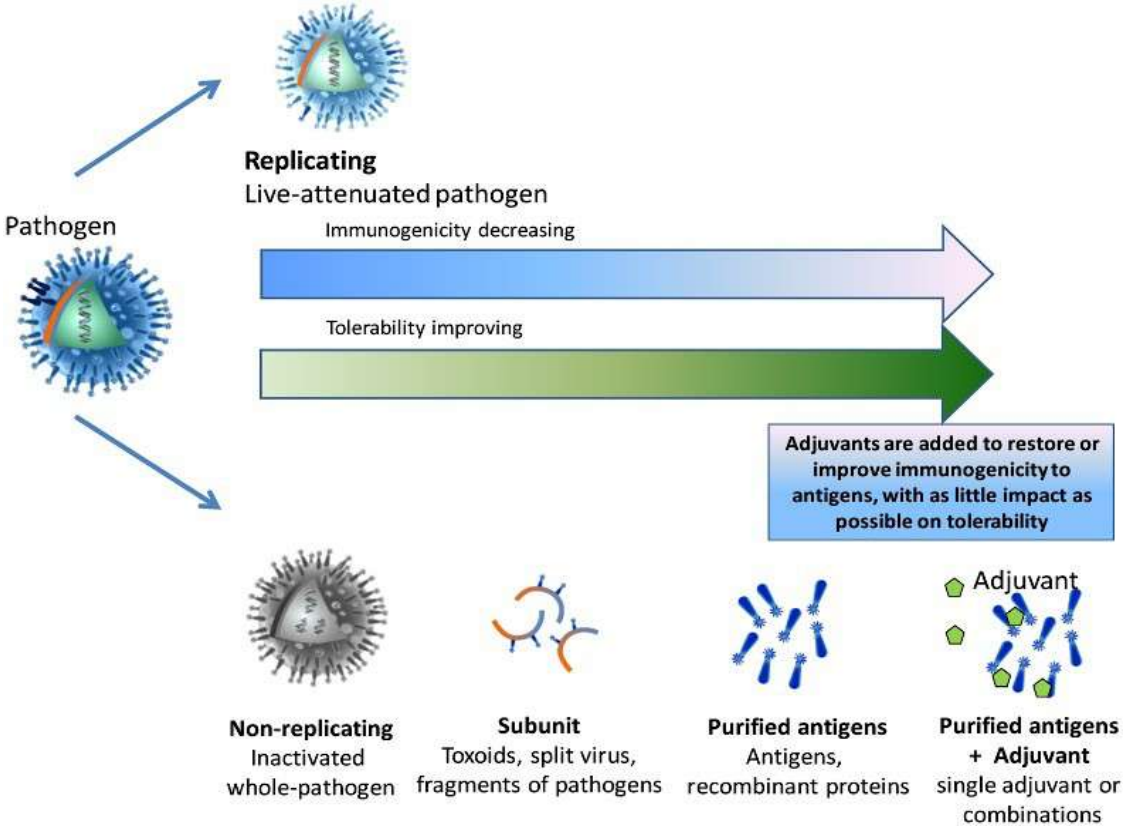


VACCINE ADJUVANTS

Adjuvants are substances added to vaccines to increase the immunogenicity of antigens with insufficient immunostimulatory capacity and have been in use in human vaccines for more than 90 years.

While early adjuvants (aluminum, oil-in-water emulsions) were used empirically, rapidly increasing knowledge about how the immune system interacts with pathogens is leading to a greater understanding of the role of adjuvants and more targeted formulations.[362]

Although vaccines containing a limited number of purified antigens generally have improved safety profiles compared with live attenuated and whole pathogen vaccines, they are also often less immunogenic because of the removal of pathogenic features of the microorganism. [363]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4494348/
Balancing immunogenicity and tolerability.

THE IMMUNE RESPONSE TO THE VACCINE

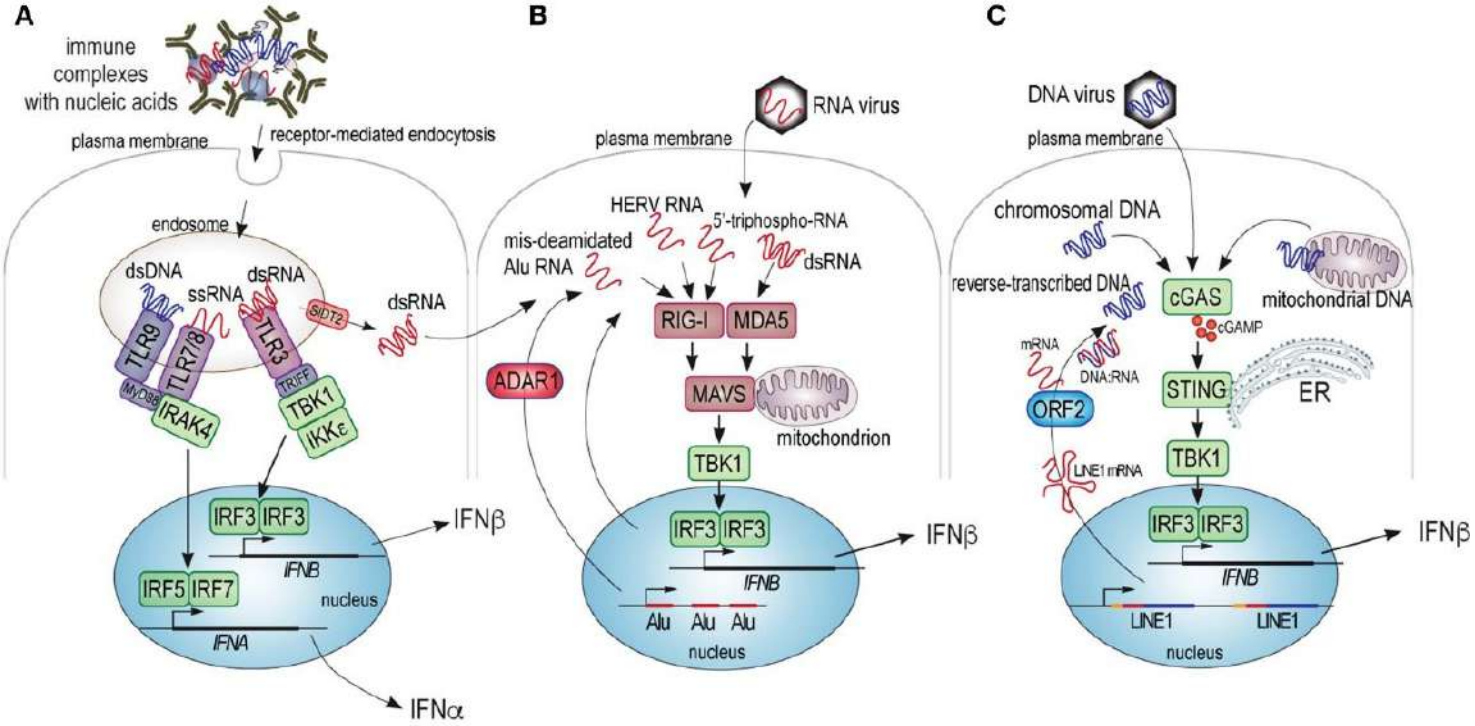
The immune response to an infectious agent (or immunization) can basically be divided into two phases: the innate response and the adaptive response.

[362] Di Pasquale A, Preiss S, Tavares Da Silva F, Garçon N.
Vaccine Adjuvants: from 1920 to 2015 and Beyond.
Vaccines (Basel). 2015;3(2):320-343. Published 2015 Apr 16. doi:10.3390/vaccines3020320
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4494348/

Shi S, Zhu H, Xia X, Liang Z, Ma X, Sun B.
Vaccine adjuvants: Understanding the structure and mechanism of adjuvanticity.
Vaccine. 2019 May 27;37(24):3167-3178. doi: 10.1016/j.vaccine.2019.04.055. Epub 2019 Apr 29. PMID: 31047671.
https://pubmed.ncbi.nlm.nih.gov/31047671/

[363] Geeraedts F, Goutagny N, Hornung V, et al.
Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signaling.
PLoS Pathog. 2008;4(8):e1000138. Published 2008 Aug 29. doi:10.1371/journal.ppat.1000138
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2516931/

When exposure to foreign agents occurs, the cellular effectors of the innate immune response, such as macrophages, monocytes, neutrophils, and dendritic cells, are able to recognize specific surface patterns (pathogen-associated molecular patterns or PAMPs) using pattern recognition receptors (PRRs) that classify the agent as a danger signal or as harmless. [364]



https://www.researchgate.net/publication/332955385_Sources_of_Pathogenic_Nucleic_Acids_in_Systemic_Lupus_Erythematosus
Schematic illustration of cellular sensors of pathogenic DNA and RNA and their signaling pathways leading to type I IFN production. (A) Extracellular nucleic acids present in immune complexes, free mitochondria or other structures can be internalized into cells by receptor-mediated endocytosis and transferred to endosomes containing TLR3, 7, 8 or 9, which recognize dsRNA, ssRNA and dsDNA, respectively.
(B) Cytosolic RNA from exogenous viruses or endogenous transcripts improperly deaminated by ADAR1, or containing recognizable retroviral motifs (HERV RNA) or other potentially aberrant RNA species can trigger RIG-I or MDA5, which bind mainly ssRNA and dsRNA, respectively.
(C) Cytosolic DNA from exogenous viruses, chromatin fragments, mitochondrial DNA, or reverse transcribed RNA, triggers the dimerization and activation of cGAMP leading to the synthesis of cGAMP, which activates the STING adaptor on the surface of the endoplasmic reticulum (ER) . STING, in turn, activates the kinase TBK, which activates IRF3.

Recognition of a potential pathogen triggers a complex series of events that can include phagocytosis, release of inflammatory mediators including chemokines and cytokines, complement activation, and cellular recruitment; all of which can lead to the development of signs and symptoms of local inflammation in the individual. [365]

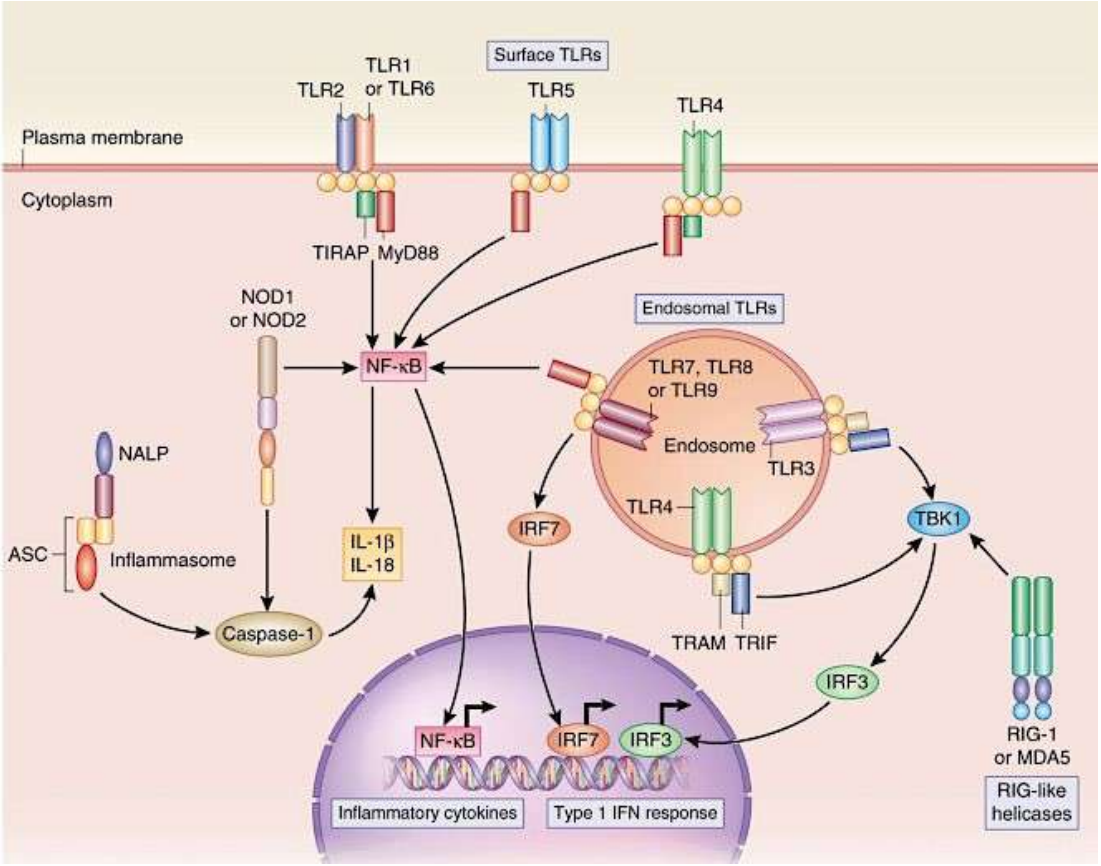
Antigen taken up by innate cells, such as dendritic cells, is processed, with cellular differentiation into APCs. APCs migrate to the region of the T-cell draining lymph node, where binding between the innate and adaptive immune response occurs.
The development of immune memory is predominantly an adaptive response, although the formation of innate immunity memory (trained immunity) has also been demonstrated recently, which will occur only if the correct signals are provided by the effectors of the innate response.

[364] Jensen S, Thomsen AR.
Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion. J Virol. 2012;86(6):2900-2910. doi:10.1128/JVI.05738-11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3302314/

[365] Reed, S., Orr, M. & Fox, C.
Key roles of adjuvants in modern vaccines.
Nat Med 19, 1597-1608 (2013). https://doi.org/10.1038/nm.3409
https://www.nature.com/articles/nm.3409

Therefore, how the innate immune response sets in motion the secondary or adaptive immune response has profound implications for the type of secondary response, the quality of the response, and the induction of immunological memory.

For an in-depth discussion of the immune system, see the paper
Clinical presentation and immunopathogenesis from p. 24 [366]



https://www.nature.com/articles/nm.3409
Several pattern recognition receptors (PRRs) that activate an innate immune response can be targeted by adjuvants, and details of their downstream signaling pathways are shown. TLRs, located on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) or the endosome (TLR3, TLR7, TLR8, and TLR9) are targets for adjuvants and when activated stimulate signaling leading to activation of the key
transcription factors, such as nuclear factor-κB (NF-κB). These transcription factors then stimulate gene expression programs that lead to
to the production of chemokines and cytokines that help target particular immune responses. Adjuvants can also target cytosolic PRRs such as NLRs and RIG-like helicases. The NLR NALP3 is part of a macromolecular assembly, the inflammasome, that leads to caspase 1 activation and production of the proinflammatory cytokines IL-1β and IL-18. ASC, apoptosis-associated speck-like protein containing CARD; IRF3, interferon-regulatory factor; MDA5, melanoma differentiation-associated protein 5; MyD88, myeloid differentiation factor 88; TBK1, TANK-binding kinase 1; TIRAP, adaptor protein containing Toll-interleukin 1 receptor domain; TRAM, Trif-related adaptor molecule; TRIF, interferon β-inducing adaptor containing TIR domain

The adaptive immune response is largely driven by lymphocytes: T cells and B cells.
The relative activities of B-cell and T-cell populations determine the type of immune response generated in response to infection.
Upon recognition of a specific antigen, B cells differentiate into plasma cells and release specific antibodies (IgM) into the circulation.
However, the development of immune memory or the ability to respond rapidly to re-exposure to the same antigen occurs only when B lymphocytes have received the "help" of T lymphocytes, a so-called "T cell-dependent response."

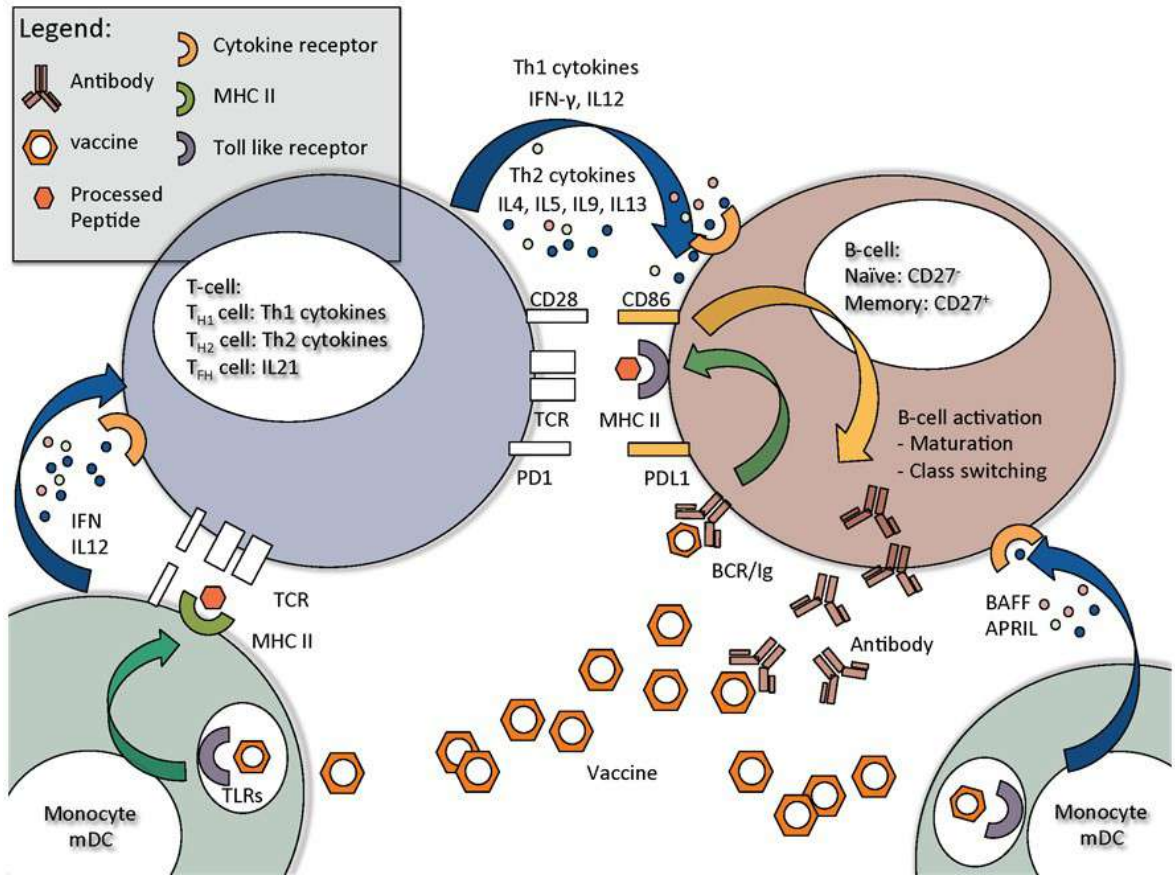
[366] https://www.studiesalute.it/salute COVID-19 immunopathology.

CD4 T-helper cells+ are unable to recognize antigen unless it is presented to them after processing by antigen-presenting cells (APCs) activated during the innate immune response.

Activated T-helper cells release inflammatory mediators that are specific to a subpopulation of T-helper cells (Th1, Th2, Th17, and Thf) [367], which has downstream implications for how effectively the pathogen is removed or contained.

In general terms, Th1 cells are required for the removal of intracellular pathogens; Th2 cells for the removal of extracellular parasites; Th17 cells for the removal of bacteria and fungi; and Thf (follicular) cells for the activation of a T-cell-dependent B-cell response.

Th1 and Th17 cells are also mediators of autoimmunity, while Th2 cells are associated with asthma and allergic diseases. CD8+ activated cytotoxic T lymphocytes can kill cells directly or through cytotoxin release.



https://smw.ch/article/doi/smw.2014.13940
Key steps in B lymphocyte activation and interaction of B lymphocytes with helper T cells and monocytes, macrophages, and monocyte-derived dendritic cells

Skeletal muscle is typically where the vaccine first enters the body: a resting muscle usually contains few immune cells, but administration of the vaccine triggers the

[367] Luckheeram RV, Zhou R, Verma AD, Xia B.
CD4+T cells: differentiation and functions.
Clin Dev Immunol. 2012;2012:925135. doi:10.1155/2012/925135
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3312336/

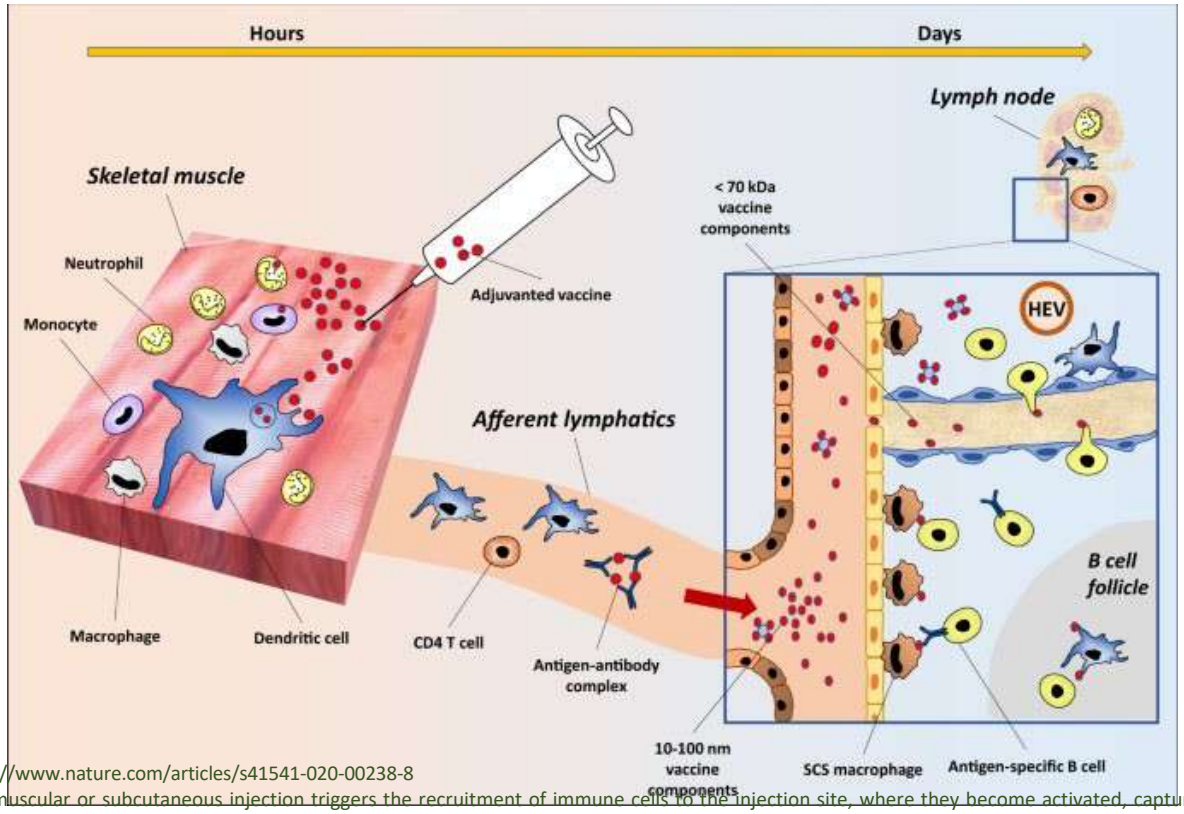
He A, et al
Vaccine adjuvants--understanding molecular mechanisms to improve vaccines.
Swiss Med Wkly. 2014 May 20;144:w13940. doi: 10.4414/smw.2014.13940. PMID: 24844935.
https://smw.ch/article/doi/smw.2014.13940

Recruitment of tissue-resident and infiltrating immune cells, including APCs, which are activated after vaccine administration [368].

Vaccine platforms that rely on DNA or RNA delivery systems employ the mechanism of muscle cell expression to express antigen at the injection site and promote the recruitment of immune cells [369].

As for subunit vaccines, antigens are generally administered with vaccine adjuvants, which help induce transient inflammation in the muscle and further promote recruitment and activation of immune cells [370].

The persistence and quality of adjuvant-induced immune stimuli, including cytokines and chemokines, represent a fingerprint of each adjuvant and provide qualitatively unique immune responses at the injection site.



https://www.nature.com/articles/s41541-020-00238-8
Intramuscular or subcutaneous injection triggers the recruitment of immune cells to the injection site, where they become activated, capture the antigen, and migrate to the draining lymph node. Antigens, adjuvants, or other components smaller than 10-100 nm in size can also diffuse into the lymphatic systems and reach the lymph node via afferent lymphatics. While smaller molecules (<70 kDa) can diffuse through subcapsular sinus windows, larger molecules are transferred to B cells with the help of subcapsular sinus macrophages. B cells and resident dendritic cells can also detect molecules in the ducts and transfer them to the B cell area to initiate germinal center responses.

[368] Lofano, G., Mallett, C.P., Bertholet, S. et al.
Technological approaches to streamline vaccination schedules, progressing toward single-dose vaccines.
npj Vaccines 5, 88 (2020). https://doi.org/10.1038/s41541-020-00238-8
https://www.nature.com/articles/s41541-020-00238-8

[369] Pardi N, Hogan MJ, Porter FW, Weissman D.
mRNA vaccines - a new era in vaccinology.
Nat Rev Drug Discov. 2018 Apr;17(4):261-279. doi: 10.1038/nrd.2017.243. Epub 2018 Jan 12. PMID: 29326426; PMCID: PMC5906799.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5906799/

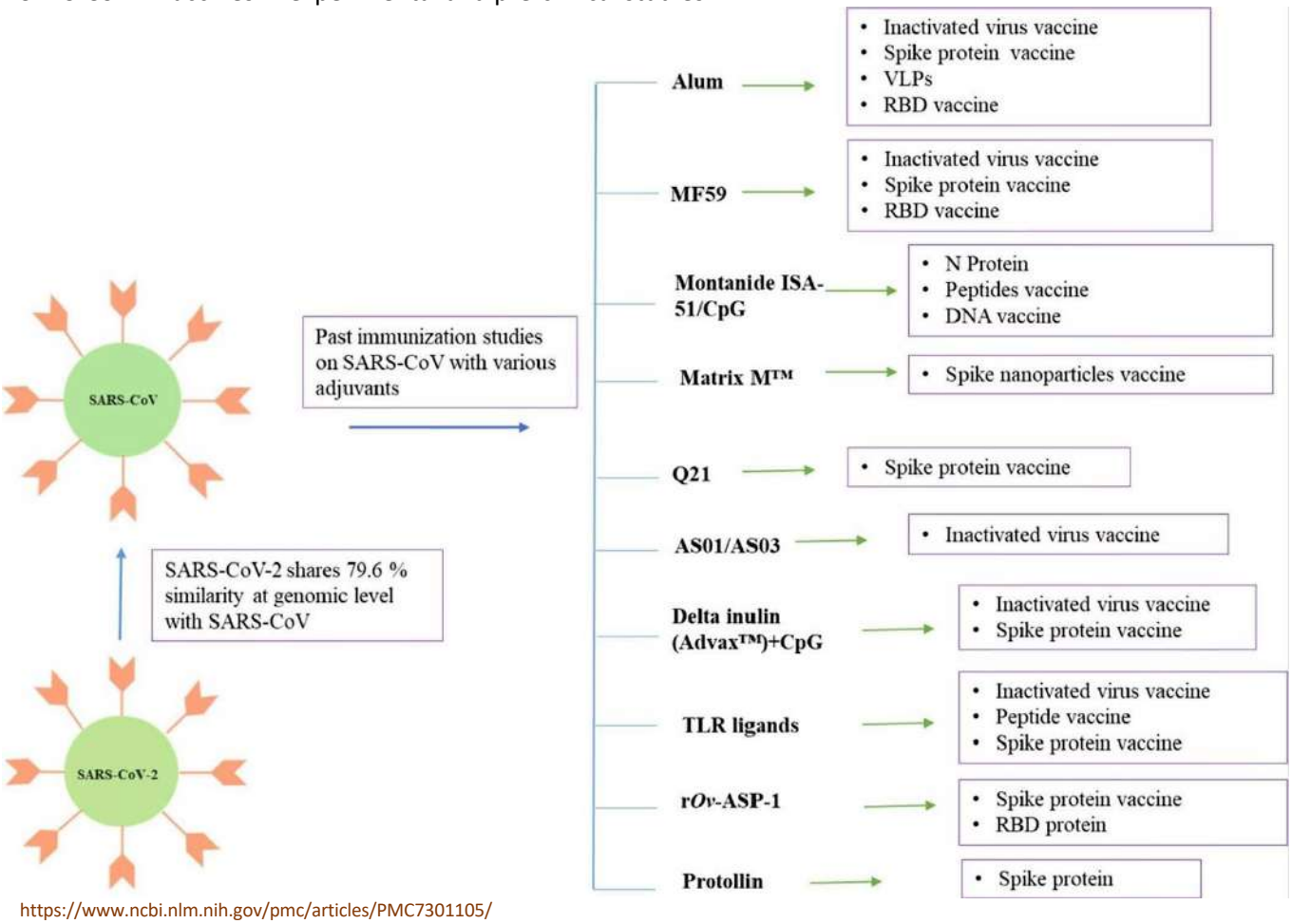
[370] Liang F, Loré K.
Local innate immune responses in the vaccine adjuvant-injected muscle.
Clin Transl Immunology. 2016;5(4):e74. Published 2016 Apr 29. doi:10.1038/cti.2016.19
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4855268/

Highly purified vaccine components often lack PAMPs, meaning that the initial innate immune response is not activated so that an effective downstream adaptive response occurs.

It is believed that the main mechanism of action of adjuvants is on the innate immune response.
Adjuvants can act as PAMPs, triggering the innate immune response through a variety of mechanisms, which identify vaccine components as a danger signal with activation and maturation of APCs and initiation of downstream adaptive immune activities.[371]

Adjuvants are critical components of both subunit vaccines and some inactivated vaccines because they induce specific immune responses that are more robust and long-lasting.

A review of the history of coronavirus vaccine development shows that only a few adjuvants, including aluminum salts, emulsions, and TLR agonists, have been formulated for SARS-CoV, MERS-CoV, and currently SARS-CoV-2 vaccines in experimental and pre-clinical studies. [372]



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7301105/
As SARS-CoV-2 is highly similar to SARS-CoV at the genomic level, the outcomes of the past vaccine studies may expedite the development of a vaccine against COVID-19.

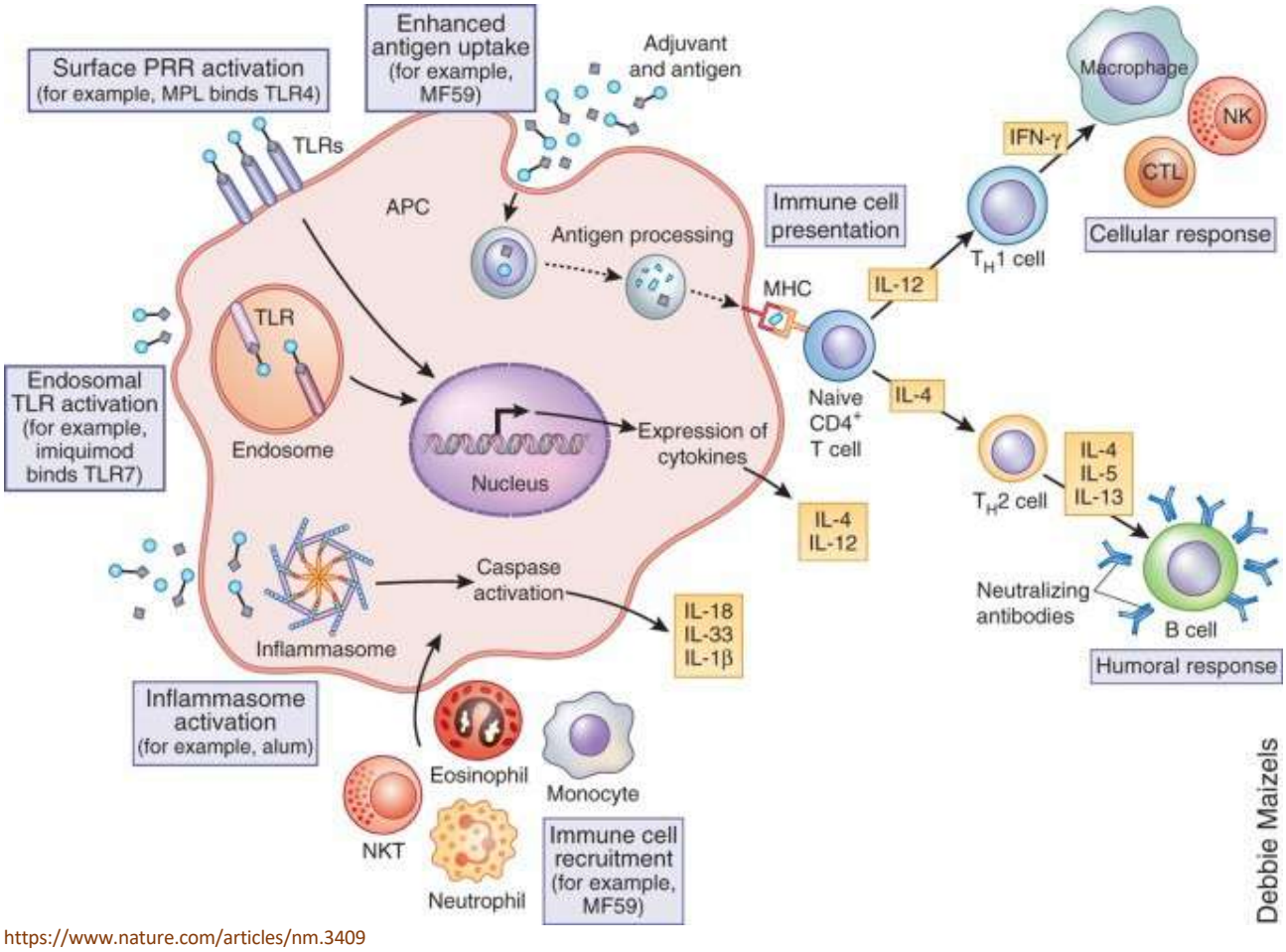
[371] Coffman RL, Sher A, Seder RA.
Vaccine adjuvants: putting innate immunity to work.
Immunity. 2010;33(4):492-503. doi:10.1016/j.immuni.2010.10.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3420356/

[372] Gupta T, Gupta SK.
Potential adjuvants for the development of a SARS-CoV-2 vaccine based on experimental results from similar coronaviruses.
Int Immunopharmacol. 2020 Sep;86:106717. doi: 10.1016/j.intimp.2020.106717. Epub 2020 Jun 18. PMID: 32585611; PMCID: PMC7301105.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7301105/

Liang Z, Zhu H, Wang X, et al.
Adjuvants for Coronavirus Vaccines.
Front Immunol. 2020;11:589833. Published 2020 Nov 6. doi:10.3389/fimmu.2020.589833

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7677582/

The following is an overview of the vaccine adjuvants that have been formulated in the reported COVID-19 protein vaccine candidates and their mechanism of action .[373]



https://www.nature.com/articles/nm.3409

Several mechanisms by which adjuvants mediate their activity have been postulated. Many adjuvants may act as ligands for PRRs that activate an innate immune response. Receptor signaling can then activate transcription factors that induce the production of cytokines and chemokines that help direct a particular immune response, such as a Th1- or Th2-type response, as well as influence the immune cells that are recruited to the injection site. Inflammasome activation has also been implicated as a mechanism for some adjuvants. Inflammasome activation leads to the production of the proinflammatory cytokines IL-1β and IL-18. Some adjuvants also influence antigen presentation by the MHC. It is possible that some adjuvants act through multiple mechanisms; for example, it has been suggested that alum may influence antigen uptake, PRR signaling, inflammasome activation, and immune cell recruitment. NK, natural killer cell

ALUMINUM HYDROXIDE (ALUM)

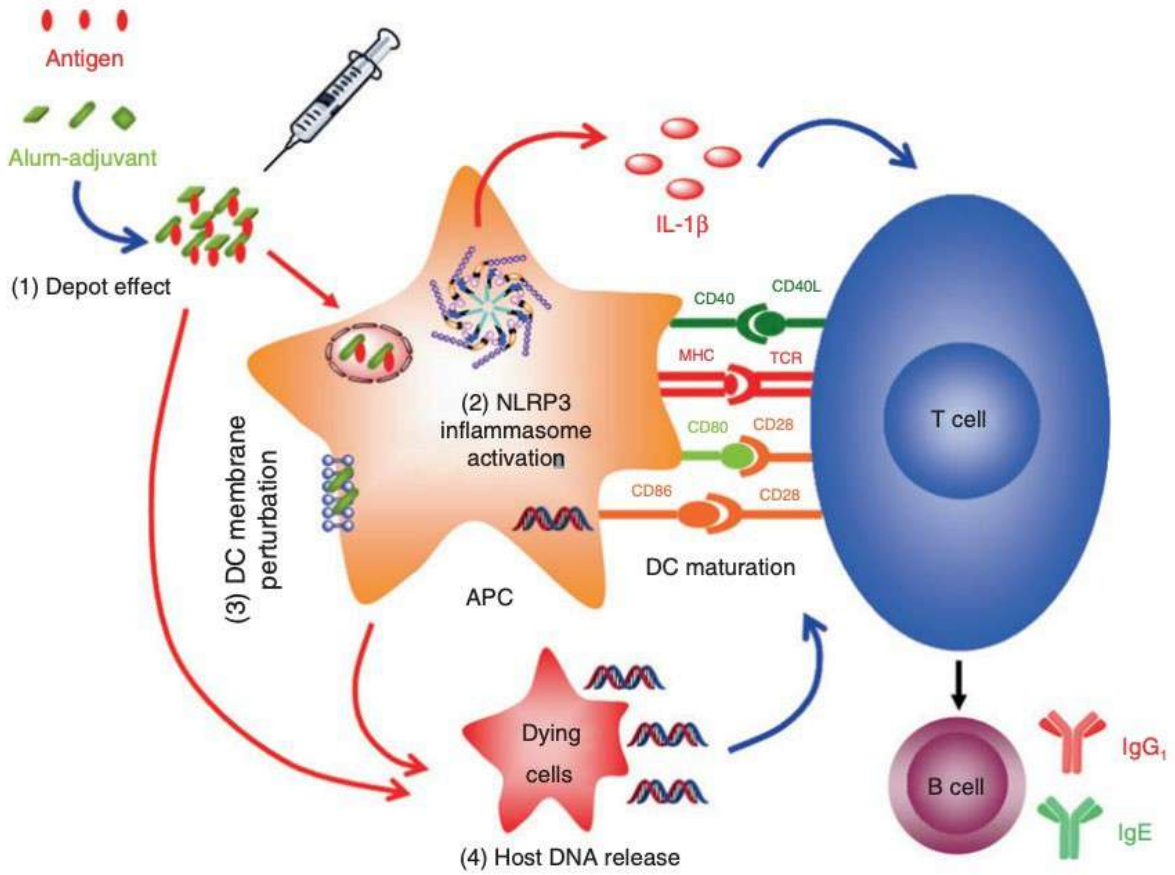
Semicrystalline aluminum suspensions are the most commonly used adjuvants in vaccine development worldwide [374].

Aluminum salts have a high binding capacity and typically adsorb antigens onto their surface. Although hundreds of millions of people have been vaccinated with aluminum-based vaccines, there is still debate about the exact mechanism of action.

[373] Pollet J, Chen WH, Strych U.
Recombinant protein vaccines, a proven approach against coronavirus pandemics
[published online ahead of print, 2021 Jan 7]. Adv Drug Deliv Rev. 2021;170:71-82. doi:10.1016/j.addr.2021.01.001
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7788321/

[374] Shardlow E, Mold M, Exley C.
Unraveling the enigma: elucidating the relationship between the physicochemical properties of aluminum-based adjuvants and their immunological mechanisms of action.
Allergy Asthma Clin Immunol. 2018;14:80. Published 2018 Nov 7. doi:10.1186/s13223-018-0305-2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6223008/

The most widely accepted explanations include a possible depot effect, enhancement of antigen phagocytosis, and activation of the pro-inflammatory NLRP3 pathway [375].
Aluminum-based formulations generally induce a strong humoral immune response in combination with the secretion of Th2-polarized inflammatory cytokines (e.g., IL-4, IL-6, IL-10).



https://link.springer.com/referenceworkentry/10.1007%2F978-94-017-9780-1_100901
Aluminum-based nanoadjuvants. Mechanisms of immune responses induced by aluminum-based nanoadjuvants, including (1) the depot effect, (2) activation of NLRP3 inflammasome, (3) membrane disruption of DCs, and (4) release of host DNA, which promotes DC maturation and enhances adaptive immune responses

Some investigations have observed the occurrence of increased antibody-dependent disease (ADE) and eosinophilic immunopathology in the lungs in response to challenge testing with infectious viruses after immunization with inactivated viral vaccine [376].

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[375] He P, Zou Y, Hu Z.
Advances in aluminum hydroxide-based adjuvant research and its mechanism.
Hum Vaccin Immunother. 2015;11(2):477-488. doi:10.1080/21645515.2014.1004026
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4514166/

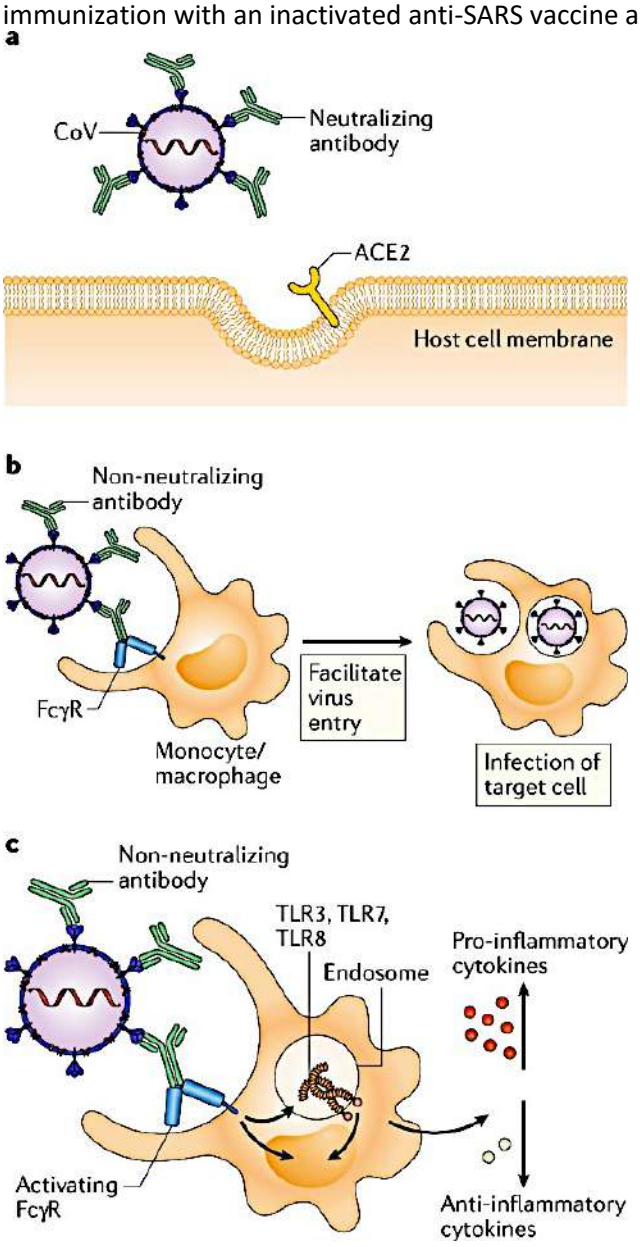
Sun B., Ji Z., Xia T. (2016)
Aluminum-Based Nano-adjuvants.
In: Bhushan B. (eds) Encyclopedia of Nanotechnology. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-9780-1_100901
https://link.springer.com/referenceworkentry/10.1007%2F978-94-017-9780-1_100901

[376] Agrawal AS, Tao X, Algaissi A, et al.
Immunization with inactivated Middle East Respiratory Syndrome coronavirus vaccine leads to lung immunopathology on challenge with live virus.
Hum Vaccin Immunother. 2016;12(9):2351-2356. doi:10.1080/21645515.2016.1177688
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5027702/

Bolles M, Deming D, Long K, et al.
A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge.
J Virol. 2011;85(23):12201-12215. doi:10.1128/JVI.06048-11
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3209347/

Tseng CT, Sbrana E, Iwata-Yoshikawa N, et al.
Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus
[published correction appears in PLoS One. 2012;7(8). doi:10.1371/annotation/2965cfae-b77d-4014-8b7b-236e01a35492]. PLoS One. 2012;7(4):e35421. doi:10.1371/journal.pone.0035421
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3335060/

Further studies have attributed the occurrence of immunopathological changes or ADEs to the presence of nonneutralizing anti-N antibodies and low-affinity neutralizing antibodies or suboptimal levels induced by immunization with an inactivated anti-SARS vaccine alone or with alum [377].



Potential outcomes of antibody response to coronavirus.

a) In antibody-mediated viral neutralization, neutralizing antibodies that bind to the receptor-binding domain (RBD) of the viral spike protein, as well as other domains, prevent the virus from attaching to its entry receptor, ACE2.

b) In antibody-dependent enhancement of infection, low-quality, low-quantity nonneutralizing antibodies bind to viral particles through Fab domains. Fc receptors (FcRs) expressed on monocytes or macrophages bind to the Fc domains of antibodies and facilitate viral entry and infection.

c) In antibody-mediated immune enhancement, low-quality, low-quantity nonneutralizing antibodies bind to viral particles. After engagement of Fc domains on antibodies, activation of FcR by ITAM initiates signaling to upregulate pro-inflammatory cytokines and downregulate anti-inflammatory cytokines. Immune complexes and viral RNA in endosomes can signal through Toll-like receptor 3 (TLR3), TLR7 and/or TLR8 to activate host cells, resulting in immunopathology.

For an in-depth discussion of vaccine immunopathology, see the papers
COVID-19 THE VACCINE from p. 34

[377] Yasui F, et al
Prior immunization with severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV.
J Immunol. 2008 Nov 1;181(9):6337-48. doi: 10.4049/jimmunol.181.9.6337. PMID: 18941225.
https://www.jimmunol.org/content/181/9/6337.long

Iwasaki A, Yang Y.
The potential danger of suboptimal antibody responses in COVID-19.
Nat Rev Immunol. 2020;20(6):339-341. doi:10.1038/s41577-020-0321-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7187142/

Deming D, et al
Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants.
PLoS Med. 2006 Dec;3(12):e525. doi: 10.1371/journal.pmed.0030525. Erratum in: PLoS Med. 2007 Feb;4(2):e80. PMID: 17194199; PMCID:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1716185/

RESPIRATORY COMPLICATIONS - PART SECOND - Immunopathology from page 48 [378]

Inclusion of an adjuvant that promotes a predominantly Th1 response to the inactivated vaccine may alleviate the problem of Th2-type immunopathology caused by aluminum [379].

Based on this evidence, most recombinant COVID-19 protein vaccine formulations containing aluminum hydroxide (alum) include a second adjuvant, such as CpG, in order to balance the immune response and also stimulate proliferation of $CD4^+$ Th1-type cells.

ADJUVANTS BASED ON OIL-IN-WATER EMULSIONS

MF59

MF59® is an oil-in-water emulsion developed by Novartis. The adjuvant contains squalene oil and two surfactants, Tween-80 and Span-85, emulsified in a citric acid buffer [380].
MF59 adjuvanted vaccines have been approved for pandemic and seasonal influenza in more than 38 countries worldwide . [381] In oil-in-water formulations, the antigen typically remains in the aqueous phase and does not interact with oil droplets.
It provides neither direct transport nor depot effect for antigen. Antigens and MF59 are taken up by neutrophils and monocytes, and subsequently followed by dendritic cells (DCs) and B lymphocytes, and transferred to draining lymph nodes [382].

MF59 affects apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and stimulates IL-4 and Stat-6 signaling, but is independent of any type 1 interferon or inflammasome signaling cascade.

Emulsion has also been shown to significantly increase the levels of IL-5 and IL-6 [383].
As with aluminum, an immunopathologic pulmonary reaction, as well as an increase in IL-5 and IL-13 cytokines, was observed in animal studies with inactivated and MF59-adjuvanted MERS-CoV vaccines.

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[378] Part 2 Pulmonary Complications Immunopathology studiesalute.co.uk/health.

[379] Deng Y, Lan J, Bao L, et al.
Enhanced protection in mice induced by immunization with inactivated whole viruses compare to spike protein of middle east respiratory syndrome coronavirus.
Emerg Microbes Infect. 2018;7(1):60. Published 2018 Apr 4. doi:10.1038/s41426-018-0056-7
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5884803/

Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N.
Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide enhanced protection while ameliorating lung eosinophilic immunopathology.
J Virol. 2015;89(6):2995-3007. doi:10.1128/JVI.02980-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4337527/

[380] Ko EJ, Kang SM.
Immunology and efficacy of MF59-adjuvanted vaccines.
Hum Vaccin Immunother. 2018;14(12):3041-3045. doi:10.1080/21645515.2018.1495301
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6343625/

[381] O'Hagan DT.
MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection.
Expert Rev Vaccines. 2007 Oct;6(5):699-710. doi: 10.1586/14760584.6.5.699.
https://pubmed.ncbi.nlm.nih.gov/17931151/

[382] Dupuis M, Denis-Mize K, LaBarbara A, Peters W, Charo IF, McDonald DM, Ott G.
Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis.
Eur J Immunol. 2001 Oct;31(10):2910-8. doi: 10.1002/1521-4141(2001010)31:10<2910::aid-immu2910>3.0.co;2-3. PMID: 11592066.
https://onlinelibrary.wiley.com/doi/10.1002/1521-4141(2001010)31:10%3C2910::AID-IMMU2910%3E3.0.CO;2-3

[383] Valensi JP, Carlson JR, Van Nest GA.
Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants.
J Immunol. 1994 Nov 1;153(9):4029-39. PMID: 7930610.
https://pubmed.ncbi.nlm.nih.gov/7930610/

Eosinophil infiltration associated with increased production of Th2-type cytokines has been shown to exacerbate lung immunopathology due to hypersensitivity in animals vaccinated with MF59-adjuvanted inactivated virus vaccines compared with vaccines with non-adjuvanted inactivated viruses. [384]
Added to this is an increased risk of autoimmune reactions in genetically predisposed individuals. [385]

AS01 and AS03

These are oil-in-water adjuvant systems developed by GlaxoSmithKline (GSK). **AS01** (adjuvant system 01) is a liposome-based adjuvant consisting of monophosphoryl-lipid A (MPL) and a saponin molecule (QS- 21), while **AS03** (adjuvant system 03) is an α-tocopherol and squalene-based adjuvant that was used in the pandemic influenza A/H1N1 vaccine.
MPL is extracted from *Salmonella minnesota* and QS-21 * is purified from the bark of the South American tree *Quillaja saponaria* Molina.
MPL acts through the Toll-like receptor-4 (TLR4) signaling pathway, which results in activation of APCs and production of cytokines and interferons (IFNs).
It has been reported that Q-21 induces an antigen-specific antibody response and cell-mediated immunity[386].
When co-administered with recombinant SARS-CoV protein S, Q-21 induced high titers of antigen-specific serum antibodies in the challenge test [387]. I
he AS01 system was also used in the preparation of inactivated SARS-CoV vaccine in mice and hamsters, and it should be reported that the study did not observe enhancing disease (ADE) in the lungs or liver of hamsters following stimulation with SARS-CoV [388].

> * **Saponins** are naturally occurring glycosidic compounds. Their unique ability to stimulate both Th1 immune response and cytotoxic T lymphocyte (CTL) production against exogenous antigens makes them ideal for use in subunit vaccines, vaccines directed against intracellular pathogens, as well as therapeutic cancer vaccines. However, Quillaja saponins have serious drawbacks such as high toxicity, undesirable hemolytic effect, and instability in the aqueous phase, which limits their use as an adjuvant in vaccination. [389]

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[384] Agrawal AS, Tao X, Algaissi A, et al.
Immunization with inactivated Middle East Respiratory Syndrome coronavirus vaccine leads to lung immunopathology on challenge with live virus.
Hum Vaccin Immunother. 2016;12(9):2351-2356. doi:10.1080/21645515.2016.1177688
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5027702/

[385] Yau, A.C.Y., Lönnblom, E., Zhong, J. et al.
Influence of hydrocarbon oil structure on adjuvanticity and autoimmunity.
Sci Rep 7, 14998 (2017). https://doi.org/10.1038/s41598-017-15096-z
https://www.nature.com/articles/s41598-017-15096-z

[386] Didierlaurent AM, Laupèze B, Di Pasquale A, Hergli N, Collignon C, Garçon N.
Adjuvant system AS01: helping to overcome the challenges of modern vaccines.
Expert Rev Vaccines. 2017 Jan;16(1):55-63. doi: 10.1080/14760584.2016.1213632. Epub 2016 Aug 2. PMID: 27448771.
https://pubmed.ncbi.nlm.nih.gov/27448771/

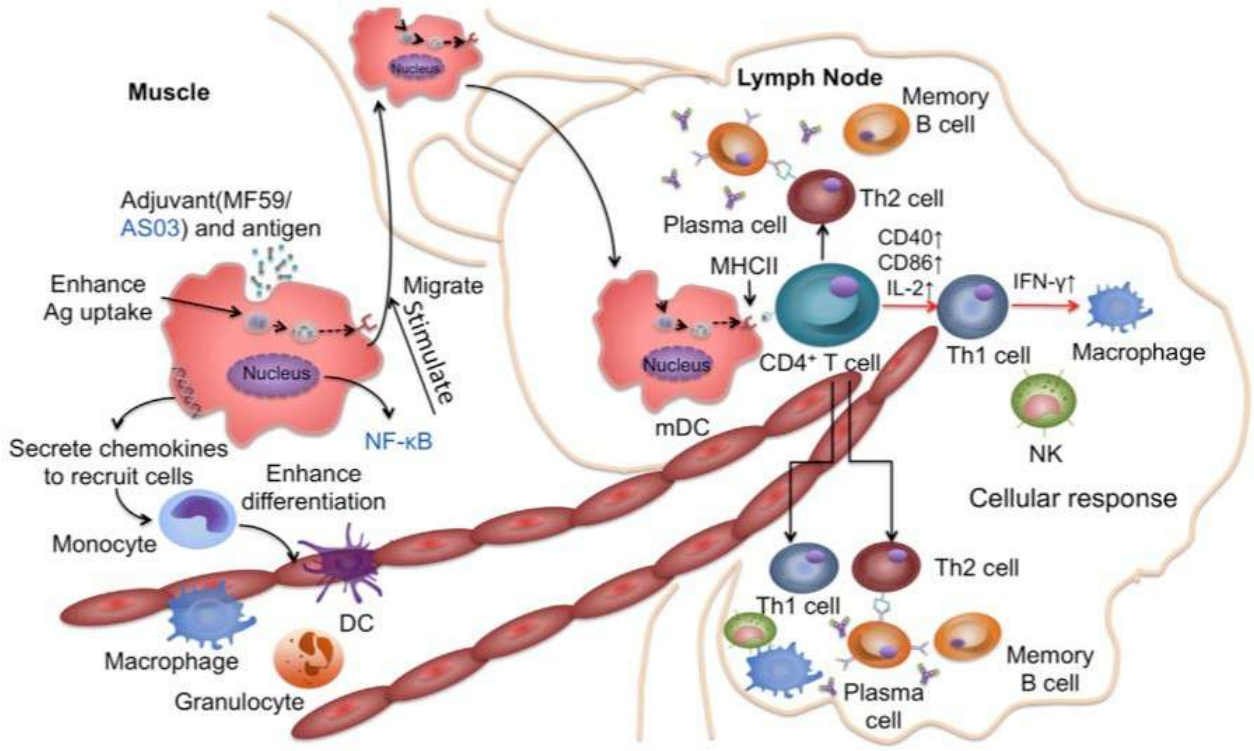
Coccia M, Collignon C, Hervé C, et al.
Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFNγ response promoting vaccine immunogenicity
[published correction appears in NPJ Vaccines. 2018 Mar 21;3:13].
NPJ Vaccines. 2017;2:25. Published 2017 Sep 8. doi:10.1038/s41541-017-0027-3
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5627273/

[387] Bisht H, Roberts A, Vogel L, Subbarao K, Moss B.
Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N- terminal segment of the spike glycoprotein.
Virology. 2005;334(2):160-165. doi:10.1016/j.virol.2005.01.042
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111832/

[388] Roberts A, Lamirande EW, Vogel L, et al.
Immunogenicity and protective efficacy in mice and hamsters of a β-propiolactone inactivated whole virus SARS-CoV vaccine.
Viral Immunol. 2010;23(5):509-519. doi:10.1089/vim.2010.0028
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2967819/

[389] Sun HX, Xie Y, Ye YP.
Advances in saponin-based adjuvants.
Vaccine. 2009 Mar 13;27(12):1787-96. doi: 10.1016/j.vaccine.2009.01.091. Epub 2009 Feb 7. PMID: 19208455.
https://pubmed.ncbi.nlm.nih.gov/19208455/

Novavax's patented **Matrix-M** adjuvant consists of two separate nanometer particles made from a different saponin fraction (Fraction-A and Fraction-C).
Saponin particles are stabilized with cholesterol and phospholipids. [390].
As part of several vaccine formulations, Matrix-M has been shown to increase Th1- and Th2-type responses by inducing high levels of neutralizing antibodies and enhancing immune cell trafficking [391].



https://pubmed.ncbi.nlm.nih.gov/31047671/
Models for the activation mechanism of MF59 and AS03. Both MF59 and AS03 create a local and transient immunocompetent environment a f t e r injection. They promote cytokine and chemokine production and cell recruitment at the injection site. Activated antigen-loaded APCs migrate to draining lymph nodes where APCs could trigger naive CD4⁺ T cells. Chemokine-driven immune cell recruitment is the key feature of the mechanism for both MF59 and AS03.

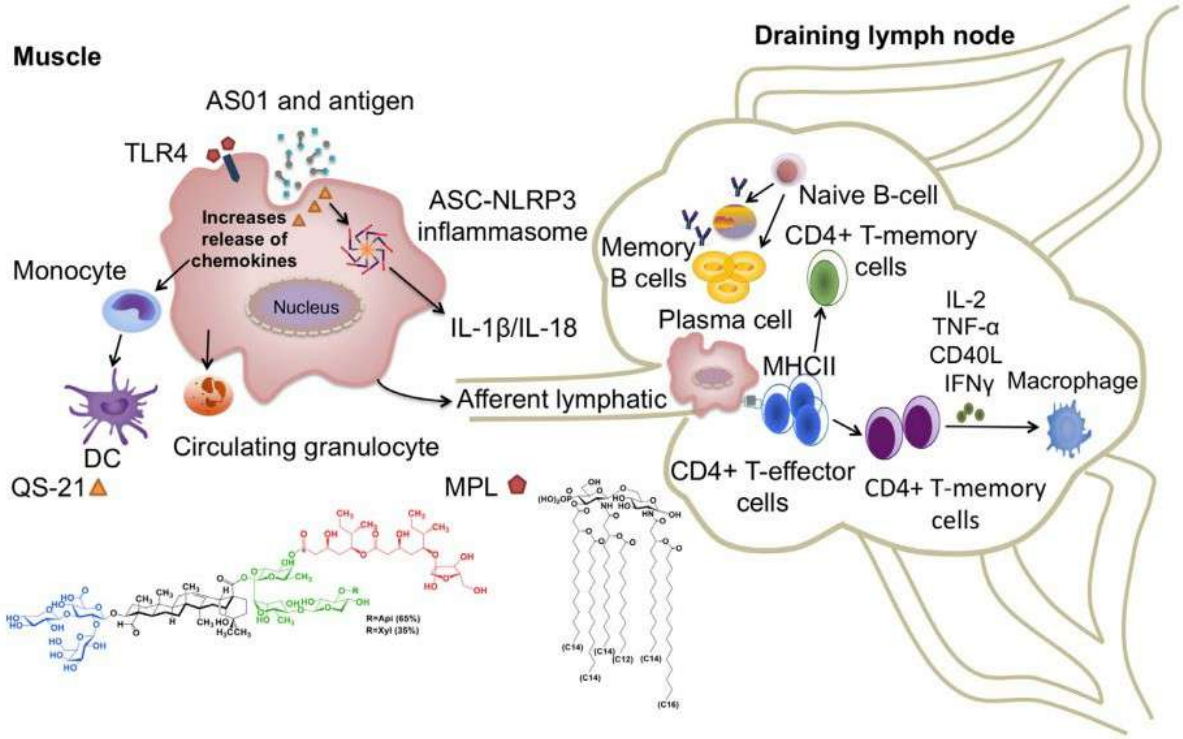
Podolak I, Galanty A, Sobolewska D.
Saponins as cytotoxic agents: a review.
Phytochem Rev. 2010 Sep;9(3):425-474. doi: 10.1007/s11101-010-9183-z. Epub 2010 Jun 25.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2928447/

[390] Magnusson SE, Altenburg AF, Bengtsson KL, Bosman F, de Vries RD, Rimmelzwaan GF, Stertman L.
Matrix-M™ adjuvant enhances immunogenicity of both protein- and modified vaccinia virus Ankara-based influenza vaccines in mice.
Immunol Res. 2018 Apr;66(2):224-233. doi: 10.1007/s12026-018-8991-x. PMID: 29594879; PMCID: PMC5899102.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5899102/

[391] Magnusson SE, Reimer JM, Karlsson KH, Lilja L, Bengtsson KL, Stertman L.
Immune enhancing properties of the novel Matrix-M™ adjuvant leads to potentiated immune responses to an influenza vaccine in mice.
Vaccine. 2013 Mar 25;31(13):1725-33. doi: 10.1016/j.vaccine.2013.01.039. epub 2013 Feb 4. PMID: 23384754.
https://pubmed.ncbi.nlm.nih.gov/23384754/

Osterhaus AD, Rimmelzwaan GF.
Induction of virus-specific immunity by iscoms.
Dev Biol Stand. 1998;92:49-58. PMID:9554259.
https://pubmed.ncbi.nlm.nih.gov/9554259/

Reimer JM, Karlsson KH, Lövgren-Bengtsson K, Magnusson SE, Fuentes A, Stertman L. Matrix-M™ adjuvant induces local recruitment, activation and maturation of central immune cells in absence of antigen.
PLoS One. 2012;7(7):e41451. doi: 10.1371/journal.pone.0041451. Epub 2012 Jul 23. PMID: 22844480; PMCID: PMC3402407.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3402407/



https://pubmed.ncbi.nlm.nih.gov/31047671/
Mechanisms of action of AS01B. AS01B adjuvant and antigen are injected into the muscle and taken up by APCs. MPL activates APCs via TLR4. QS-21 activates the NLRP3 inflammasome, resulting in the release of IL-1b and IL-18. MPL and QS-21 act synergistically to increase chemokine release, circulate granulocytes, and enhance recruitment of monocytes and dendritic cells. In lymph node drainage, highly activated dendritic cells effectively induce the differentiation of $CD4^+$ naïve T cells into CD4 memory T $cells^+$ and CD4 T-effector $cells^+$. Cytokines secreted by CD4 T-effector $cells^+$ such as IL-2, TNF-alpha, CD40L and IFN-c could stimulate the division of naïve B cells into plasma cells and memory B cells.

In addition to MF59 and AS03, other emulsion-based adjuvants such as Freund's adjuvant* (only in comparative preclinical studies between adjuvants) and Montanide ISA51 have also been formulated in CoV vaccines [392].

Montanide in particular is a water-in-oil (w/o) emulsion that contains mineral oil and surfactant from the monooleate mannide family.
It possesses immunostimulatory activity and acts on deposit formation at the injection site, resulting in slower antigen release, local inflammation, and recruitment of antigen-presenting cells (APCs).
The combination of Montanide ISA-51 and CpG ODN (oligodeoxynucleotide) has been widely used in many vaccines and to emulsify recombinant SARS-CoV protein N, as the combination exerts a synergistic effect in enhancing the Th1 immune response. [393]

* **Freund's adjuvant** is one of the most commonly used adjuvants in research for the induction of severe autoimmune and inflammatory diseases. [394], so its use in humans is not permitted.

[392] Zhang N, Channappanavar R, Ma C, et al.
Identification of an ideal adjuvant for receptor-binding domain-based subunit vaccines against Middle East respiratory syndrome coronavirus.
Cell Mol Immunol. 2016;13(2):180-190. doi:10.1038/cmi.2015.03
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4786625/

[393] Gupta T, Gupta SK.
Potential adjuvants for the development of a SARS-CoV-2 vaccine based on experimental results from similar coronaviruses.
Int Immunopharmacol. 2020;86:106717. doi:10.1016/j.intimp.2020.106717
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7301105/

[394] Billiau A, Matthys P.
Modes of action of Freund's adjuvants in experimental models of autoimmune diseases.
J Leukoc Biol. 2001 Dec;70(6):849-60. PMID: 11739546.
https://pubmed.ncbi.nlm.nih.gov/11739546/

Constantinescu C.S., Hilliard B.A.

It is used as a water-in-oil emulsion and is prepared from non-metabolizable oils (kerosene oil and mannide monooleate).
If it also contains killed *Mycobacterium tuberculosis it* is known as **Freund's complete adjuvant**, without the bacteria it is **Freund's incomplete adjuvant**.
First developed by Jules Freund in the 1940s, it is designed to provide continuous release of the antigens needed to stimulate a strong and persistent immune response.

TLR AGONIST ADJUVANTS

As seen above, TLRs are pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs).
These are present on the surface of cells and in endosomal compartments.
Interaction with the appropriate ligand triggers the release of proinflammatory cytokines and type -1 IFNs (interferons), which activate cells of the innate and adaptive immune systems leading to humoral and cell-mediated responses enhanced by the specific antigen.

Therefore, TLR ligands have been extensively studied and tested as adjuvants in many human and veterinary vaccine preparations against infectious diseases.
Different TLR ligands induce a different type of immune response (Th1/Th2/Th0) depending on the signaling pathway involved.
The TLRs evaluated in studies on vaccine adjuvants and their ligands are as follows: TLR3 (dsRNA), TLR4 (LPS - MPL), TLR5 (Flagellin), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (unmethylated CpG oligonucleotide) [395].
Of these, only the TLR4 ligand, MPL, has been approved for use in human vaccine formulations such as human papillomavirus vaccine (Cervarix ™), hepatitis vaccine (Fendrix®, GSK Biologicals), and malaria vaccine (RTS, S/AS01 or Mosquirix).

CPG

After the first work in 1995 showing that cytosine-phosphoguanosine (CpG) motifs in bacterial DNA could enhance immune stimulation, extensive studies were conducted on bacterial DNA and its synthetic analogs, the CpG oligodeoxynucleotides (CpG ODNs) [396].

ODN CpGs are synthetic DNA molecules consisting of a phosphorothioate ODN structure containing unmethylated CpG motifs [397].
CpG motifs occur more frequently in bacterial and viral DNA than in vertebrate DNA [398] and depending on their structure and biological functions, CpG-containing sequences can

Adjuvants in EAE. In: Lavi E., Constantinescu C.S. (eds) (2005) Experimental Models of Multiple Sclerosis. Springer, Boston, MA.
https://doi.org/10.1007/0-387-25518-4_5
https://link.springer.com/chapter/10.1007/0-387-25518-4_5

[395] Duthie MS, Windish HP, Fox CB, Reed SG.
Use of defined TLR ligands as adjuvants within human vaccines.
Immunol Rev. 2011;239(1):178-196. doi:10.1111/j.1600-065X.2010.00978.x
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5872835/

[396] Anderson, R. B.. "Incorporation of CpG oligodeoxynucleotides into α2-macroglobulin: development of a novel vaccine adjuvant delivery mechanism." (2007).
https://pdfs.semanticscholar.org/2eaf/2cad7de8b9cd058634a245abcae55ebe3332.pdf?_ga=2.174222948.714159447.1611069892-1565440789.1605823056

[397] Scheiermann J, Klinman DM.
Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer.
Vaccine. 2014;32(48):6377-6389. doi:10.1016/j.vaccine.2014.06.065
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4252359/

[398] Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM.
CpG DNA as a vaccine adjuvant.
Expert Rev Vaccines. 2011;10(4):499-511. doi:10.1586/erv.10.174
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3108434/

Be divided into the different classes A, B, C, P and S [399]. Of these, class CpG-B is the most commonly used in pre-clinical and clinical studies. [400].

ODN type	Representative sequence	Structural characteristics	Immune effects
D- also referred to as A-class	GGTGCATCGATGCAGGGGGG	Mixed phosphodiester/phosphorothioate backbone Single CpG motif CpG flanking region forms a palindrome Poly G tail at 3′ end	Induces strong pDC IFN-α secretion APC maturation
K- also referred to as B-class	TCCATGGACGTTCCTGAGCGTT	Phosphorothioate backbone Multiple CpG motifs 5′ motif most stimulatory	Induces strong B-cell activation pDC maturation Preferentially supports the production of TNF-α and IL-6
C	TCGTCGTTCGAACGACGTTGAT	Phosphorothioate backbone Multiple CpG motifs TCG dimer at 5′ end CpG motif imbedded in a central palindrome	Induces B-cell and pDC proliferation and differentiation Induces production of IL-6 and IFN-α
P	TCGTCGACGATCGGCGCGCGCCG	Phosphorothioate backbone Two palindromes Multiple CpG motifs	Stimulates pDC and B cells Strong IFN-α secretion

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3108434/
Comparison of ODN classes D, K, C and P.
Bold letters in ODN sequences indicate self-complementary palindromes; CpG motifs are underlined. APC: antigen-presenting cell; ODN: oligodeoxynucleotide; pDC: plasmacytoid dendritic cell.

CpG ODNs are strong adjuvants capable of inducing Th1 responses, cytotoxic T lymphocyte (CTL) generation, and IFN-gamma secretion [401].

The signal cascade triggered by the interaction of TLR-9s with CpG ODNs culminates in the activation of genes that mediate an inflammatory response.
This signal pathway proceeds through stimulation of MyD88, IRAK and TRAF-6.
Subsequently, the recruitment of various MAP kinases and transcription factors (including NF-κB, AP1, and IRF-7) upregulates the expression of proinflammatory genes [402].

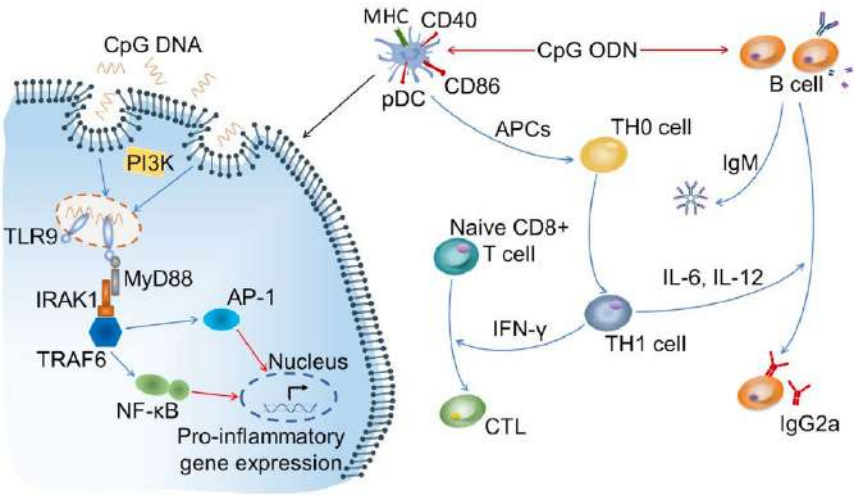
The DNA of CpGs directly activates pDCs and B cells, contributing to the induction of both innate and adaptive immune responses. The cascade of events initiated by CpGs indirectly supports the maturation, differentiation, and proliferation of natural killer cells, T cells, and monocytes/macrophages.

[399] Vollmer J, Krieg AM.
Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists.
Adv Drug Deliv Rev. 2009 Mar 28;61(3):195-204. doi: 10.1016/j.addr.2008.12.008. Epub 2009 Jan 13. PMID: 19211030.
https://pubmed.ncbi.nlm.nih.gov/19211030/

[400] Campbell JD.
Development of the CpG Adjuvant 1018: A Case Study.
Methods Mol Biol. 2017;1494:15-27. doi: 10.1007/978-1-4939-6445-1_2. PMID: 27718183.
https://pubmed.ncbi.nlm.nih.gov/27718183/

[401] Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H.
CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses.
J Immunol. 2000 Aug 1;165(3):1228-35. doi: 10.4049/jimmunol.165.3.1228. PMID: 10903720.
https://www.jimmunol.org/content/165/3/1228.long

[402] Klinman DM.
Immunotherapeutic uses of CpG oligodeoxynucleotides.
Nat Rev Immunol. 2004 Apr;4(4):249-58. doi: 10.1038/nri1329. PMID: 15057783.
https://www.nature.com/articles/nri1329



https://pubmed.ncbi.nlm.nih.gov/31047671/
Mechanisms of action of CpG ODNs. As a new type of adjuvant, synthetic oligodeoxynucleotides (ODNs) containing immunestimulatory CpG motifs are supportive of Th1 cell responses. After initial uptake of CpG ODNs within antigen-presenting cells, PI3K facilitates translocation into TLR9-containing endosomal vesicles. The interaction between TLR9 and CpG ODNs transduces the cytoplasmic activation signal. CpG ODNs directly activate B cells and plasmacytoid dendritic cells, producing an environment rich in proinflammatory and T helper 1 (Th1) cytokines. CpG ODNs could facilitate the maturation of pDCs and enhance antigen processing and presentation. CpG ODNs induce T cells to promote CTL development via IFN-c and increase production of IL-6, IL-12 to support IgG2a antibody secretion

Although vaccines are typically administered prophylactically to reduce host susceptibility to infection, there are situations in which pathogen-specific immunity is needed after exposure (e.g., following the release of pathogens for bioterrorism purposes).

In these cases, vaccines are required to accelerate the induction of immunity, and several studies indicate that ODN CpGs accelerate the development of vaccine-induced responses.

For example, mice vaccinated with AVA (anthrax adosrbite vaccine) adjuvanted with CpG developed an immune response three times faster than those immunized with AVA alone, with significant vaccine antibody production observed within 5 days versus 15 days ($p < 0.05$).
The combination of CpG ODN with AVA accelerated the anti-PA IgG serum response, producing anti-PA serum titers that were 10-fold higher and significantly more effective by day 10 at challenge test ($p < 0,05$). [403]

Regarding the safety profile, preclinical and clinical studies performed suggest the possibility that CpG ODNs may increase host susceptibility to autoimmune diseases or predispose to toxic shock. Immune stimulation induced by CpG motifs may reduce death by apoptosis of stimulated lymphocytes, induce activation of polyclonal B lymphocytes, increase production of auto-antibodies and proinflammatory cytokines [404] and consequently increase the risk of autoimmune diseases. [405]

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[403] Klinman DM, Currie D, Lee G, Grippe V, Merkel T.
Systemic but not mucosal immunity induced by AVA prevents inhalational anthrax.
Microbes Infect. 2007;9(12-13):1478-1483. doi:10.1016/j.micinf.2007.08.002
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2117355/

[404] Gilkeson GS, Ruiz P, Howell D, Lefkowith JB, Pisetsky DS.
Induction of immune-mediated glomerulonephritis in normal mice immunized with bacterial DNA.
Clin Immunol Immunopathol. 1993 Sep;68(3):283-92. doi: 10.1006/clin.1993.1129. PMID: 8370182.
https://pubmed.ncbi.nlm.nih.gov/8370182/

Gilkeson GS, Pippen AM, Pisetsky DS.
Induction of cross-reactive anti-dsDNA antibodies in preautoimmune NZB/NZW mice by immunization with bacterial DNA.
J Clin Invest. 1995 Mar;95(3):1398-402. doi: 10.1172/JCI117793. PMID: 7883986; PMCID: PMC441482.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC441482/pdf/jcinvest00491-0478.pdf

Steinberg AD, Krieg AM, Gourley MF, Klinman DM.
Theoretical and experimental approaches to generalized autoimmunity.
Immunol Rev. 1990 Dec;118:129-63. doi: 10.1111/j.1600-065x.1990.tb00815.x. PMID: 2079325.
https://pubmed.ncbi.nlm.nih.gov/2079325/

[405] Klinman DM.
Polyclonal B cell activation in lupus-prone mice precedes and predicts the development of autoimmune disease.
J Clin Invest. 1990;86(4):1249-1254. doi:10.1172/JCI114831
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC296855/pdf/jcinvest00076-0241.pdf

Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR.
Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis.
J Immunol. 1991 Jul 1;147(1):117-23. PMID: 2051017.
https://pubmed.ncbi.nlm.nih.gov/2051017/

For organ-specific autoimmune diseases, which are typically promoted by a Th1-like response elicited by CpG DNAs, an IL-12-dependent model of experimental allergic encephalomyelitis (mimicking multiple sclerosis) was studied.

Animals treated with CpG DNA and then stimulated with self-antigens developed disease-causing autoreactive Th1 effector cells, while mice injected only with self-antigen remained disease-free [406].

In a molecular mimicry model, CpG DNA administered concomitantly with *Chlamydia-derived* antigen promoted the induction of autoimmune myocarditis [407]. CpG ODNs also increased the susceptibility of mice to interventions that can induce arthritis [408]. These results indicate that CpG motifs may promote the development of deleterious autoimmune reactions under certain circumstances.

Toxic shock can be induced by repeatedly exposing the host to TNF-α-inducing agents, such as lipopolysaccharide and d-galactosamine [409]. In this context, toxic shock has been observed in murine studies involving the administration of CpG ODN in combination with sublethal doses of lipopolysaccharide or animals pre-sensitized with d-galactosamine. [410]

In clinical trials, an increase in the frequency and/or severity of local adverse events and systemic symptoms (including flu-like symptoms) from CpG-adjuvanted vaccines has been detected.

Krieg AM.
CpG DNA: a pathogenic factor in systemic lupus erythematosus?
J Clin Immunol. 1995 Nov;15(6):284-92. doi: 10.1007/BF01541318. PMID: 8576314.
https://pubmed.ncbi.nlm.nih.gov/8576314/

[406] Segal BM, Klinman DM, Shevach EM.
Microbial products induce autoimmune disease by an IL-12-dependent pathway.
J Immunol. 1997 Jun 1;158(11):5087-90. PMID: 9164922.
https://pubmed.ncbi.nlm.nih.gov/9164922/

Segal BM, Chang JT, Shevach EM.
CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo.
J Immunol. 2000 Jun 1;164(11):5683-8. doi: 10.4049/jimmunol.164.11.5683. PMID: 10820244.
https://www.jimmunol.org/content/164/11/5683.long

[407] Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM.
Chlamydia infections and heart disease linked through antigenic mimicry.
Science. 1999 Feb 26;283(5406):1335-9. doi: 10.1126/science.283.5406.1335. PMID: 10037605.
https://pubmed.ncbi.nlm.nih.gov/10037605/

[408] Zeuner RA, Verthelyi D, Gursel M, Ishii KJ, Klinman DM.
Influence of stimulatory and suppressive DNA motifs on host susceptibility to inflammatory arthritis.
Arthritis Rheum. 2003 Jun;48(6):1701-7. doi: 10.1002/art.11035. PMID: 12794839.
https://onlinelibrary.wiley.com/doi/full/10.1002/art.11035

[409] Opal MS.
Endotoxins and other sepsis triggers.
Contrib Nephrol. 2010;167:14-24. doi: 10.1159/000315915. Epub 2010 Jun 1. PMID: 20519895.
https://pubmed.ncbi.nlm.nih.gov/20519895/

[410] Sparwasser T, Miethke T, Lipford G, Borschert K, Häcker H, Heeg K, Wagner H. Bacterial DNA causes septic shock.
Nature. 1997 Mar 27;386(6623):336-7. doi: 10.1038/386336a0. PMID: 9121548.
https://pubmed.ncbi.nlm.nih.gov/9121548/

Cowdery JS, Chace JH, Yi AK, Krieg AM.
Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides.
J Immunol. 1996 Jun 15;156(12):4570-5. PMID: 8648098.
https://pubmed.ncbi.nlm.nih.gov/8648098/

Hartmann G, Krug A, Waller-Fontaine K, Endres S.
Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by heparin.
Mol Med. 1996;2(4):429-438.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2230162/pdf/molmed00040-0039.pdf

Specifically, in a sample of subjects vaccinated with CpG ODN plus PPV-23, 75.6 percent developed adverse events classified as moderate-to-severe. In contrast, flu-like symptoms were less frequent among those who received PPV-23 alone.
It is possible that the increased reactogenicity seen with the adjuvanted vaccine reflects the synergy between CpG ODN and bacterial cell wall PAMPs present in the PPV-23 vaccine.[411]

AS04

Aluminum salts have been used as platforms for the discovery of new adjuvants consisting of various Toll-like receptor (TLR) agonists adsorbed on them.
One of these, known as adjuvant system 04 (AS04), has been used in HPV and HBV vaccines.
Among them, Cervarix, a bivalent human papillomavirus vaccine, is the first AS04-adjuvanted vaccine approved by the FDA in 2009.

AS04 is prepared from 3'-O-monophosphoryl-lipid A (MPL) and an aluminum salt.
MPL is a detoxified lipopolysaccharide (LPS) that has been reported to be a specific agonist of TLR4 [412].
However, the signaling properties of MPL through TLR4 activation are not exactly the same as those of LPS.
The difference could be due to the absence of the 1-phosphate in the MPL molecule. [413].
AS04, compared with an adjuvant containing only aluminum salt, has been shown to induce a long-term effective immune response in HPV vaccines [414].
Studies have found rapid cytokine production and recruitment of various immune cells in muscles and lymph node drainage at the injection site within 3-6 h when adjuvanted with MPL or AS04.

MPL has been shown to be the major component of the vaccine that mediated the early immune response. Although aluminum salts did not synergize with MPL, their presence prolonged the immune response because of the function of the depot effect.

In addition, a recent study indicates that levels of IFN-gamma, a marker of polarized Th1 response, were higher when HPV-16 and HPV-18 VLP antigens were adjuvanted with AS04 compared with aluminum hydroxide alone. These results indicate that AS04 is more efficient in inducing amplification and differentiation of CD4 T lymphocytes^{+} and promoting a polarized Th1 response. [415]

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[411] Sen G, Khan AQ, Chen Q, Snapper CM.
In vivo humoral immune responses to isolated pneumococcal polysaccharides are dependent on the presence of associated TLR ligands.
J Immunol. 2005 Sep 1;175(5):3084-91. doi: 10.4049/jimmunol.175.5.3084. PMID: 16116197.
https://www.jimmunol.org/content/175/5/3084.long

[412] Garçon N, Chomez P, Van Mechelen M.
GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives.
Expert Rev Vaccines. 2007 Oct;6(5):723-39. doi: 10.1586/14760584.6.5.723. PMID: 17931153.
https://pubmed.ncbi.nlm.nih.gov/17931153/

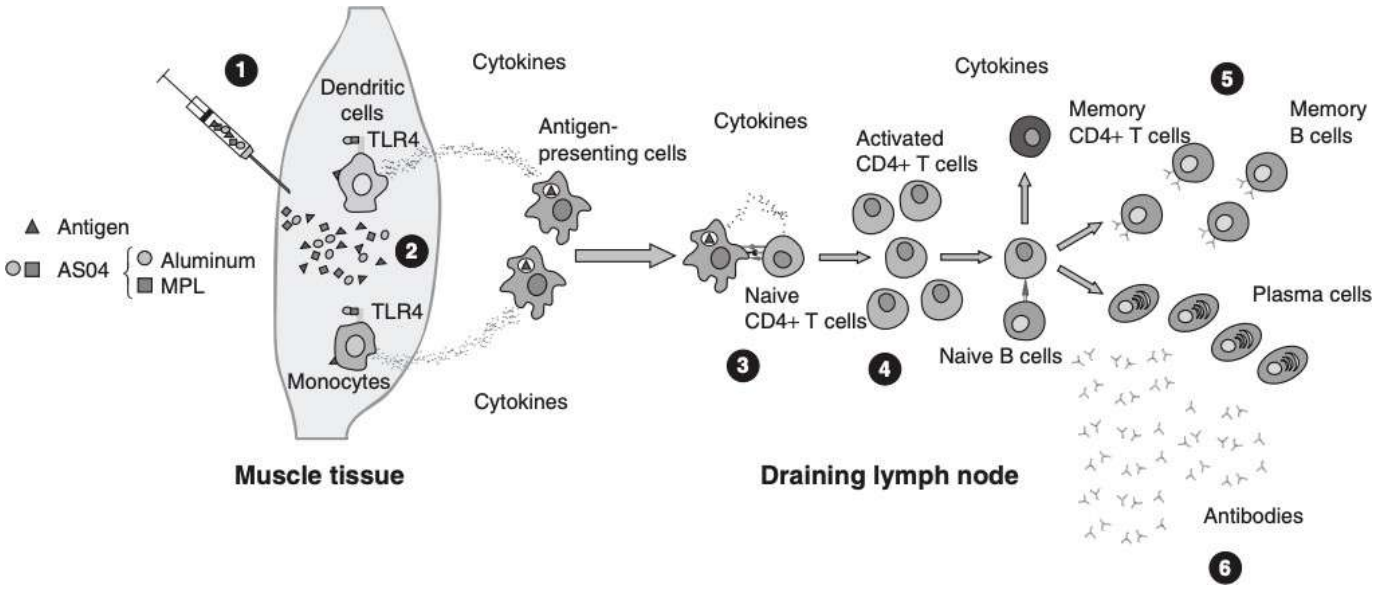
Garçon N, Morel S, Didierlaurent A, Descamps D, Wettendorff M, Van Mechelen M.
Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach.
BioDrugs. 2011 Aug 1;25(4):217-26. doi: 10.2165/11591760-000000000-00000. PMID: 21815697.
https://pubmed.ncbi.nlm.nih.gov/21815697/

[413] Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO.
The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.
Nature. 2009 Apr 30;458(7242):1191-5. doi: 10.1038/nature07830. Epub 2009 Mar 1. PMID: 19252480.
https://pubmed.ncbi.nlm.nih.gov/19252480/

Park BS, Lee JO.
Recognition of lipopolysaccharide pattern by TLR4 complexes.
Exp Mol Med. 2013;45(12):e66. Published 2013 Dec 6. doi:10.1038/emm.2013.97
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3880462/

[414] Giannini SL et al
Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only.
Vaccine. 2006 Aug 14;24(33-34):5937-49. doi: 10.1016/j.vaccine.2006.06.005. Epub 2006 Jun 19.
https://pubmed.ncbi.nlm.nih.gov/16828940/

[415] Del Giudice G, Rappuoli R, Didierlaurent AM.



https://sci-hub.se/10.1016/j.vaccine.2019.04.055
Mechanism of action of AS04 adjuvant. **Step (1)**: the vaccine is injected into the muscle. AS04 contains aluminum salts and monophosphoryl lipid A (MPL), a Toll-like receptor 4 (TLR4) agonist. Cells expressing TLR4 in muscle, such as dendritic cells or resident or recruited monocytes, are activated and induce a local and transient response. This response is mainly driven by MPL. AS04 allows rapid recruitment and activation of monocytes and dendritic cells. **Phase (2)**: AS04 and virus-like particles co-localize. **Phase (3)**: Activated monocytes and dendritic cells, loaded with antigen, migrate to the draining lymph node. AS04 allows for better activation of those antigen-presenting cells, e.g., increased expression of co-stimulatory molecules, which results in a greater ability to present the antigen to CD4+ T cells. **Stage (4)**: Generation of more and/or enhanced CD4+ T cells leads to improved B-cell differentiation. **Phase (5)**: AS04 increases the frequency of antigen-specific memory B cells. Phase **(6):** high levels of antibodies are released into the circulation.

ADVAX

Advax manufactured by Vaxine (Australia) is a microcrystalline polysaccharide particle composed of **delta inulin** [416] and has recently been successfully tested in several human studies, including vaccine studies to prevent seasonal and pandemic influenza, hepatitis B, and hyperallergic reactions to insect venom[417].

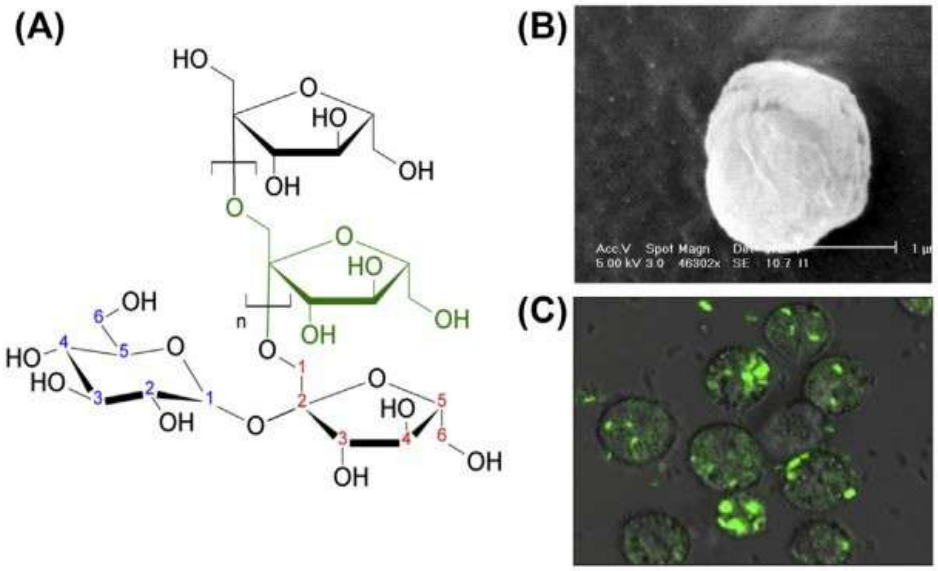
Structure of delta inulin. Schematic diagram of a single polymer chain of inulin composed of multiple fructose units with a terminal glucose (A). A single particle of delta inulin imaged by scanning electron microscopy of freezing fracture (B). Fluorescent particles of delta inulin endocytosed by human monocytes after overnight co-culture (C).

Correlates of adjuvanticity: A review on adjuvants in licensed vaccines.
Semin Immunol. 2018 Oct;39:14-21. doi: 10.1016/j.smim.2018.05.001. Epub 2018 May 23. PMID: 29801750.
https://www.sciencedirect.com/science/article/pii/S1044532318300514?via%3Dihub

Didierlaurent AM et al
AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity.
J Immunol. 2009 Nov 15;183(10):6186-97. doi: 10.4049/jimmunol.0901474. Epub 2009 Oct 28
https://www.jimmunol.org/content/183/10/6186.long

[416] Petrovsky N, Cooper PD.
Advax™, a novel microcrystalline polysaccharide particle engineered from delta inulin, provides robust adjuvant potency together with tolerability and safety.
Vaccine. 2015 Nov 4;33(44):5920-6. doi: 10.1016/j.vaccine.2015.09.030. Epub 2015 Sep 25.
https://pubmed.ncbi.nlm.nih.gov/26407920/

[417] Heddle R, Smith A, Woodman R, Hissaria P, Petrovsky N.
Randomized controlled trial demonstrating the benefits of delta inulin adjuvanted immunotherapy in patients with bee venom allergy.
J Allergy Clin Immunol. 2019;144(2):504-513.e16. doi:10.1016/j.jaci.2019.03.035
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7112352/



https://www.sciencedirect.com/science/article/pii/B9780128040195000104

Compared with controls, adjuvant Advax [418] appears to enhance antibody and T-cell response, and is safe and well tolerated [419].

Advax does not induce the production of inflammatory cytokines, a factor that distinguishes it from other adjuvants. Although its mechanism of action remains the subject of intensive ongoing studies, it is hypothesized to act by modulating the function of antigen-presenting cells in a noninflammatory manner, thereby enhancing t h e co-stimulation and activation of antigen-specific T helper cells, which in turn trigger the expansion of memory B cells and CD8 T cells.[420]

As already seen, coronavirus vaccines present a peculiar safety problem in that immunized individuals when infected with the virus can develop very severe eosinophilic lung disease, a problem that is further compounded by the formulation of SARS-CoV vaccines with alum-based adjuvants.

To overcome this unacceptable adverse reaction, formulations of the SARS-CoV spike protein were tested in inactivated whole virus and subunit vaccines with new polysaccharide adjuvants based on delta

[418] Hayashi M, Aoshi T, Haseda Y, et al.
Advax, a Delta Inulin Microparticle, Potentiates In-built Adjuvant Property of Co-administered Vaccines.
EBioMedicine. 2017;15:127-136. doi:10.1016/j.ebiom.2016.11.015
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5233800/

Petrovsky N, Cooper PD.
Advax™, a novel microcrystalline polysaccharide particle engineered from delta inulin, provides robust adjuvant potency together with tolerability and safety.
Vaccine. 2015 Nov 4;33(44):5920-6. doi: 10.1016/j.vaccine.2015.09.030. Epub 2015 Sep 25.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4639457/

[419] Gordon D, Kelley P, Heinzel S, Cooper P, Petrovsky N.
Immunogenicity and safety of Advax™, a novel polysaccharide adjuvant based on delta inulin, when formulated with hepatitis B surface antigen: a randomized controlled Phase 1 study.
Vaccine. 2014;32(48):6469-6477. doi:10.1016/j.vaccine.2014.09.034
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4253909/

Skwarczynski M.
Inulin: A New Adjuvant With Unknown Mode of Action.
EBioMedicine. 2017;15:8-9. doi:10.1016/j.ebiom.2016.11.019
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5233799/

[420] N. Petroski,
Chapter 10 - Advax Adjuvant: A Potent and Safe Immunopotentiator Composed of Delta Inulin,
Editor(s): Virgil E.J.C. Schijns, Derek T. O'Hagan, Immunopotentiators in Modern Vaccines (Second Edition), Academic Press, 2017, Pages 199-210,
ISBN 9780128040195, https://doi.org/10.1016/B978-0-12-804019-5.00010-4.
http://www.sciencedirect.com/science/article/pii/B9780128040195000104

inulin, and it was found that this adjuvant increases neutralizing antibody titers and protection against clinical disease, but at the same time also protects against the development of pulmonary eosinophilic immunopathology in challenge tests.
They also showed that the immunity achieved with delta inulin adjuvants is long-lasting, thus overcoming the natural tendency for immunity to the rapidly declining coronavirus. [421]

REGULATORY ASPECTS FOR THE AUTHORIZATION OF NEW ADJUVANTS

The regulatory agencies (FDA and EMA) define adjuvants as one of the constituent materials of the vaccine, so unless the adjuvant has a "stand-alone" indication, adjuvants are usually not evaluated and approved on their own but rather as part of a vaccine formulation.

The guidelines state that "*an adjuvant should not be introduced into a product unless there is satisfactory evidence that it does not adversely affect the safety or potency of the product.*"[422]

Although it is not necessary to demonstrate the safety of adjuvant administered alone, the safety of an adjuvanted vaccine formulation must be demonstrated with adequate and well-controlled studies.
WHO published a guideline in 2013 and described the nonclinical, quality, pharmacological, toxicological, and other information needed to support the initiation of clinical trials with a vaccine combined with a new adjuvant [423].

In addition to appropriate safety studies, to support approval, the guideline emphasizes the importance of demonstrating the need for the use of an adjuvant with a defined mechanism of action (MOA) in well-defined human in vitro animal models. [424]

TOXICOLOGY OF ADJUVANTS

Although adjuvants are added to many vaccines for their immunostimulatory effects, they can potentially induce unwanted reactogenicity at the same time, with physical manifestations of the immunomodulatory and/or inflammatory response occurring within 72 hours of vaccination.[425]

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[421] Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N.
Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide enhanced protection while ameliorating lung eosinophilic immunopathology.
J Virol. 2015;89(6):2995-3007. doi:10.1128/JVI.02980-14
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4337527/

McPherson C, Chubet R, Holtz K, Honda-Okubo Y, Barnard D, Cox M, Petrovsky N. Development of a SARS Coronavirus Vaccine from Recombinant Spike Protein Plus Delta Inulin Adjuvant.
Methods Mol Biol. 2016;1403:269-84. doi: 10.1007/978-1-4939-3387-7_14. PMID: 27076136; PMCID: PMC7139448.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7139448/

[422] Guidance for industry for the evaluation of combination vaccines for preventable diseases: production, testing and clinical studies
https://www.fda.gov/media/77191/download

GUIDELINE ON ADJUVANTS IN VACCINES FOR HUMAN USE
https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-adjuvants-vaccines-human-use-see-also-explanatory-note_en.pdf

[423] Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines
https://www.who.int/biologicals/areas/vaccines/TRS_987_Annex2.pdf?ua=1
https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1

[424] Nanishi E, Dowling DJ, Levy O.
Toward precision adjuvants: optimizing science and safety.
Curr Opin Pediatr. 2020;32(1):125-138. doi:10.1097/MOP.0000000000000868
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6970548/

[425] Hervé C, Laupèze B, Del Giudice G, Didierlaurent AM, Tavares Da Silva F.
The how's and what's of vaccine reactogenicity.
NPJ Vaccines. 2019;4:39. Published 2019 Sep 24. doi:10.1038/s41541-019-0132-6
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6760227/

Reactivity can be divided into local and systemic depending on the site of symptoms.
Local reactogenicity includes erythema, swelling, pain, soreness or induration at the injection site, while **systemic reactogenicity**, often referred to as "flu-like" symptoms, includes chills, fever, fatigue, nausea, arthritis, myalgia and headache.

Thus, reactogenicity refers to acute inflammatory reactions after vaccination, while the term "**safety**" refers to all adverse events attributable to vaccination that could potentially be caused, triggered, or worsened after vaccine administration.

In addition to the symptoms described above as reactogenicity, safety includes adverse events such as anaphylactic reactions after administration of an adjuvanted vaccine and the potential risk of the adjuvanted vaccine to induce or worsen autoimmune diseases [426] and other long-term conditions that may not be seen in clinical phases before marketing.

The post-marketing clinical phase is therefore of paramount importance for reporting new adverse reactions, which may occur in the population consisting of individuals who are heterogeneous in terms of genetics, existing diseases, age, gender, ect...
However, passive surveillance, carried out through reporting by the vaccinated person or health care provider to the appropriate authorities of suspected vaccine injury, leads to a greatly underestimated result of the incidence and type of harm that actually occurred in the medium to long term. [427]

From the perspective of the mechanism of induction of reactogenicity, the same inflammatory events with cytokine release, already seen and necessary to trigger strong acquired antigen-specific immune responses, can also lead to the development of signs and symptoms of inflammation at the injection site (pain, redness, and swelling) in the vaccinated individual.

Mediators and products of circulating inflammation can affect other body systems causing systemic side effects (such as fever, fatigue, and headache). It follows that to maintain reactogenicity at clinically acceptable levels, it is necessary to balance the beneficial and harmful effects of these inflammatory events.

Batista-Duharte A, Martínez DT, Carlos IZ.
Efficacy and safety of immunological adjuvants. Where is the cut-off?
Biomed Pharmacother. 2018 Sep;105:616-624. doi: 10.1016/j.biopha.2018.06.026. epub 2018 Jun 9. PMID: 2989496262.
https://pubmed.ncbi.nlm.nih.gov/29894962/.

[426] Guimarães LE, Baker B, Perricone C, Shoenfeld Y.
Vaccines, adjuvants and autoimmunity.
Pharmacol Res. 2015 Oct;100:190-209. doi: 10.1016/j.phrs.2015.08.003. Epub 2015 Aug 12.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7129276/

Ruiz JT, Luján L, Blank M, Shoenfeld Y.
Adjuvants- and vaccines-induced autoimmunity: animal models.
Immunol Res. 2017 Feb;65(1):55-65. doi: 10.1007/s12026-016-8819-5. PMID: 27417999.
https://pubmed.ncbi.nlm.nih.gov/27417999/

Vaccines and Autoimmunity
Editor(s): Yehuda Shoenfeld, Nancy Agmon-Levin, Lucija Tomljenovic
First published:May 15, 2015 Print ISBN:9781118663431 |Online ISBN:9781118663721 |DOI:10.1002/9781118663721 2015 Wiley-Blackwell

[427] AUDITIONS DDL 363 AND DDL 770 ON VACCINAL PREVENTION Sitting of January 17, 2019
http://www.paolobellavite.it/files/190117Senato-Bellavite-Depositata.pdf

Bellavite P.
Causality assessment of adverse events following immunization: the problem of multifactorial pathology.
F1000Res. 2020 Mar 9;9:170. doi: 10.12688/f1000research.22600.2.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7111503/

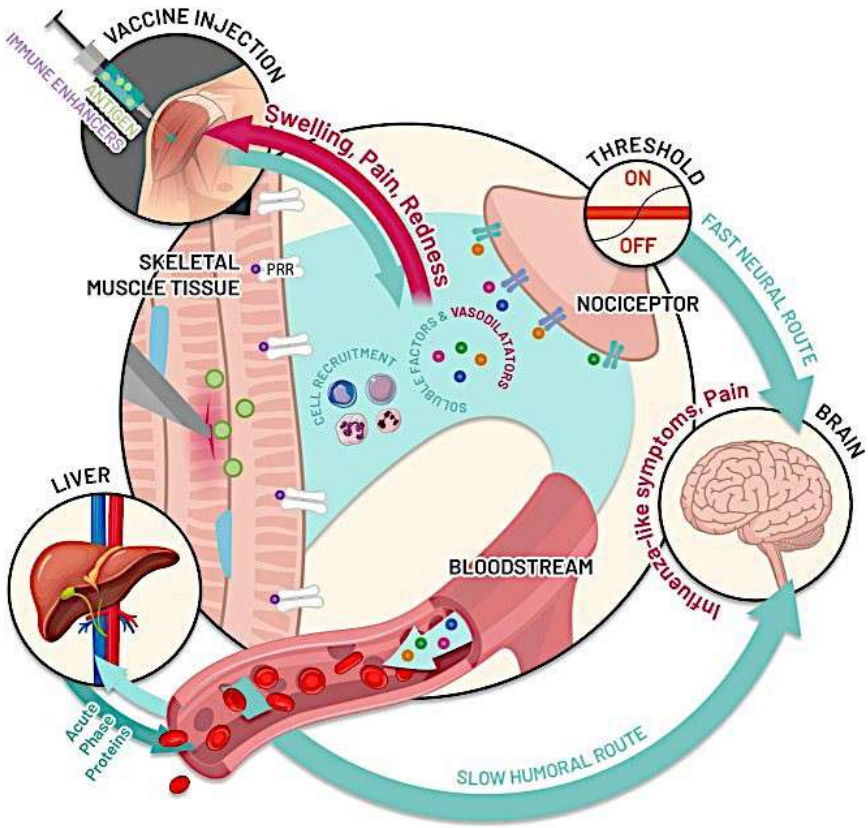
Biological mechanisms of reactogenicity

All vaccines share the ability to activate PRRs that will lead to the production of different mediators.
PRRs are expressed by immune cells, including monocytes, macrophages, mast cells, and resident dendritic and stromal cells, as well as keratinocytes and skeletal muscle cells.

Resident cells, particularly macrophages and mast cells, are key target cells that initiate the response within minutes of vaccination, releasing pro-inflammatory cytokines, chemokines, effectors of the complement cascade (C3a and C5a) and vasodilators, including vasoactive amines and bradykinin.

Vasodilators and chemokine gradient promote cell recruitment from the blood, but also lead to the development of redness and swelling.
Neutrophils, monocytes, and lymphocytes flowing in from the blood adhere to the vessel walls and accumulate at the site of injury by extravasation.

These immune cells can contribute to peripheral nociceptive sensitization by releasing soluble factors, such as cytokines, prostaglandins, or ATP, and interacting directly with nociceptors (sensory neurons that respond to potentially damaging stimuli) to cause pain if the pain threshold is reached. Pain sensation is transmitted through fast-conducting myelinated neurons (fast neural pathway).



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6760227/
Hypothesized link between vaccination-induced innate immune response and reactogenicity. After vaccination, inflammation is triggered by innate immune activation of pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) that recognize and bind antigens (green circle in skeletal muscle) and potential immune enhancers (purple circle in skeletal muscle) present in the vaccine formulation. Resident immune cells, mast cells, monocytes, and macrophages become activated within minutes of injection and release soluble factors (proinflammatory cytokines, chemokines, effectors of the complement cascade) and vasodilators, which allow cell recruitment from the blood but also lead to the development of redness and symptoms of swelling. These newly recruited immune cells, composed mainly of blood-born neutrophils, monocytes, and T lymphocytes, also contribute to the sensation of pain by releasing soluble factors, such as cytokines, prostaglandins, or ATP, which can interact directly with local sensory receptors called nociceptors and cause pain through the fast neural pathway if the threshold is reached. Once produced, cytokines act both locally in an autocrine and paracrine manner and can act systemically on distant organs, leading to the production of C-reactive protein and other acute phase proteins by the liver. Several immune system signaling pathways to the brain can propagate an inflammatory response to the central nervous system after peripheral activation of the innate immune system (slow humoral pathway), leading to the development of febrile and disease behaviors

The following table shows the most relevant mechanisms involved in the immunotoxicity of adjuvants.

Mechanisms	Consequence	Clinical manifestations	Adjuvant examples
Associated with local reactions			
Direct cytolytic	Direct lytic effects on cells in the inoculation site. Damage-associated molecular patterns (DAMPs) release from injured cells.	Local irritation and inflammation	Alum Saponins
Depot effect and slow degradation	Excessive recruitment of immune cells and Th1-biased response	Long lasting local inflammation, granuloma (delayed-type hypersensitivity)	Gels Emulsions
Inflammation-associated oncogenesis	Tumorigenesis	Tumors in the inoculation site	Alum
Associated with local and (or) systemic reactions			
Profuse release of inflammatory cytokine/chemokines	Excessive stimulation and/or suboptimal downregulation of innate immune system	Local inflammation, acute phase response Vascular leak syndrome Aplastic-like bone marrow	Multiple Cytokines pATRex
Disturbs in hepatic cytochrome P450 expression/activity mediated. Changes in drug transporters	Changes in pharmacokinetics (including metabolism) and pharmacodynamics of drugs mediated by cytokines	Toxicity of some drugs administered during or shortly after vaccination	Freund´s adjuvants Alum LPS
Off-target effect	Expression of innate immune receptors by cell types not involved in the immune response	Inflammation in non-immune tissues. Autoimmune/inflammatory symdrome associated to adjuvants?	Alum
Failure in the contraction of adaptive immune response	Homeostatic disturbances in several immune mechanisms	Hypersensibility reactions and autoimmune disorders	Freund´s adjuvants, Alum. Regulatory T cells modulators
Loss of peripheral immunotolerance	Immune response against own tissues	Autoimmune process	Freund´s adjuvants Alum
Excessive Th2-biased response	Excessive stimulation of IgE response and allergy mediators	Immediate-type hypersensitivity reactions	Alum
Formation and deposition of immune complex (IC)	Local or systemic inflammatory reactions mediated by IC	Arthus reactions, vasculitis	Alum

https://pubmed.ncbi.nlm.nih.gov/29894962/

Transient local inflammation [428]

Immediately after the first contact of the adjuvant formulation with the tissue, chemical irritation may occur due to non-physiological pH, osmolarity, or direct cytotoxicity [429]

Adjuvants such as alum, saponins (e.g., Quil A and QS21 saponin fractions); immunostimulatory complexes (ISCOM); Iscomatrix) and some emulsions, produce direct cytolysis at the site of inoculation associated with immediate local pain congestion and focal inflammation. [430]

Results of a recent study showed that the direct irritant effect of cytotoxic adjuvants detected in vitro is directly associated with severe local reactions at the site of inoculation in vivo. [431]

[428] Batista-Duharte A, Martínez DT, Carlos IZ.
Efficacy and safety of immunological adjuvants.
Where is the cut-off? Biomed Pharmacother. 2018 Sep;105:616-624. doi: 10.1016/j.biopha.2018.06.026. Epub 2018 Jun 9.
https://pubmed.ncbi.nlm.nih.gov/29894962/

[429] Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK.
Adjuvants--a balance between toxicity and adjuvanticity.
Vaccine. 1993;11(3):293-306. doi: 10.1016/0264-410x(93)90190-9. PMID: 8447157.
https://pubmed.ncbi.nlm.nih.gov/8447157/

Goto N, Kato H, Maeyama J, Shibano M, Saito T, Yamaguchi J, Yoshihara S.
Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties.
Vaccine. 1997 Aug-Sep;15(12-13):1364-71. doi: 10.1016/s0264-410x(97)00054-6. PMID: 9302746.
https://pubmed.ncbi.nlm.nih.gov/9302746/

[430] Waite DC, Jacobson EW, Ennis FA, Edelman R, White B, Kammer R, Anderson C, Kensil CR.
Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21.
Vaccine. 2001 Jul 16;19(28-29):3957-67. doi: 10.1016/s0264-410x(01)00142-6. PMID: 11427271.
https://pubmed.ncbi.nlm.nih.gov/11427271/

[431] Waite DC, Jacobson EW, Ennis FA, Edelman R, White B, Kammer R, Anderson C, Kensil CR.

For this reason, cytotoxic adjuvants are particularly contraindicated for mucosal vaccination. The cytotoxicity of emulsions containing mineral salts is due to the presence of short-chain hydrocarbons with a detergent effect, which dissolve the lipid bilayer of the cell membrane.
Mineral oils are a mixture of hydrocarbons with carbon chains of different lengths. Short chains induce local reactions, while longer chains (> C14) are safer but less efficient as adjuvants.

Emulsifiers used in water/oil emulsions, such as mannide monooleate, can produce cytotoxic effects through enzymatic breakdown of native lipid chains that release toxic fatty acids [432].
Other known cytotoxic adjuvants are the aforementioned saponins that interact with cell membranes leading to cell lysis.[433]
The surface activity responsible for the foaming properties, as well as some other biological functions, including the hemolytic activity of saponins, are attributed to their amphiphilic nature , which results from the presence of a hydrophilic sugar moiety and a hydrophobic genin (called sapogenin).
This structure facilitates complexation with cholesterol in the cell membrane, which leads to pore formation and cell permeabilization. [434]

Adjuvants that cause cytotoxicity can activate the innate immune response through molecules released from damaged cells (DAMPs). Some of the best-known DAMPs include high mobility group 1 (HMGb1) with chromatin-associated proteins, heat shock proteins (HSPs), and purine metabolites such as ATP and uric acid.
In addition, there are also extracellular localized DAMPs generated after matrix proteolysis by enzymes released from dying cells that include matrix fragments, such as hyaluronan, heparan sulfate, and biglican. [435]

Adjuvants that cause DAMP release are known as **DAMP-type adjuvants**. [436] They act on monocytes, macrophages or granulocytes to induce cytokines that generate a local immunostimulatory environment, eventually leading to dendritic cell activation.
Specifically, alum produces a direct cytotoxic effect, and DNA, uric acid, and other intracellular molecules released from dying cells mediate the adjuvant action of alum through the pro-inflammatory receptor family, i.e., the pryin-3 domain-containing inflammasome pathway (NLRP3). [437]

Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21.
Vaccine. 2001 Jul 16;19(28-29):3957-67. doi: 10.1016/s0264-410x(01)00142-6. PMID: 11427271.
https://pubmed.ncbi.nlm.nih.gov/11427271/

[432] Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK.
Adjuvants--a balance between toxicity and adjuvanticity.
Vaccine. 1993;11(3):293-306. doi: 10.1016/0264-410x(93)90190-9. PMID: 8447157.
https://pubmed.ncbi.nlm.nih.gov/8447157/

[433] Sun HX, Xie Y, Ye YP.
Advances in saponin-based adjuvants.
Vaccine. 2009 Mar 13;27(12):1787-96. doi: 10.1016/j.vaccine.2009.01.091. Epub 2009 Feb 7. PMID: 19208455.
https://pubmed.ncbi.nlm.nih.gov/19208455/

[434] Podolak I, Galanty A, Sobolewska D.
Saponins as cytotoxic agents: a review.
Phytochem Rev. 2010;9(3):425-474. doi:10.1007/s11101-010-9183-z
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2928447/

[435] Chen GY, Nuñez G.
Sterile inflammation: sensing and reacting to damage.
Nat Rev Immunol. 2010;10(12):826-837. doi:10.1038/nri2873
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3114424/

[436] Powell BS, Andrianov AK, Fusco PC.
Polyionic vaccine adjuvants: another look at aluminum salts and polyelectrolytes.
Clin Exp Vaccine Res. 2015;4(1):23-45. doi:10.7774/cevr.2015.4.1.23
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4313107/

[437] He P, Zou Y, Hu Z.
Advances in aluminum hydroxide-based adjuvant research and its mechanism.

It is worth noting that SARS-CoV-2 could directly activate the NLRP3 inflammasome resulting in endogenous adjuvant activity capable of inducing an adaptive immune response against the virus, and patients with a reduced immune form may demonstrate dysregulated inflammasome activity resulting in a severe form of COVID-19 with tissue damage and a cytokine storm. [438]

Another important group of adjuvants, called **PAMP-type adjuvants**, contain pathogen-associated molecular patterns in their composition and can directly interact with pattern recognition receptors (PRRs) in dendritic cells for their activation. [439]
Due to the activation of dendritic cells, pro-inflammatory cytokines are released, including interleukin-1 (IL-1), tumor necrosis factor (TNF)-α, IL-6, and C-X-C motif chemokines that recruit neutrophils, such as CXCL1, CXCL2 , CXCL5, and CXCL8, which promote neutrophil egress from the vascular system and migration into the tissue. [440]
If the initial neutrophil response is insufficient to clear the inoculum, a second cascade of chemotactic signals is stimulated to recruit additional inflammatory cells by releasing C- C motif chemokines such as CCL3, CCL4, CCL8, and CCL20.
In this way, neutrophils, monocytes, and macrophages cooperate to remove foreign entities [441] . Simultaneously, structural and functional changes are induced on local draining lymphatic vessels to allow trafficking of dendritic cells carrying antigens to regional lymph nodes. [442]

These early events occur during the first 24-72 hours after inoculation and are accompanied by a transient local inflammatory reaction characterized by redness, mild pain, and swelling.
It is the most frequent adverse event after vaccination. [443] Once the inflammatory stimulus has been eliminated, the ongoing inflammatory response must be resolved to prevent excessive tissue damage.

Uptake of apoptotic neutrophils by macrophages (efferocytosis) promotes anti-inflammatory signaling characterized by elevated production of IL-10, transforming growth factor (TGF)
-β and by low production of IL-12p40.

Hum Vaccin Immunother. 2015;11(2):477-488. doi:10.1080/21645515.2014.1004026
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4514166/

[438] van den Berg DF, Te Velde AA.
Severe COVID-19: NLRP3 Inflammasome Dysregulated.
Front Immunol. 2020 Jun 26;11:1580. doi: 10.3389/fimmu.2020.01580.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7332883/

[439] Olafsdottir T, Lindqvist M, Harandi AM.
Molecular signatures of vaccine adjuvants.
Vaccine. 2015 Sep 29;33(40):5302-7. doi: 10.1016/j.vaccine.2015.04.099. Epub 2015 May 16.
https://www.sciencedirect.com/science/article/pii/S0264410X15005964?via%3Dihub

[440] Turner MD, Nedjai B, Hurst T, Pennington DJ.
Cytokines and chemokines: At the crossroads of cell signaling and inflammatory disease.
Biochim Biophys Acta. 2014 Nov;1843(11):2563-2582. doi: 10.1016/j.bbamcr.2014.05.014. Epub 2014 Jun 2.
https://www.sciencedirect.com/science/article/pii/S0167488914001967?via%3Dihub

[441] Soehnlein O, Lindbom L.
Phagocyte partnership during the onset and resolution of inflammation.
Nat Rev Immunol. 2010 Jun;10(6):427-39. doi: 10.1038/nri2779.
https://www.nature.com/articles/nri2779

[442] Swartz MA, Hubbell JA, Reddy ST.
Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design.
Semin Immunol. 2008 Apr;20(2):147-56. doi: 10.1016/j.smim.2007.11.007. Epub 2008 Jan 16.
https://core.ac.uk/reader/147939644?utm_source=linkout

Neeland MR, Elhay MJ, Powell DR, Rossello FJ, Meeusen ENT, de Veer MJ.
Transcriptional profile in afferent lymph cells following vaccination with liposomes incorporating CpG.
Immunology. 2015 Mar;144(3):518-529. doi: 10.1111/imm.12401.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4557688/

[443] Batista-Duharte A, Portuondo D, Carlos IZ, Pérez O.
An approach to local immunotoxicity induced by adjuvanted vaccines.
Int Immunopharmacol. 2013 Nov;17(3):526-36. doi: 10.1016/j.intimp.2013.07.025. Epub 2013 Aug 20.
https://pubmed.ncbi.nlm.nih.gov/23968848/

These macrophages suppress the local inflammatory response by decreasing the production of proinflammatory cytokines and reactive oxygen species (ROS) and leading to resolution ll inflammation with tissue restoration. [444]

Infrequent acute reactions at the site of inoculation Arthus reaction:
is a type III local hypersensitivity reaction, resulting in the deposition of antigen/antibody immune complexes (IC) in the walls of blood vessels, causing vasculitis with severe local inflammatory reaction.
This reaction may begin 2-8 hours after antigen injection and occur in the presence of elevated levels of preformed antibodies in a previously vaccinated person.

IC deposition triggers Fc-gamma receptor-dependent inflammation, in which macrophages recognize IC and release migration inhibitory factor (MIF) that damages surrounding tissue.[445] Repeated administration of the same adjuvant in different vaccines could induce high levels of antibodies directed toward the adjuvant itself, leading to the possibility of inducing an Arthus reaction. [446]
However, with the exception of squalene, most adjuvants do not stimulate antibody responses against themselves [447].
Arthus reaction has been reported after repeated administration of vaccines such as recombinant hepatitis B and diphtheria/tetanus anatoxins.[448]

Type 1 local hypersensitivity reactions:
Aluminum-based adjuvants stimulate a Th2 profile and thus can potentially induce an IgE-mediated response, particularly in genetically predisposed individuals . [449]

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[444] Greenlee-Wacker MC.
Clearance of apoptotic neutrophils and resolution of inflammation. I
mmunol Rev. 2016 Sep;273(1):357-70. doi: 10.1111/imr.12453.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5000862/

Elliott MR, Koster KM, Murphy PS.
Efferocytosis Signaling in the Regulation of Macrophage Inflammatory Responses.
J Immunol. 2017;198(4):1387-1394. doi:10.4049/jimmunol.1601520
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5301545/

[445] Paiva CN, Arras RH, Magalhães ES, Alves LS, Lessa LP, Silva MH, Ejzemberg R, Canetti C, Bozza MT.
Migration inhibitory factor (MIF) released by macrophages upon recognition of immune complexes is critical to inflammation in Arthus reaction. J Leukoc Biol. 2009 May;85(5):855-61. doi: 10.1189/jlb.0108009. Epub 2009 Feb 2.
https://pubmed.ncbi.nlm.nih.gov/19188484/

[446] Peng B, Wei M, Zhu FC, Li JX.
The vaccines-associated Arthus reaction.
Hum Vaccin Immunother. 2019;15(11):2769-2777. doi:10.1080/21645515.2019.1602435
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6930064/

[447] Asa PB, Wilson RB, Garry RF.
Antibodies to squalene in recipients of anthrax vaccine.
Exp Mol Pathol. 2002 Aug;73(1):19-27. doi: 10.1006/exmp.2002.2429.
https://pubmed.ncbi.nlm.nih.gov/12127050/

[448] Froehlich H, Verma R.
Arthus reaction to recombinant hepatitis B virus vaccine.
Clin Infect Dis. 2001 Sep 15;33(6):906-8. doi: 10.1086/322585. Epub 2001 Aug 21.
https://pubmed.ncbi.nlm.nih.gov/11512098/

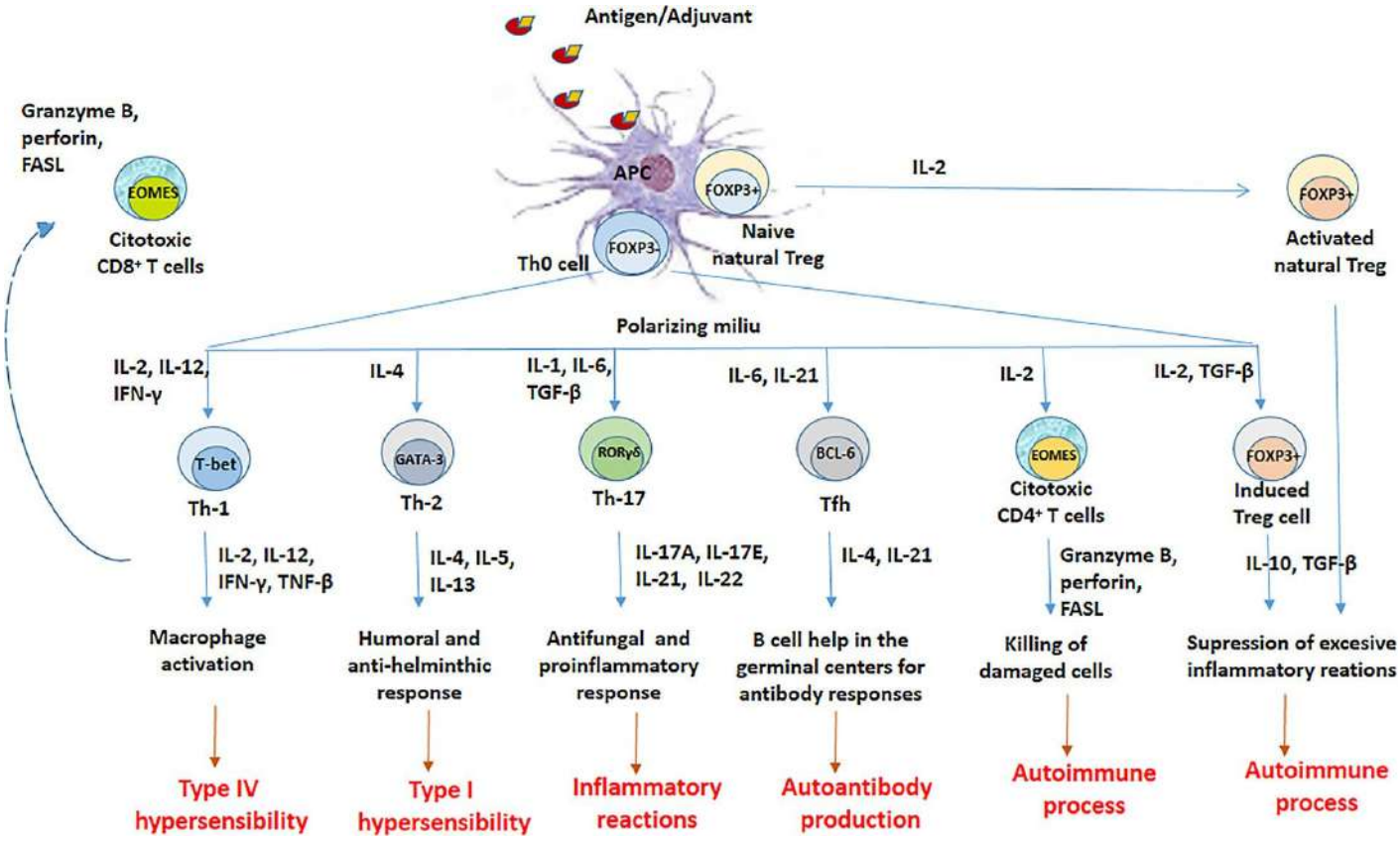
Ponvert C.
Les réactions d'hypersensibilité allergique et non allergique aux vaccins contenant des anatoxines [Allergic and non-allergic hypersensitivity reactions toxoid-containing vaccines].
Arch Pediatr. 2009 Apr;16(4):391-5. French. doi: 10.1016/j.arcped.2009.01.002. Epub 2009 Feb 27.
https://pubmed.ncbi.nlm.nih.gov/19250809/

[449] Terhune TD, Deth RC.
How aluminum adjuvants could promote and enhance non-target IgE synthesis in a genetically-vulnerable sub-population. J Immunotoxicol. 2013 Apr-Jun;10(2):210-22. doi: 10.3109/1547691X.2012.708366. Epub 2012 Sep 11.
https://pubmed.ncbi.nlm.nih.gov/22967010/

Urticarial recall (RU), also known as fixed drug recall reaction, is a localized response that occurs at the site of previous antigen injection after reexposure to that antigen at a remote site and is manifested by immediate swelling, urticaria and intense itching. [450]
This reaction has been observed in association with peptide-based vaccines that include alum or QS-21 saponin. [451]

Circulating excess unbound antigen and subsequent cross-linking with antigen-specific IgE, which binds to skin mast cells previously deposited at old immunization sites with subsequent histamine release, is a possible mechanism involved in RU.[452]

Nicolau syndrome (NiS):
NiS is a rare local reaction that can occur after intramuscular injection of vaccine by accidental intravascular or perivascular inoculation, causing vasospasm secondary to needle puncture, embolization of the injected material, or pressure of the injected material around the vessel.
NiS is characterized by a sudden onset of painful swelling, followed by liveoid erythema, circumscribed hemorrhagic spots, and finally tissue necrosis[35] .[453]



[450] Karaayvaz M, Ozangüç N.
Recall urticaria: a case report.
J Allergy Clin Immunol. 1996 Jun;97(6):1419-20. doi: 10.1016/s0091-6749(96)70215-4.
https://pubmed.ncbi.nlm.nih.gov/8648043/

[451] Rinn K, Schiffman K, Otero HO, Disis ML.
Antigen-specific recall urticaria to a peptide-based vaccine.
J Allergy Clin Immunol. 1999 Jul;104(1):240-2. doi: 10.1016/s0091-6749(99)70143-0.
https://pubmed.ncbi.nlm.nih.gov/10400869/

[452] Batista-Duharte A, Portuondo D, Carlos IZ, Pérez O.
An approach to local immunotoxicity induced by adjuvanted vaccines.
Int Immunopharmacol. 2013 Nov;17(3):526-36. doi: 10.1016/j.intimp.2013.07.025. Epub 2013 Aug 20.
https://pubmed.ncbi.nlm.nih.gov/23968848/

[453] Kienast AK, Mentze D, Hoeger PH.
Nicolau's syndrome induced by intramuscular vaccinations in children: report of seven patients and review of the literature.
Clin Exp Dermatol. 2008 Aug;33(5):555-8. doi: 10.1111/j.1365-2230.2008.02861.x. Epub 2008 Jul 9
https://pubmed.ncbi.nlm.nih.gov/18627396/

https://pubmed.ncbi.nlm.nih.gov/29894962/
Polarization of CD4+ T cells into functionally distinct cell lines after stimulation with antigen/adjuvant and associated immunotoxicity reactions. After antigen/adjuvant interaction with antigen-presenting cells (APCs) and presentation to naive T lymphocytes, natural Treg cells are activated while Th0 cells can be polarized into different cells of the Th subset. Polarization of CD4 T cells
+ is determined by the nature of the antigen and adjuvant, mode of administration, and genetic background. Immune polarization optimizes the immune response, but under unregulated conditions, several immunotoxic responses can be induced (highlighted in red)

Acute systemic reactions

Acute phase response (APR)

Acute phase response (APR) is a transient syndrome that summarizes several endocrine, metabolic, and neurological changes as a consequence of an inflammatory response.
APR is initiated when pro-inflammatory cytokines are produced in sufficient levels to reach the bloodstream, causing systemic effects especially on the hypothalamic-pituitary-adrenal axis, liver, and hemolymphatic system.[454]

Flu-like symptoms are observed d u r i n g APR and usually appear within a few hours of vaccination and generally disappear without complications.
Flu-like symptoms typically consist of moderate fever of 38-39°C, in some cases above 40°C, chills, fatigue, myalgia, headache and nausea.[455]
The cytokines IL-1β, IL-6, IL-8, TNF-α, interferon (IFN)-β, IFN-γ, prostaglandin E2 (PGE2) and various chemokines, act as pyrogens and cause other reactions at a distance. [456]

These mediators, which spill into the systemic circulation, can access the brain through saturable transport systems, and enter circumventricular organs through fenestrated capillaries, where they induce the production of prostaglandins, such as PGE2, a centrally controlled mediator of fever.[457]

Genetic susceptibility can influence the extent of flu-like symptoms.
Stanley et al. identified eight haplotypes in the IL1A, IL1B, IL1R1 and IL18 genes that were associated with an increased or decreased risk of developing fever after smallpox vaccine inoculation [458], and recently, a significant association was discovered between single nucleotide polymorphisms/haplotypes in the IL18R1 and IL18 genes and IFN-γ cytokine release in the adaptive immune response induced by smallpox vaccine.[459]

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[454] Batista-Duharte A, Portuondo D, Pérez O, Carlos IZ.
Systemic immunotoxicity reactions induced by adjuvanted vaccines.
Int Immunopharmacol. 2014 May;20(1):170-80. doi: 10.1016/j.intimp.2014.02.033. Epub 2014 Mar 6.
https://pubmed.ncbi.nlm.nih.gov/24607449/

[455] Christian LM, Porter K, Karlsson E, Schultz-Cherry S.
Proinflammatory cytokine responses correspond with subjective side effects after influenza virus vaccination.
Vaccine. 2015 Jun 26;33(29):3360-6. doi: 10.1016/j.vaccine.2015.05.008. Epub 2015 May 28.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4467994/

[456] Talaat KR, Halsey NA, Cox AB, Coles CL, Durbin AP, Ramakrishnan A, Bream JH.
Rapid changes in serum cytokines and chemokines in response to inactivated influenza vaccination.
Influenza Other Respiratory Viruses. 2018 Mar;12(2):202-210. doi: 10.1111/irv.12509. Epub 2018 Jan 4. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5820426/

[457] Conti B, Tabarean I, Andrei C, Bartfai T.
Cytokines and fever.
Front Biosci. 2004 May 1;9:1433-49. doi: 10.2741/1341.
https://pubmed.ncbi.nlm.nih.gov/14977558/

[458] Stanley SL Jr, Frey SE, Taillon-Miller P, Guo J, Miller RD, Koboldt DC, Elashoff M, Christensen R, Saccone NL, Belshe RB.
The immunogenetics of smallpox vaccination.
J Infect Dis. 2007 Jul 15;196(2):212-9. doi: 10.1086/518794. Epub 2007 Jun 4
https://pubmed.ncbi.nlm.nih.gov/17570108/

[459] Ovsyannikova IG, Haralambieva IH, Kennedy RB, O'Byrne MM, Pankratz VS, Poland GA.
Genetic variation in IL18R1 and IL18 genes and Inteferon γ ELISPOT response to smallpox vaccination: an unexpected relationship.
J Infect Dis. 2013 Nov 1;208(9):1422-30. doi: 10.1093/infdis/jit341. Epub 2013 Jul 30.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3789570/

APR is manifested by the production of acute phase proteins and altered drug metabolism in the liver. [460] Hepatocytes respond to proinflammatory cytokines, mainly through altered gene transcription, to increase the production of acute phase proteins.
Many of these proteins such as C-reactive protein, fibrinogen, serum amyloid protein A, and albumin increase in serum after immunization and can be used as biomarkers of post-vaccinal APR. [461]

Another effect of APR is the inhibition of hepatic cytochrome p450 (CYP450) isoenzymes due to immunostimulation, which is involved in the disturbances of metabolism and elimination of concomitantly administered drugs with increased toxicity. [462]
Cytokines such as IL1, IL-2, IL-6, TNF, TGF-β, and IFN are involved in modulating the expression of different CYP450 isoforms. [463]

Prandota reported an underregulation of CYP450 isoforms through a direct reduction in mRNA levels, protein content, and catalytic activity in rats treated with Freund's complete adjuvant and suggested that polymorphisms of drug-metabolizing enzymes and cytokines may influence drug-induced hepatotoxicity and drug pharmacokinetics in genetically susceptible subjects. [464]
Interestingly, there are drugs such as acetaminophen whose toxicity in overdose depends on the integrity of hepatic CYP450, and inflammation can increase its toxicity.[465]

Another mechanism that may be involved in drug toxicity after vaccination is inflammation-mediated reduction in drug transporter expression/activity. IL-1β, TNF-α, and IL-6 that

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[460] Gribble EJ, Sivakumar PV, Ponce RA, Hughes SD.
Toxicity as a result of immunostimulation by biologics.
Expert Opin Drug Metab Toxicol. 2007 Apr;3(2):209-34. doi: 10.1517/17425255.3.2.209.
https://pubmed.ncbi.nlm.nih.gov/17428152/

[461] Green MD.
Acute Phase Responses to Novel, Investigational Vaccines in Toxicology Studies: The Relationship Between C-Reactive Protein and Other Acute Phase Proteins.
Int J Toxicol. 2015 Sep-Oct;34(5):379-83. doi: 10.1177/1091581815598750. Epub 2015 Aug 12.
https://journals.sagepub.com/doi/pdf/10.1177/1091581815598750

[462] Levine M, Jones MW, Gribble M.
Increased serum phenytoin concentration following influenza vaccination.
Clin Pharm. 1984 Sep-Oct;3(5):505-9.
https://pubmed.ncbi.nlm.nih.gov/6488730/

Renton KW, Gray JD, Hall RI.
Decreased elimination of theophylline after influenza vaccination.
Can Med Assoc J. 1980 Aug 23;123(4):288-90. PMID: 7260771
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1704744/pdf/canmedaj01464-0042.pdf

Pellegrino P, Carnovale C, Perrone V, Salvati D, Gentili M, Brusadelli T, Pozzi M, Antoniazzi S, Clementi E, Radice S.
On the possible interaction between vaccines and drugs.
Eur J Clin Pharmacol. 2014 Mar;70(3):369-71. doi: 10.1007/s00228-013-1616-3. Epub 2013 Dec 5.
https://pubmed.ncbi.nlm.nih.gov/24306497/

[463] Renton KW.
Alteration of drug biotransformation and elimination during infection and inflammation.
harmacol Ther. 2001 Nov-Dec;92(2-3):147-63. doi: 10.1016/s0163-7258(01)00165-6.
https://pubmed.ncbi.nlm.nih.gov/11916535/

[464] Prandota J.
Important role of proinflammatory cytokines/other endogenous substances in drug-induced hepatotoxicity: depression of drug metabolism during infections/inflammation states, and genetic polymorphisms of drug-metabolizing enzymes/cytokines may markedly contribute to this pathology. Am J Ther. 2005 May-Jun;12(3):254-61.
https://pubmed.ncbi.nlm.nih.gov/15891270/

[465] Jaeschke H, Ramachandran A.
Mechanisms and pathophysiological significance of sterile inflammation during acetaminophen hepatotoxicity.
F ood Chem Toxicol. 2020 Apr;138:111240. doi: 10.1016/j.fct.2020.111240. Epub 2020 Mar 4.
https://pubmed.ncbi.nlm.nih.gov/32145352/

are released during an acute inflammatory process, greatly alter the expression profile of hepatic transporters in rodents and humans.[466]

Infrequent acute systemic reactions

Vascular leakage syndrome (VLS):
is a major dose-limiting toxicity of cytokine therapy, including IL-2, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-15, and other cytokines, used in cancer immunotherapy. [467]

VLS is a serious adverse reaction to the use of cytokines as adjuvants in vaccines. This reaction is characterized by increased vascular permeability resulting in tissue edema, weight gain, oliguria, hypotension, dyspnea, and multiorgan failure. [468]
Several mechanisms have been proposed for VLS that include activation or damage of endothelial cells and leukocytes, release of cytokines and mediators of inflammation (e.g, IL-1, TNF-α, components of the complement cascade), cytotoxicity of lymphocyte-activated killer cells on vascular endothelial cells, release of perforins, and alterations in cell-cell interactions, cell-matrix adhesion, and cytoskeleton function resulting in disruption of vascular integrity.[469]

In another study, therapeutic vaccination inducing antibodies against P277 (a 24 aa fragment of the HSP60 molecule with effective action on insulin-dependent diabetes mellitus) was reported to mediate endothelial cell damage and induce VLS.[470]

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[466] Fardel O, Le Vée M.
Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines.
Expert Opin Drug Metab Toxicol. 2009 Dec;5(12):1469-81. doi: 10.1517/17425250903304056.
https://pubmed.ncbi.nlm.nih.gov/19785515/

Cressman AM, Petrovic V, Piquette-Miller M.
Inflammation-mediated changes in drug transporter expression/activity: implications for therapeutic drug response
Expert Rev Clin Pharmacol. 2012 Jan;5(1):69-89. doi: 10.1586/ecp.11.66.
https://pubmed.ncbi.nlm.nih.gov/22142160/

[467] R.G. Baluna,
Cytokine-induced vascular leak syndrome, in: R.V. House, J. Descotes (Eds.),Cytokines in human health: immunotoxicology, pathology, and therapeutic applications, Humana Press, 2007, pp. Totowa, NJ, 2007, pp. 205-231. Vaccine 30 (26) (2012) 3885-3890.
https://www.springer.com/gp/book/9781588294678

[468] Batista-Duharte A, Portuondo D, Pérez O, Carlos IZ.
Systemic immunotoxicity reactions induced by adjuvanted vaccines.
Int Immunopharmacol. 2014 May;20(1):170-80. doi: 10.1016/j.intimp.2014.02.033. Epub 2014 Mar 6.
https://pubmed.ncbi.nlm.nih.gov/24607449/

Damle NK, Doyle LV.
IL-2-activated human killer lymphocytes but not their secreted products mediate increase in albumin flux across cultured endothelial monolayers. Implications for vascular leak syndrome.
J Immunol. 1989 Apr 15;142(8):2660-9.
https://pubmed.ncbi.nlm.nih.gov/2522965/

[469] Li J, Zhang JK.
LHRH-PE40-Induced Vascular Leak Syndrome.
Toxicol Mech Methods. 2006;16(8):473-6. doi: 10.1080/15376520600735196
https://pubmed.ncbi.nlm.nih.gov/20021022/

Baluna R, Vitetta ES.
Vascular leak syndrome: a side effect of immunotherapy.
Immunopharmacology. 1997 Oct;37(2-3):117-32. doi: 10.1016/s0162-3109(97)00041-6.
https://pubmed.ncbi.nlm.nih.gov/9403331/

[470] Ma YJ, Lu Y, Hou J, Dong YK, Du MZ, Xing Y, Ge CY, Xu ML, Jin L, Cao RY, Li TM, Wu J, Liu JJ.
Vaccination of non-obese diabetic mice with a fragment of peptide P277 attenuates insulin-dependent diabetes mellitus.
Int Immunopharmacol. 2011 Sep;11(9):1298-302. doi: 10.1016/j.intimp.2011.04.012. Epub 2011 Apr 28.
https://pubmed.ncbi.nlm.nih.gov/21530685/

Delayed post-vaccinal reactions

Delayed post-vaccinal reactions are those that last longer than 72 h. Some of them may appear several weeks, months or even years after vaccination and may be observed at the site of inoculation or systemically.

Delayed reactions at the site of inoculation
When vaccine inocula are not rapidly removed in the first 72 h, chronic local inflammation may occur due to a delayed-type hypersensitivity (DTH) response, especially in an already sensitized individual.

Several properties of the adjuvant can promote a depot effect, such as poor biodegradability, high viscosity and particle size. Adjuvants, such as aluminum salts, oil emulsions, liposomes, biodegradable polymer microspheres, and attenuated carriers, all induce long-term antigen persistence at the site of administration.[471]

The development of a typical DTH reaction involves four steps.[472]

1) *Initiation*: after the initial inflammatory reaction, macrophages are unable to eliminate the inoculum, manifesting incomplete phagocytosis and macrophage fusion (giant cells).
2) *Accumulation*: CD4 T cells$^+$ are recruited to activate macrophages, B cells and eosinophils.
3) *Effector phase*: Th1 cells secrete interferon-γ (IFN-γ) and TNF-β to activate microbicidal mechanisms such as reactive oxygen species and nitric oxide in macrophages and to enhance recruitment of effector cells such as natural killer and CD8 T cells$^+$.
   Histologic changes include a localized area of tissue necrosis containing foreign material believed to be adjuvant or vaccine components.
   The central zone of foreign and necrotic material is bordered by macrophages and multi- nucleated giant cells, with a peripheral zone of lymphocytes and varying numbers of plasma cells and eosinophils. This lesion is often referred to as a foreign body granuloma,
4) *Resolution*: when the previously mentioned mechanisms fail to eliminate the inoculum, a process to prevent the expansion of tissue damage begins, and the granuloma is surrounded by fibrosis. Cytokines such as TGF-β and IL-13, have been implicated in granulomatous fibrosis, which is produced by a resident T-cell population of the granuloma.
   During the resolution phase of the infection, tissue remodeling occurs, directed by the innate immune response [473]

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[471] Petrovsky N.
Comparative Safety of Vaccine Adjuvants: A Summary of Current Evidence and Future Needs.
Drug Saf. 2015 Nov;38(11):1059-74. doi: 10.1007/s40264-015-0350-4.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4615573/

Batista-Duharte A, Portuondo D, Carlos IZ, Pérez O.
An approach to local immunotoxicity induced by adjuvanted vaccines.
Int Immunopharmacol. 2013 Nov;17(3):526-36. doi: 10.1016/j.intimp.2013.07.025. epub 2013 Aug 20. PMID: 23968848.
https://pubmed.ncbi.nlm.nih.gov/23968848/

[472] Schrijvers R, Gilissen L, Chiriac AM, Demoly P.
Pathogenesis and diagnosis of delayed-type drug hypersensitivity reactions, from bedside to bench and back.
Clin Transl Allergy. 2015 Sep 3;5:31. doi: 10.1186/s13601-015-0073-8. PMID: 26339470; PMCID: PMC4558726.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4558726/

Jeffrey K. Actor,
Chapter 8 - Immune Hypersensitivities, Editor(s): Jeffrey K. Actor, Introductory Immunology (Second Edition), Academic Press, 2019, Pages 103-110,
ISBN 9780128165720, https://doi.org/10.1016/B978-0-12-816572-0.00008-5.
http://www.sciencedirect.com/science/article/pii/B9780128165720000085

Co DO, Hogan LH, Il-Kim S, Sandor M.
T cell contributions to the different phases of granuloma formation.
Immunol Lett. 2004 Mar 29;92(1-2):135-42. doi: 10.1016/j.imlet.2003.11.023.
https://pubmed.ncbi.nlm.nih.gov/15081537/

[473] Micera A, Balzamino BO, Di Zazzo A, Biamonte F, Sica G, Bonini S.
Toll-Like Receptors and Tissue Remodeling: The Pro/Cons Recent Findings.

Rare chronic local reactions

Macrophage myofascitis (MMF):
MMF is a local histopathological reaction that has been observed in human deltoid muscle and is associated with the long-term persistence of vaccine-derived aluminum hydroxide within the muscle.
The MMF lesion consists of focal infiltration of the epimysium, perimysium, and perifascicular endomysium by well-circumscribed, cohesive sheets of large mononuclear cells of the monocyte/macrophage lineage. These cells are usually mixed with a minor lymphocytic population and aggregates of macrophages containing aluminum hydroxide spicules.[474]

Tumorigenesis:
a causal relationship between post-vaccinal inflammation and the development of several types of sarcomas, histiocytomas, and cutaneous lymphomas at injection sites has been reported in association with veterinary vaccines containing alum in genetically predisposed dogs, ferrets, and cats. [475]

The exact mechanism of vaccine-induced tumorigenesis is unknown, but it is hypothesized that fibroblasts or myofibroblasts are stimulated by local inflammation, triggering inactive oncogenes. [476]

Rare cases of cutaneous and subcutaneous pseudolymphoma have been documented in humans after immunization with alum-adjuvanted hepatitis vaccine.
Histopathologic studies showed dermal and hypodermal lymphocytic follicular infiltrates with formation of germinal centers.

J Cell Physiol. 2016 Mar;231(3):531-44. doi: 10.1002/jcp.25124. Epub 2015 Sep 1.
https://pubmed.ncbi.nlm.nih.gov/26248215/

[474] Gherardi RK, Crépeaux G, Authier FJ.
Myalgia and chronic fatigue syndrome following immunization: macrophagic myofasciitis and animal studies support linkage to aluminum adjuvant persistence and diffusion in the immune system.
Autoimmun Rev. 2019 Jul;18(7):691-705. doi: 10.1016/j.autrev.2019.05.006. Epub 2019 May 4.
https://pubmed.ncbi.nlm.nih.gov/31059838/

Gherardi RK, Authier FJ.
Macrophagic myofasciitis: characterization and pathophysiology.
Lupus. 2012;21(2):184-189. doi:10.1177/0961203311429557
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3623725/

Preusse C, Goebel HH, Pehl D, Rinnenthal JL, Kley RA, Allenbach Y, Heppner FL, Vorgerd M, Authier FJ, Gherardi R, Stenzel W.
Th2-M2 immunity in lesions of muscular sarcoidosis and macrophagic myofasciitis.
Neuropathol Appl Neurobiol. 2015 Dec;41(7):952-63. doi: 10.1111/nan.12231. Epub 2015 May 19. PMID: 25711697.
https://pubmed.ncbi.nlm.nih.gov/25711697/.

[475] Roccabianca P, Avallone G, Rodriguez A, Crippa L, Lepri E, Giudice C, Caniatti M, Moore PF, Affolter VK.
Cutaneous Lymphoma at Injection Sites: Pathological, Immunophenotypical, and Molecular Characterization in 17 Cats. Vet Pathol. 2016 Jul;53(4):823-32. doi: 10.1177/0300985815623620. Epub 2016 Mar 1.
https://journals.sagepub.com/doi/pdf/10.1177/0300985815623620

Porcellato I, Menchetti L, Brachelente C, Sforna M, Reginato A, Lepri E, Mechelli L.
Feline Injection-Site Sarcoma.
Vet Pathol. 2017 Mar;54(2):204-211. doi: 10.1177/0300985816677148. Epub 2016 Dec 22.
https://pubmed.ncbi.nlm.nih.gov/28005492/

Munday JS, Stedman NL, Richey LJ.
Histology and immunohistochemistry of seven ferret vaccination-site fibrosarcomas.
Vet Pathol. 2003 May;40(3):288-93. doi: 10.1354/vp.40-3-288.
https://journals.sagepub.com/doi/pdf/10.1354/vp.40-3-288

Vascellari M, Melchiotti E, Bozza MA, Mutinelli F.
Fibrosarcomas at presumed sites of injection in dogs: characteristics and comparison with non-vaccination site fibrosarcomas and feline post-vaccinal fibrosarcomas.
J Vet Med A Physiol Pathol Clin Med. 2003 Aug;50(6):286-91. doi: 10.1046/j.1439-0442.2003.00544.x.
https://pubmed.ncbi.nlm.nih.gov/12887620/

[476] Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A.
Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.
Carcinogenesis. 2009 Jul;30(7):1073-81. doi: 10.1093/carcin/bgp127. Epub 2009 May 25.
https://pubmed.ncbi.nlm.nih.gov/19468060/

The follicles were composed of B cells without atypia, while CD4 T cells+ were predominant at the periphery.
Molecular analysis revealed a polyclonal pattern of B and T cell subsets.
Histochemical staining in all cases, and by microanalysis and ultrastructural studies in one case, identified aluminum deposits.
Associated manifestations included vitiligo and chronic fatigue with myalgia in some cases. [477]

Delayed systemic reactions

Delayed systemic reactions induced by immunological adjuvants have been observed under experimental conditions, whereas autoimmune reactions are considered rare adverse events of preventive human vaccines.

Induction or worsening of autoimmune diseases
Induction or worsening of autoimmune diseases is one of the best examples of immunotoxic reaction by combined adjuvant/antigen effect. However, it should be mentioned that there are examples of experimental adjuvant-induced autoimmunity without any joint-specific antigen. [478]

Other clinical examples such as siliconosis, MMF, Gulf War syndrome, and post-vaccination phenomena, which are part of the "adjuvant-induced autoimmune/inflammatory syndrome" (ASIA) , highlight the role of adjuvants in the mechanisms of induction or worsening of autoimmune diseases.[479]

In general, a classic vaccine formulation may contain all the elements necessary to trigger *de novo* or worsen an existing autoimmune disease in susceptible individuals. [480]
The cryptic antigens in the vaccine, may contain epitopes mimetic with structures of the self, while the co-administered adjuvant stimulates over-regulation of co-stimulatory molecules and cytokines, which may promote polyclonal activation of specific and resistant anergic autoreactive lymphocytes, and thus reactivate their potential to trigger autoimmune reactions.
In addition, an epitope spreading mechanism may occur due to the continuous damage and release of self peptides during the inflammatory process.[481]

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[477] Maubec E, Pinquier L, Viguier M, Caux F, Amsler E, Aractingi S, Chafi H, Janin A, Cayuela JM, Dubertret L, Authier FJ, Bachelez H.
Vaccination-induced cutaneous pseudolymphoma.
J Am Acad Dermatol. 2005 Apr;52(4):623-9. doi: 10.1016/j.jaad.2004.12.021.
https://pubmed.ncbi.nlm.nih.gov/15793512/

Cerroni L, Borroni RG, Massone C, Chott A, Kerl H.
Cutaneous B-cell pseudolymphoma at the site of vaccination.
Am J Dermatopathol. 2007 Dec;29(6):538-42. doi: 10.1097/DAD.0b013e3181591bea.
https://pubmed.ncbi.nlm.nih.gov/18032948/

[478] Whitehouse MW.
Adjuvant arthritis 50 years on: The impact of the 1956 article by C. M. Pearson, 'Development of arthritis, periarthritis and periostitis in rats given adjuvants'.
Inflamm Res. 2007 Apr;56(4):133-8. doi: 10.1007/s00011-006-6117-8.
https://pubmed.ncbi.nlm.nih.gov/17522809/

Whiteley PE, Dalrymple SA.
Models of inflammation: adjuvant-induced arthritis in the rat.
Curr Protoc Pharmacol. 2001 Aug;Chapter 5:Unit5.5. doi: 10.1002/0471141755.ph0505s13.
https://pubmed.ncbi.nlm.nih.gov/21959761/

[479] Vaccines and Autoimmunity
Editor(s): Yehuda Shoenfeld, Nancy Agmon-Levin, Lucija Tomljenovic
First published:May 15, 2015 Print ISBN:9781118663431 |Online ISBN:9781118663721 |DOI:10.1002/9781118663721 2015 Wiley-Blackwell

[480] Segal Y, Shoenfeld Y.
Vaccine-induced autoimmunity: the role of molecular mimicry and immune crossreaction.
Cell Mol Immunol. 2018;15(6):586-594. doi:10.1038/cmi.2017.151
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6078966/

[481] Pellegrino P, Clementi E, Radice S.
On vaccine's adjuvants and autoimmunity: Current evidence and future perspectives.
Autoimmun Rev. 2015 Oct;14(10):880-8. doi: 10.1016/j.autrev.2015.05.014. Epub 2015 May 29.

However, a clear distinction must be made between autoimmunity and autoimmune disease. Autoimmune reactions are present in many normal immunological processes, and these events rarely develop into clinical disease due to existing mechanisms of immune regulation.

However, there are numerous reports of suspected autoimmune clinical manifestations associated with some prophylactic vaccines, many of them obtained from the Vaccine Adverse Event Reporting System (VAERS) [482]; several cases, however, cannot be confirmed epidemiologically*, which has created much debate and disagreement, so much so that in 2015 a panel of experts concluded that there is no convincing evidence to support the association between the use of vaccine adjuvants and signs of autoimmunity, however, they agreed that future biomarkers related to autoimmune diseases could help provide a better understanding and management of risk in the susceptible subpopulation.[483]

* It is important to note that the epidemiological data denying causation come from the comparison of two groups of vaccinated population, and to date, appropriate long-term studies with active pharmacovigilance with a negative control group of never vaccinated have not been carried out. Given the onset in the medium to long term, the study of autoimmune diseases is never carried out in the clinical trials conducted to obtain marketing authorization, and in post-marketing pharmacovigilance the underestimation of reporting is such that the incidence of autoimmune-type adverse reactions cannot be properly assessed.

In the case of therapeutic vaccines, other common observations show that the potential risk of a post-vaccinal autoimmune event is real.
One of the most obvious examples is the development of vitiligo (an autoimmune skin reaction) in patients receiving therapeutic melanoma vaccines. Fortunately, vitiligo is a self-limited reaction and is associated with a good prognosis in terms of therapeutic efficacy.[484]
However, other manifestations of systemic autoimmunity have been reported during adjuvant cancer immunotherapy. [485]

[482] Orbach H, Agmon-Levin N, Zandman-Goddard G.
Vaccines and autoimmune diseases of the adult.
Discov Med. 2010 Feb;9(45):90-7. PMID: 20193633.
https://www.discoverymedicine.com/Hedi-Orbach/2010/02/04/vaccines-and-autoimmune-diseases-of-the-adult/

Geier DA, Geier MR.
Quadrivalent human papillomavirus vaccine and autoimmune adverse events: a case-control assessment of the vaccine adverse event reporting system (VAERS) database.
Immunol Res. 2017;65(1):46-54. doi:10.1007/s12026-016-8815-9
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5406441/

Geier DA, Geier MR.
A case-control study of serious autoimmune adverse events following hepatitis B immunization.
Autoimmunity. 2005 Jun;38(4):295-301. doi: 10.1080/08916930500144484. PMID: 16206512.
https://pubmed.ncbi.nlm.nih.gov/16206512/

[483] van der Laan JW, Gould S, Tanir JY;
ILSI HESI Vaccines and Adjuvants Safety Project Committee. Safety of vaccine adjuvants: focus on autoimmunity.
Vaccine. 2015 Mar 24;33(13):1507-14. doi: 10.1016/j.vaccine.2015.01.073. Epub 2015 Feb 7.
https://www.sciencedirect.com/science/article/pii/S0264410X15001309?via%3Dihub

[484] Teulings HE, Limpens J, Jansen SN, Zwinderman AH, Reitsma JB, Spuls PI, Luiten RM.
Vitiligo-like depigmentation in patients with stage III-IV melanoma receiving immunotherapy and its association with survival: a systematic review and meta-analysis.
J Clin Oncol. 2015 Mar 1;33(7):773-81. doi: 10.1200/JCO.2014.57.4756. Epub 2015 Jan 20.
https://pubmed.ncbi.nlm.nih.gov/25605840/

[485] Gogas H, et al.
Prognostic significance of autoimmunity during treatment of melanoma with interferon.
N Engl J Med. 2006 Feb 16;354(7):709-18. doi: 10.1056/NEJMoa053007. PMID: 16481638.
https://pubmed.ncbi.nlm.nih.gov/16481638/

Embryonic immunotoxicity

Pregnancy is a complex immunological state in which a shift toward Th2s protects the fetus and is important for a successful pregnancy, while the profile of Th1 cytokines during pregnancy may increase the risk of miscarriage and fetal morphological defects. [486]

In addition, there is evidence to suggest that proinflammatory cytokines increase the risk of adverse neonatal outcomes, independent of the direct effect on preterm labor.[487]
Cytokines, natural killer cells, and gamma/delta T cells of maternal origin appear to be involved in processes such as fetal recognition, placental development, and regulation of gene expression during organogenesis. [488]

Studies revealed that injection of high doses of CpG ODN, an adjuvant that induces strong Th1 responses, to pregnant C57BL/6 mice resulted in markedly increased fetal resorption and craniofacial/limb defects, while lower doses had little or no effect. Histological examination showed placental cell necrosis with mixed inflammation and calcification in the spongiotrophoblast layer and dysregulation of labyrinthine vascular development. [489]

Another study showed that fetal resorption and preterm birth are rapidly induced in mice after intraperitoneal injection of CpG in the 10th to 14th gestational days.
In contrast, TLR9 -/- mice or mice receiving oral administration of the TLR9 inhibitor chloroquine were protected from these effects. [490]

In theory, an adjuvanted vaccine administered early in pregnancy could influence embryofetal development through Th1 type immunity.[491]

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[486] Raghupathy R.
Th1-type immunity is incompatible with successful pregnancy.
Immunol Today. 1997 Oct;18(10):478-82. doi: 10.1016/s0167-5699(97)01127-4.
https://pubmed.ncbi.nlm.nih.gov/9357139/

Makhseed M, Raghupathy R, Azizieh F, Omu A, Al-Shamali E, Ashkanani L.
Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions.
Hum Reprod. 2001 Oct;16(10):2219-26. doi: 10.1093/humrep/16.10.2219. PMID: 11574519.
https://pubmed.ncbi.nlm.nih.gov/11574519/

[487] Sykes L, MacIntyre DA, Yap XJ, Ponnampalam S, Teoh TG, Bennett PR.
Changes in the Th1:Th2 cytokine bias in pregnancy and the effects of the anti-inflammatory cyclopentenone prostaglandin 15-deoxy-Δ(12,14)-prostaglandin J2.
Mediators Inflamm. 2012;2012:416739. doi: 10.1155/2012/416739. Epub 2012 May 29.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3368617/

[488] Szekeres-Bartho J.
Immunological relationship between the mother and the fetus.
Int Rev Immunol. 2002 Nov-Dec;21(6):471-95. doi: 10.1080/08830180215017.
https://pubmed.ncbi.nlm.nih.gov/12650238/

[489] Prater MR, Johnson VJ, Germolec DR, Luster MI, Holladay SD.
Maternal treatment with a high dose of CpG ODN during gestation alters fetal craniofacial and distal limb development in C57BL/6 mice.
Vaccine. 2006 Jan 16;24(3):263-71. doi: 10.1016/j.vaccine.2005.07.105. Epub 2005 Aug 22. https://pubmed.ncbi.nlm.nih.gov/16143434/

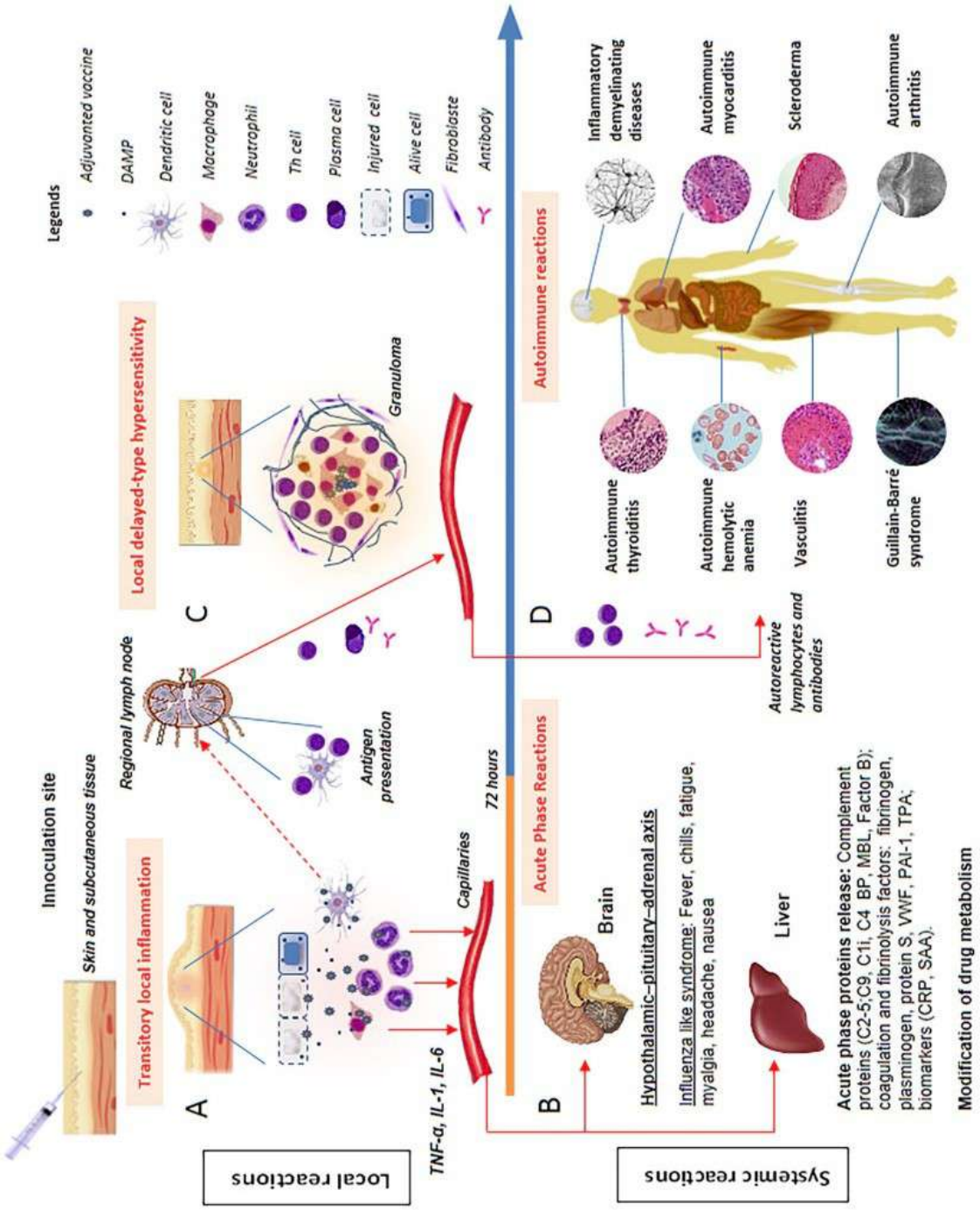
Thaxton JE, Romero R, Sharma S.
TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes.
J Immunol. 2009;183(2):1144-1154. doi:10.4049/jimmunol.0900788
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2785500/

[490] Scharfe-Nugent A, Corr SC, Carpenter SB, Keogh L, Doyle B, Martin C, Fitzgerald KA, Daly S, O'Leary JJ, O'Neill LA.
TLR9 provokes inflammation in response to fetal DNA: mechanism for fetal loss in preterm birth and preeclampsia.
J Immunol. 2012 Jun 1;188(11):5706-12. doi: 10.4049/jimmunol.1103454. Epub 2012 Apr 27.
https://pubmed.ncbi.nlm.nih.gov/22544937/

[491] Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J.
T Helper (Th) Cell Profiles in Pregnancy and Recurrent Pregnancy Losses: Th1/Th2/Th9/Th17/Th22/Tfh Cells.
Front Immunol. 2020;11:2025. Published 2020 Aug 18. doi:10.3389/fimmu.2020.02025
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7461801/

A recent review of adverse events after hepatitis B vaccination of pregnant women reported to the Vaccine Adverse Event Reporting System (VAERS) revealed that among 192 reports describing an adverse event, the most common pregnancy-specific outcomes included miscarriage in 23 reports, preterm delivery in 7 reports, and elective termination in 5 reports.[492]

Despite the reports described above, there is little information available on developmental harm caused by immunotoxicity due to adjuvant administration, so teratogenic effects that may result from exposure to vaccine adjuvants require special attention.



https://pubmed.ncbi.nlm.nih.gov/29894962/
Overview of immunotoxic reactions induced by adjuvants.
A) transient local inflammation and B) acute phase response, both occurring during the first few hours after vaccination. C) delayed-type local hypersensitivity and D) selected autoimmune reactions occurring 72 hours after vaccination *. * Autoimmune reactions after prophylactic human vaccine are considered rare adverse events and the causal relationship in many cases is still under discussion.

[492] Moro PL, Zheteyeva Y, Barash F, Lewis P, Cano M.
Assessing the safety of hepatitis B vaccination during pregnancy in the Vaccine Adverse Event Reporting System (VAERS), 1990-2016.
Vaccine. 2018 Jan 2;36(1):50-54. doi: 10.1016/j.vaccine.2017.11.039. Epub 2017 Nov 27.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6626541/