

## Review

# Antigen presentation of mRNA based and viral vector corona vaccines

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**Abstract:** Infection with Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) causes Coronavirus Disease 2019 (COVID-19), which has reached pandemic proportions. A number of effective vaccines have been produced, including mRNA vaccines and viral vector vaccines, which are now being implemented on a large scale in order to control the pandemic. The mRNA vaccines are composed of the Spike S1 protein encoding mRNA, incorporated in a lipid nanoparticle, stabilized by polyethylene glycol (PEG). mRNA vaccines are novel in many respects, including cellular uptake, the intracellular routing, processing, and secretion of the viral protein. Viral vector vaccines have incorporated DNA sequences encoding the SARS-CoV-2 Spike S1 protein into (attenuated) adenoviruses. The antigen presentation routes in MHC class I and class II, in relation to induction of virus neutralizing antibodies and cytotoxic T-lymphocytes will be reviewed. In rare cases, mRNA vaccines induce unwanted immune mediated side effects. mRNA based vaccines may lead to an anaphylactic reaction. This reaction may be triggered by PEG. The intracellular routing of PEG, and potential presentation in the context of CD1 will be discussed. Adenovirus vector based vaccines have been associated with thrombocytopenic thrombosis events. The anti-platelet factor 4 antibodies found in these patients could be generated due to conformational changes of relevant epitopes presented to the immune system.

**Keywords:** mRNA vaccine; viral vector vaccine; Spike protein; antigen presentation; polyethylene glycol; platelet factor 4; thrombosis

## 1. Introduction

The high morbidity and mortality rate of coronavirus disease of 2019 (COVID-19) has triggered the rapid development of vaccines against its causative agent, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). Vaccines are the most effective way to eliminate and control the virus [1, 2]. Most of the vaccines developed for COVID-19 have shown very high level of protection. Within one year after the outbreak of the pandemic and identification of the genomic structure of SARS-CoV-2, a number of highly effective vaccines were approved and used globally, as over 2.5 billion vaccine doses have been administered [3](dated June 25, 2021; World Health Organization). The two major categories of SARS-CoV-2 vaccines are mRNA based vaccines and viral vector vaccines, both targeting the Spike S1protein of the virus [4]. World-wide, the most used mRNA vaccines are those of

Pfizer/BioNTech (BNT162b2, brand name Comirnaty) and of Moderna (mRNA-1273, brand name COVID-19 Vaccine Moderna). Most used adenovirus vector vaccines are the ones of Oxford/AstraZeneca (ChAdOx1 nCoV-19, brand names Vaxzevria and Covishield) and Jansen/Johnson and Johnson (Ad26.COV2.S, brand name Janssen COVID-19 Vaccine).

Both mRNA vaccines for SARS-CoV-2 as well as viral vector based vaccines have turned out to be highly effective in protection against mild and severe COVID-19. After vaccination, high titers of IgG and IgA antibodies against the S1 Spike protein are generated, which *in vitro* show virus neutralizing capacity, and cytotoxic T cells are activated [5-7].

The aim of this review is to delineate the molecular pathways, outside and inside of the cell, which ultimately lead to presentation of Spike S1 peptides to the immune system. Both the classical antigen presentation routes via MHC class I to CD8+ T cells and via MHC class II to CD4+ T cells, as well as the antigen presenting routes for presentation to non-conventional T cells will be reviewed and discussed.

While SARS-CoV-2 vaccines are protecting from the severe illness and deaths due to COVID-19, after large scale implementation rare, immune mediated side-effects became apparent. Especially anaphylactic reactions and various thrombotic/abnormal bleeding have raised concern [8, 9]. These side effects may be due to abnormal handling and/or presentation of the vaccine, or vaccine additives to the immune system of which the potential scenarios will be discussed.

## 2. SARS-CoV-2 antigen presentation

### 2.1. Presentation of SARS-CoV-2 antigens during COVID-19

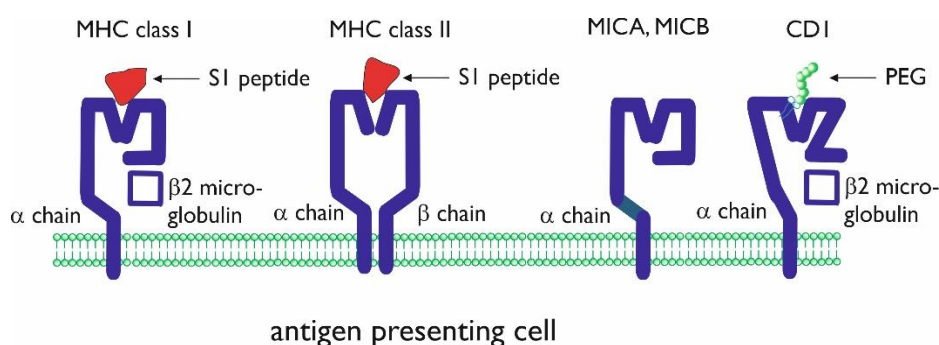
SARS-CoV-2, like the other coronaviruses (e.g. SARS and MERS) are enveloped, positive sense, single stranded RNA viruses of ~30kB. The life cycle of the virus within the host consist of 5 steps: (1) attachment, (2) penetration, (3) replication, (4) maturation and (5) release. Attachment occurs through the binding of a virus to host receptors, penetration occurs through the endocytosis of membrane fusion. Once the virus enters the host cytoplasm, viral contents are released and enter the nucleus for replication. The virus takes over the host's protein synthesizing mechanisms to produce viral proteins (replication), which are subsequently produced (maturation) and released [10].

Coronaviruses consists of four structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N). In the mechanism of infection, the Spike protein is one of the key players [10](Yuki et al., 2020). On a mature coronavirus, Spike protein is present as a trimer with three receptor-binding S1 heads sitting on top of a trimeric membrane fusion S2 stalk [11]. These two functional subunits have different functions: the S1 subunit binds to the host cell receptor and the S2 subunit is responsible for the fusion of viral and cellular membranes [10]. The SARS-CoV-2 S1 unit contains a receptor binding domain (RBD) that specifically recognizes angiotensin-converting enzyme 2 (ACE2) as its receptor. To fuse membranes, the Spike protein needs to be proteolytically activated at the S1/S2 boundary so that the SA1 dissociates and S2 undergoes a structural change [11]. Together with host derived factors such as the cell surface serine protease TMPRSS2 (Transmembrane protease, serine 2) the viral uptake and cellular fusion with the host membrane is promoted [12].

Once having entered the cytoplasm of the host cell, the virus is uncoated and viral genomic RNA is released. Translation of the two large open reading frames, ORF1a and ORF1b, of the viral RNA is initiated immediately. These reading frames encode 15-16 nonstructural proteins (nsp) which compose the viral replication and transcription complex which includes RNA-processing and RNA-modifying enzymes and a proofreading function

necessary for maintaining the coronavirus genome. ORFs that encode structural and accessory proteins are transcribed from the 3' one-third of the genome to form a set of sub genomic mRNAs (sg mRNAs) [12]. Translated structural proteins transit through the ER-to-Golgi intermediate compartment (ERGIC). Here the interaction with N-encapsidated, newly synthesized viral RNA takes place and results in the budding of the lumen of secretory vesicular compartments. Completed virions are secreted from the infected cells by exocytosis [12].

Throughout the viral infection cycle, the infected cells present viral peptides within major histocompatibility complex (MHC) class I antigens. Class I presented viral peptides will lead to activation of CD8<sup>+</sup> T cells, which are capable of lysing virus-infected tissue cells [13, 14]. CD8<sup>+</sup> T cells become activated, proliferate and differentiate into virus-specific effector and memory T cells. In the early stages of infection, professional antigen-presenting cells (dendritic cells, macrophages, and also B-lymphocytes) present viral peptides to CD4<sup>+</sup> T cells through the MHC class II molecules (Figure 1) [14].



**Figure 1.** Schematic presentation of conventional and non-conventional antigen presenting molecules. Major Histocompatibility Complex (MHC) class I and class II molecules are shown with (different) Spike S1 peptides in the antigen presenting groove. MHC class I related molecules A and B (MICA, MICB) and CD1 are non-conventional antigen presenting molecules consisting of a single α chain. The light blue domain in the MICA, MICB α chain can be cut by metalloproteases resulting in soluble MICA and MICB. CD1 is composed of an α chain, associated with β2 microglobulin. CD1 can present lipid antigens and (potentially) lipid bound polyethylene glycol (PEG).

## 2.2. mRNA vaccines

Nucleic acid vaccines containing antigens encoded by either DNA or RNA and are delivered through the use of viral vectors (such as adenoviruses) or non-viral delivery systems (e.g. electroporation or lipid nanoparticles) [15]. These types of vaccines offer solutions for issues caused by more traditional vaccines, such as the risk of a reversion to a virulence in live-attenuated vaccines or the need for additional adjuvants [16]. Nucleic acid vaccines can also be very effective, as they mimic a live, in situ infection by expressing antigens after immunization. This primes both B and T cell responses and builds an adaptive immune response directed towards the encoded target antigen [15].

Within RNA vaccines, two general classes of mRNAs are commonly used as vaccine vectors: non-replicating and self-amplifying mRNA [16]. Although both utilize the host cell translational machinery for the production of antigen target and launch of an adaptive immune response, non-replicating mRNA only encodes protein antigen(s) of interest, while self-amplifying mRNA is also capable of encoding proteins allowing for RNA replication [17]. The current COVID-19 mRNA vaccines are non-replicating vaccines.

mRNA vaccines against SARS-CoV-2 Spike protein were developed by Moderna and Pfizer/BioNtech in record time, with initial vaccinations occurring less than a year after this novel coronavirus was sequenced [18-21]. Even though mRNA vaccines appear to be simple (consisting of a lipid envelop surrounding mRNA molecules encoding for the protein of interest), the foundation to optimize their safety and efficacy profiles was previously established through the pioneering work of multiple individuals, in part enabling this success story. Four key technical aspects will be briefly discussed below in the context of antigen uptake and presentation.

### 2.2.1. mRNA.

The mRNA molecule in both vaccines consists of the following elements: 5' Cap attached to the 5' UTR, followed by the coding sequence for SARS-CoV-2 Spike protein, a 3' UTR and a long poly-A tail [22, 23]. In general, elements within the mRNA were optimized to increase its stability, maximize protein translation and reduce unwanted side effects due to innate immune activation. However, side-by-side comparison of each company's individual approach will be possible once currently undisclosed information becomes available upon protection of intellectual property rights. Importantly, 'minor' variations in mRNA elements impacting mRNA stability, translation and Spike protein expression efficacy, could potentially explain differences in immunogenicity profiles described for both vaccines, as discussed below. The following is a description of the methodology to optimize some mRNA elements and is based on references cited in publications reporting initial results for SARS-CoV-2 vaccine trials.

In both vaccines, the mRNA is synthesized by *in vitro* transcription of a DNA fragment encoding for all elements, except the 5' Cap. A Cap1 structure is covalently attached to the 5' UTR either co- or post-transcriptionally via different capping enzymes used by Pfizer/BioNtech and Moderna, respectively [22, 24]. Thereafter, the final product is purified using affinity chromatography on oligo-dT, in order to remove impurities generated during transcription (such as double-stranded RNA), which could potentially activate the innate immune response [25]. Moderna optimized the 5' UTR using machine learning techniques trained on ribosomal loading profiles of a reporter gene library in which the 5' UTRs contained completely random sequences [26]. This was further tested and validated on thousands of human 5' UTR and variants associated with diseases in humans. BioNtech also used a library to optimize the 3' UTR [27]. Specifically, the reporter gene was linked at the 3' end to random cDNAs obtained by reverse-transcription of fragmented mRNA isolated from human dendritic cells. Surprisingly, upon several rounds of enrichment for highly expressing constructs, the most effective 3' UTR corresponded to a mitochondrial, non-coding rRNA. Adding a second 3' UTR from a different gene further enhanced mRNA stability and protein expression. Finally, BioNtech optimized the poly-A tail to 120 bp in length by testing levels of a reporter protein expressed from mRNAs differing in poly-A tail length [28]. This study also characterized an idoneous poly-A tail as being unmasked, i.e., no other nucleotides except adenines should be present in the 3' end [28].

Perhaps the most important aspect of mRNA vaccine optimization, consisted of replacing pseudouridine ( $\Psi$ ) or N1-methyl- $\Psi$  for uridine during *in vitro* transcription. Karikó and co-workers correctly hypothesized that unmodified nucleotide bases, such as those present in RNA transcribed *in vitro*, were responsible for RNA's strong activation of the innate immune system [29]. In contrast to the ubiquitous presence of modified bases in mammalian RNA (excluding mitochondrial RNA), innate immune mechanisms evolved to detect and become activated upon encounter with RNA containing unmodified bases [29]. Indeed, significant reduction in dendritic cell activation was observed when transfected with RNA transcribed *in vitro* in the presence of  $\Psi$  or N1-methyl- $\Psi$ , instead of uridine [30, 31]. Similar

results were observed *in vivo* in animals injected parentally with mRNA synthesized in the presence of modified bases [32, 33]. Furthermore, higher and longer expression levels for the protein of interest were observed in animals injected with mRNA containing modified bases. As recently reported, higher translation efficacy could result from stable secondary structures occurring in mRNAs containing N1-methyl-Ψ [34], as well as greater ribosomal density associated to modified mRNA [35].

#### 2.2.2. Protein sequence.

BNT162b2 and mRNA-1273, Pfizer/BioNtech and Moderna anti-SARS-CoV-2 mRNA vaccines respectively, both encode for the full length Spike protein consisting of a signal sequence, S1 and S2 domains (comprising the large extracellular domain including the RBD), followed by a short transmembrane and cytoplasmic domain [19, 22]. Pfizer/BioNtech additionally performed clinical trials with BNT162b1, a secreted, truncated version of the Spike protein consisting of the RBD region [21]. Despite eliciting a potent humoral and cellular immune response [21, 36], BNT162b1 is not currently used due, in part, to more frequent side effects [19]. Furthermore, BNT162b2 encoding the full length Spike protein had the added benefit of including additional epitopes potentially targeted by the immune response. Upon ribosomal translation, the signal sequence targets the protein to the ER where it starts undergoing significant post-translational modifications. Besides removal of the signal sequence, glycosylation (both N and O-type), as well as disulphide bond formation occur in this organelle. As it continues through the secretory pathway en route to the cell membrane, the first of two activating proteolytic events occurs: a furin-dependent cleavage not present in the related SARS-CoV-1 Spike protein [37]. To stabilize the vaccine's pre-fusion conformation upon furin cleavage and maximize eliciting antibodies against the native viral Spike protein, two mutations for proline residues were engineered in the vaccine's S2 subunit [22, 38]. The end product in host cells expressing these mRNA vaccines is a surface-exposed, membrane-anchored, glycosylated and trimerized Spike protein resembling the 3-D structure of the native viral Spike protein, to the extent that interacts with its cognate receptor, hACE2 [22].

#### 2.2.3. Lipid nano particle (LNP).

In order for translation of exogenous mRNA to occur in the cytoplasm of the target cell, it first has to cross the barrier imposed by the hydrophobic, lipid cell membrane. Lipid nanoparticles (LNPs) are the most commonly used platforms for mRNA delivery and are mainly composed of ionizable cationic lipids, cholesterol, phospholipids (such as distearoylphosphatidylcholine) and polyethylene glycol (PEG)-lipid [39, 40]. Ionizable cationic lipids participate in nanoparticle packaging by interacting with negatively charged mRNA molecules [41]. Moreover, the amine head group plays a key role mediating endosomal uptake. Specifically, increased LNP uptake and mRNA translation was observed when LNPs included ionizable cationic lipids derivatized with amine head groups having pKa 6.6-6.8 [42]. Interestingly, efficient mRNA translation was shown to be associated with rab7-dependent late endosomes and their association with mTOR signaling [43]. Even though these formulations of LNPs + mRNA accumulated in late endosomes, only a small, yet sufficient percent of the vaccine's mRNA was translocated to the cytoplasm for translation [43]. Thus, further work is required to precisely define the mechanism wherein endosomally-localized mRNAs are shuttled cytoplasmically. Finally, the amine head group present in ionizable cationic lipids was closely associated with LNP pharmacokinetics *in vivo* [42]. Upon administration, biodegradable LNPs that were rapidly cleared from injected tissues were less likely to induce inflammation and tissue damage [44], while, importantly, conserving adequate mRNA translation levels [42].



#### 2.2.4. Immune response.

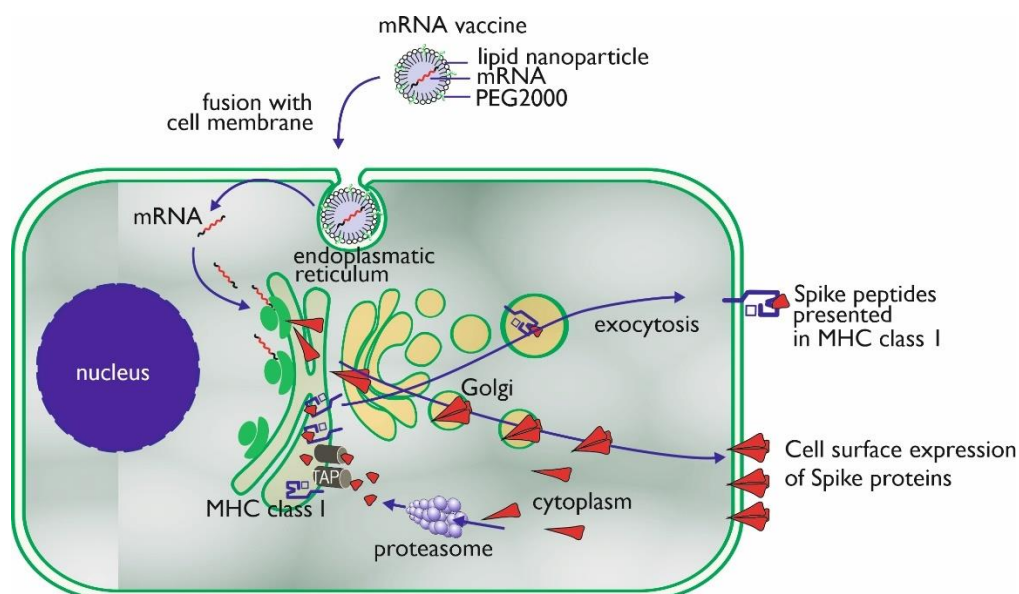
To determine the fate of mRNA vaccines upon *in vivo* administration, some studies used fluorescently labeled LNPs carrying mRNA encoding for a fluorescent or luminescent reporter proteins [45]. Using a previous iteration of LNPs, Moderna showed leukocyte migration to the site of LNP injection, but not upon control injections with PBS. Vaccine formulations were efficiently uptaken at the site of injection by phagocytic cells such as neutrophils and different types of monocytes/macrophages, however macrophages represented the major vaccine cell type at the draining lymph nodes. Interestingly, not all transfected leukocytes efficiently translated the target protein and a clear dissociation between high vaccine uptake, yet low protein expression was observed for neutrophils [45].

Further studies evaluated how mRNA vaccines induced a potent humoral response. Upon vaccination of non-human primates, germinal centers were observed in draining lymph nodes and, importantly, antigen-specific follicular helper T (T<sub>fh</sub>) cells were detected within these structures [46]. Collectively, this represents an ideal niche conducive to B cell activation, antibody isotype switching and affinity maturation, leading to long lived memory B cells and plasma cells. Indeed, a major goal of Covid-19 mRNA vaccines consisted of eliciting high titers of high affinity antibodies against Spike protein/RBD, capable of neutralizing SARS-CoV-2 infection [18, 19].

Using overlapping peptide pools covering the Spike protein, additional subsets of antigen-specific T cells were reported in humans upon vaccination with Moderna and Pfizer/BioNTech Covid-19 mRNA vaccines [24, 47]. CD4<sup>+</sup> T cells responded to peptides from both the S1 and S2 subunits, validating the decision to use mRNA encoding the full length Spike protein instead of a secreted RBD. Furthermore, based on cytokine profiles, the vaccine skewed helper CD4<sup>+</sup> T cells to Th1 [24, 47], an ideal scenario to circumvent Th2 responses responsible for vaccine-associated enhanced respiratory disease (VAERD) [48]. Intriguingly, whereas both vaccines induced helper CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells were only reported for Pfizer/BioNTech vaccine [24, 47]. The significance of this result remains to be determined as both vaccines elicited comparable levels of protection (~95%) against SARS-CoV-2 strains circulating at the time clinical trials were performed. However, this could have important repercussions against emerging viral variants with higher virulence and/or transmissibility. Pfizer/BioNTech recently characterized in greater detail human CD8<sup>+</sup> T cell responses in a small cohort of vaccinated individuals. Interestingly, CD8<sup>+</sup> T cells responding to peptides from the S2 subunit were identified in some unvaccinated individuals, possibly crossreacting to epitopes shared with seasonal coronaviruses [24]. Furthermore, some overlap was observed for epitopes recognized by CD8<sup>+</sup> T cells upon vaccination and natural infection. Clearly, additional epitope mapping is required to understand how mRNA vaccines elicit cellular immune responses.

The mechanism of action of an mRNA vaccine is very similar to the mechanism of viral infection. Prior to vaccination, *in vitro* transcribed (IVT) mRNA is produced from a linear DNA template or PCR products using a T3, T7 or Sp6 phage RNA polymerase. The resulting product optimally contains an open reading frame (ORF) that encodes the antigen of interest, 5' and 3' untranslated regions (UTRs), a 5' cap and a poly-A tail [17]. Efficient mRNA delivery and endocytosis to cells is critical in order for mRNA vaccines to achieve therapeutic relevance. All components of the LNP are thought to facilitate and promote the endosomal escape of mRNA [40]. By means of the translational machinery of the host cells, the mRNA is translated into proteins. These proteins may undergo post-translational modification and either function within the cell or are secreted. Proteasomes degrade cytoplasmic proteins, thus generating antigenic peptide epitopes that are transported to the ER and loaded onto MHC class I molecules (Figure 2). The MHC class I can present these peptides on the surface of the cell to specific CD8<sup>+</sup> T cells. Alternatively, the secreted exogenous proteins can be taken up

by professional antigen presenting cells, processed, and presented in MHC class II [17]. In mRNA vaccinated individuals (BNT162b1), T-cells can be detected secreting interferon- $\gamma$  upon *in vitro* restimulation with SARS-CoV-2 peptides, which confirms the induction of CD4<sup>+</sup> Th-cells through MHC class II [36]. Professional antigen presenting cells also can present exogenous antigens, which is processed via an alternative intracellular routing and presented via MHC class I (cross-presentation) [49, 50].



**Figure 2.** Uptake, processing, and MHC class I presentation of Spike proteins, encoded by mRNA vaccine.

The mRNA of the Pfizer and Moderna vaccines encodes for the Spike protein of the virus. This viral protein is essential for attachment to membrane ACE 2 receptors and subsequent invasion of host cells. Therefore, a vaccine directed against the Spike protein prevents the proliferation and spread of SARS-CoV-2 [51]. Liu and co-workers predicted that the population coverage for the SARS-CoV-2 subunit vaccines encoding the Spike protein could generate at least 6 peptides for high affinity binding to MHC class I and class II in respectively 99.4 and 96.4% of the population [52, 53].

There are multiple advantages of the use of mRNA-based vaccines over traditional approaches. As mentioned before, mRNA vaccines, at conceptual level, combine the simplicity, safety and focused immunogenicity of subunit vaccines with the favorable immunological properties of live viral vaccines. mRNA vaccines are molecularly defined to encode only the specific antigen of interest and no other excess information. This means, that in case of a SARS-CoV-2 vaccine, the mRNA does not encode the entire virus, but only the S-protein. This greatly reduces complications associated with biological vaccine production (such as genetic variability). A downside of mRNA vaccines always has been that, because of ubiquitous RNases, the RNA would be rapidly degraded [54]. The use of modified pseudouridine was a breakthrough discovery circumventing this major obstacle [30, 55]. An important benefit of RNA based vaccines is the enormous flexibility of vaccine design and production. The antigen encoding sequence (the ORF) can be easily modified at specific locations and/or codon-optimized to improve translation or engineered to guide the antigen to the desired intracellular compartments to improve antigen presentation [56]. Modifications

such as point mutations, deletions or removal of glycosylation sites could all potentially affect antigenicity, immunogenicity and overall vaccine efficacy [56-58]. Moreover, next to additions to the coding sequence, the half-life of mRNA, the pharmacokinetics of protein expression (such as magnitude and duration), and immunogenicity are all available for fine tuning via modifications of for example the 5' and 3' UTRs and optimization of the length of the poly-A tail [56]. The mRNA could also be tailored in such a way to provide potent adjuvant stimuli to the innate immune system by direction activation of RNA-specific receptors, which may reduce the need for additional adjuvants [54].

### 2.3. Adenoviral vector vaccines

A relatively new group of vaccines are those based on viral vectors [59]. This type of vaccine gained importance for vaccination against pathogens that did not yield sufficient immune responses in the past when approached by conventional vaccines. After initial success of adenoviral vector-based therapeutic drugs in clinical settings [60-62], vaccines based on viral vectors were developed for the prophylaxis of infectious diseases as Ebola and malaria. The choice for adenoviruses as vectors is obvious, because this group of viruses is widespread and usually they do not lead to serious infections and related pathology [63]. Technologically, adenoviruses are easy to grow and multiply in tissue cultures. They are thermostable, have a broad tropism, which means they can infect a wide range of cells, and administration of these adenovirus vector-containing vaccines is easy to perform without any hurdles in muscular and mucosal tissue. Adenoviruses are non-enveloped and have double stranded DNA and their medium-sized genome is about 26-48 Kbp. This size is still acceptable for easy manipulation. Adenoviruses are widespread, species-specific and many serotypes are known, and still novel serotypes are regularly described (<http://hadvwg.gmu.edu>). In humans, the more than 80 serotypes are divided into seven species A to G [64, 65]. Most serotypes belong to species D.

Due to the abundant presence of adenoviruses in the human population, humoral and cellular immunity against these viruses is generally present [66, 67], and initially this was considered a drawback that needed to be solved [68-70]. The strategies to follow are modification of the antigenic epitopes on the viral capsid of human Ad5. One way is the chemical modification by PEGylation to improve the vaccine efficacy via shielding or hiding of the epitopes [71]. Other useful techniques are insertion of peptides or other types of modification of the capsid proteins. As an alternative, rare human adenoviral serotypes such as Ad26 and Ad35 [72], or chimpanzee adenoviruses can be used [68, 73]. In addition, all these viruses need to be genetically modified to prevent replication when administered to humans. Normally, adenoviruses attach to membrane receptors. Ad5 will bind to the so-called coxsackievirus and adenovirus receptor CAR, which belongs to the Ig superfamily of proteins, is present at nearly every human cell, and which normal function is just being discovered [74]. Via clathrin-coated pits the virus is taken into the cells and after rupture of the endosome, viral particles are dispersed into the cytoplasm. The E1 gene is transcribed in the immediate early phase, and it is this gene which is removed to prevent viral replication when the adenovirus is used as a vector.

Replication-deficient adenovirus vectors need to be produced in cells that do contain the E1 gene for their replication. Originally, human embryonic kidney (HEK293) cells, a cell line derived from a female fetus, were used as important cell for the Ad5 viral vector replication [75]. Later different cell lines with and without transfection of particular genes were also explored for other adenoviral vectors [63, 76].

The working mechanism of the adenoviral vector vaccines is based on the cellular introduction of a recombinant viral genome (cDNA) containing a promotor sequence, the



gene encoding the antigen and a poly-A tail. Upon intramuscular administration, muscle cells get infected and will subsequently present processed antigen via MHC class I and start to secrete viral antigen to induce an immune response by activating antigen presenting cells. The advantage of this mode of action is that both the innate and adaptive immune system are activated, and a humoral and cellular response will result.

Currently, the vectors of choice for Sars-CoV-2 and suitable candidates for long-term protective immunity are both human and primate adenoviruses [77, 78]. At the moment there are four approved adenovector vaccines available: Ad26.CoV2.S (Janssen-Johnson & Johnson; brand name Janssen COVID-19 Vaccine), ChAdOx1 nCoV-19 or AZD1222 (Astra-Zeneca; brand names Vaxzevria and Covishield), Gam-COVID-Vac (Gamaleya National Research Centre for Epidemiology and Microbiology; brand name Sputnik-V), and Ad5-nCOV (CanSino Biologics; brand name Convidecia).

The first one makes use of the human non-replicating vector Ad26, encoding a full length SARS-CoV-2 Spike protein (cDNA), which demonstrated to be efficient in inducing both humoral and cellular immune responses after just one administration [79, 80].

The second vaccine is based on the chimpanzee-derived E1-deficient adenoviral vector ChAdY25, which was demonstrated to have a low human seroprevalence [81]. Also this vaccine expressed the SARS-CoV-2 Spike protein and induced an efficient immune response [82, 83].

The third adenoviral vector vaccine, Gam-COVID-Vac, is a recombinant non-replicating two-vector vaccine (rAd26 and rAd5, respectively) with expression of full length Spike protein, which is introduced intramuscularly with an interval of 21 days. Also with this vaccine a high efficiency was obtained [84].

The fourth vaccine is based on the modified non-replicating Ad5 vector with the full Spike protein expressed. Also this vaccine is able to induce a significant immune response [85].

Many other viral vector vaccines are currently being developed [86], but in addition vaccines making use of other techniques are also under investigation, as there are future vaccines to be expected from amongst others the REGA institute of the Catholic University Leuven (weakened virus, based on the yellow fever vaccine), GSK-Sanofi (adjuvanted recombinant protein-based vaccine), and Medicago (a (tobacco) plant-based virus-like particle vaccine).

### 3. Presentation of Corona vaccine additives

#### 3.1. Unconventional T lymphocytes, such as NKT cells, $\gamma\delta$ T cells

To date, three classes of unconventional T cells have been described. The common denominator to classify these T cells as unconventional is that they lack the classical  $\alpha\beta$  T cell receptor and, maybe as a consequence, they do not recognize peptides presented by MHC class I or class II. In total these unconventional T cells represent no more than 10% of peripheral T cells. However, upon activation they rapidly respond with cytokine production and cytotoxic activity. Based on these properties unconventional T cells also are described as innate-like T cells [87, 88].

The three classes of unconventional T cells are 1) CD1d restricted natural killer T cells (NKT cells), 2) MR-1 restricted, mucosal-associated invariant T cells (MAIT cells) and 3) gamma-delta T cells ( $\gamma\delta$  T cells).

NKT cells express an invariant  $V\alpha\beta$  TCR and can be activated by glycolipid antigens including  $\alpha$ -galactocylceramide. Upon activation, NKT cells produce large amounts of Th1, Th2, as well as Th17 cytokines. NKT cells play a role in cancer immunotherapy [89], and are also implicated in controlling the outcome of viral infections, as demonstrated in animal models as well as in humans [90]. A number of papers have analyzed NKT cells during COVID-19. Zingaropoli et al. find a major reduction of peripheral blood NKT cell numbers in severe COVID-19 [91]. Jouan showed that NKT cells, as well as MAIT cells and  $\gamma\delta$  T cells all decrease during severe COVID-19 [92]. Probably there is more.

MAIT cells have a specificity for microbial riboflavin-derived antigens when presented by the major histocompatibility complex (MHC) class I-like protein MR1 [87, 93]. These cells express TCRs with limited diversity but broad specificity. MAIT cells have antibacterial and antifungal properties, but also are activated during viral infections, including SARS-CoV-2 [94]. Indeed, during COVID-19, MAIT cells are activated and (subsequently) depleted from peripheral blood [95, 96].

$\gamma\delta$  T cells  $\gamma\delta$  T cells express a T cell receptor made up of a  $\gamma$ - and a  $\delta$ -chain, both of which have a V segment repertoire less restricted than that of  $\alpha$  and  $\beta$  TCR genes [97]. In peripheral blood they form a small population within the T cell compartment, but at mucosal sites such as the gastrointestinal and respiratory tract the numbers may be substantially higher [97]. Out of the total number of  $\gamma\delta$  T cells,  $V\gamma9V\delta2$  cells are the dominant population in adults. In the elderly this is more variable, with some individuals  $V\gamma9V\delta2$  are almost lacking, while in others all virtual all  $\gamma\delta$  T cells are  $V\gamma9V\delta2$  [98]. It has been shown that  $V\gamma9V\delta2$  T cells have a so-called polycytotoxic profile [98]. Indeed, this subset of  $\gamma\delta$  T cells also has been implicated in influenza, as well as in corona virus infections [98, 99]. Poccia et al. previously found a selective expansion of the  $V\gamma9V\delta2$  T cell population in peripheral blood of health care workers who survived a SARS-CoV-1 infection during the 2003 outbreak [100]. We have described that SARS-CoV-2 hospitalized patients who do not survive have lower numbers of  $V\gamma9V\delta2$  T cells than COVID-19 survivors [101].  $\gamma\delta$  T cells do not recognize antigens presented by classical MHC molecules but use the alternative antigen presenting molecules such as BTN3A [99, 102] and MHC class I-related molecules MICA and MICB [103] (Figure 1).

It is attractive to postulate that NKT cells (as well as the other unconventional T cells) migrate to the lungs during the most active phase of the disease [104]. Because of the involvement of unconventional T cells in COVID-19 it is also important to consider the role of unconventional antigen presentation routes of SARS-CoV-2, the virus as well as the vaccines.

### 3.2. Unconventional antigen presentation molecules, i.e. CD1

In paragraph 2.1, the classical routes of antigen presentation of peptide antigens, in MHC class I and II to CD8+ (cytotoxic T cells) and CD4+ (T helper cells), respectively, has been described. The B cell receptor (membrane immunoglobulin) can recognize and interact with either soluble or cell-bound antigens, but does not require antigen presentation in MHC class I or II. However, the antibody response of B cells to protein antigens is dependent on T helper cells, in particular follicular helper T cells [105].

In the allergic reactions to mRNA vaccination, which occur as a rare but serious adverse event, IgE antibodies to PEG have been implicated. Although rare, cases of anaphylaxis or possible anaphylactic reactions have been reported following injection of the different COVID-19 vaccines. The frequency of anaphylaxis after injection is approximately 11.1 in 1 million for the Pfizer/BioNTech vaccine [9]. The CDC in the USA reported 21 cases of anaphylaxis out of 1.8 million doses administered. For the Moderna vaccine the CDC reported

2.5 cases per million doses administered [106]. AstraZeneca has also shown some cases of anaphylaxis. According to the European Medicines Agency, 41 out of 5 million people vaccinated with AstraZeneca showed possible signs of anaphylaxis. Jansen has also shown few hypersensitive reactions [107].

Thus, even though the Pfizer/BioNTech and Moderna vaccines are mRNA vaccines, and AstraZeneca and Janssen/Johnson and Johnson are viral vector vaccines, all COVID-19 vaccines in rare cases can induce a severe allergic response. The mechanisms behind these anaphylactic reactions are still unknown, however a hypersensitivity reaction to either polyethylene glycol (PEG) in the mRNA vaccines or to polyoxyethylene-sorbitan-20-monooleate (polysorbates 80) in the viral vector vaccines could be the vaccine components triggering the hypersensitive reaction.

Previous studies on vaccine-associated anaphylaxis showed that additives such as gelatine, egg protein, latex or polysorbate 80 all can elicit hypersensitive reactions in susceptible persons [108]. Case studies have shown that patients with a known allergy for PEG also can be hypersensitive to polysorbate based on cross reactivity between the two compounds [109]. The (cross) reaction occurs by the induction of degranulation of mast cells or basophils, but is not mediated by IgE or other immunoglobulin classes. Polysorbate 80 has however also been shown to cause IgE-mediated anaphylactic reactions.

In the mRNA vaccines the nucleoside-modified RNA is formulated in lipid nanoparticles, which contain PEG. PEG seems to be the most likely cause of hypersensitivity since the components of the mRNA-based vaccines do not include any substances that are known to cause hypersensitivity and the mRNA is introduced for the first time to the body and therefore the body is not sensitized to this specific component. Many people also have had previous exposure to PEG since it can be found in many household products, medications and food products, therefore opening the possibility of prior sensibilization.

### *3.3. Hypersensitivity and PEG antigen presentation*

It is difficult to understand why some people have an allergic response to PEG and why others do not. Previous studies have indicated that induction of hypersensitivity does depend on the dosage, the route of administration, as well as the molecular weight of PEG used [110]. Numerous cosmetic products, such as toothpaste, contain PEG and therefore everyone is continuously exposed to PEG. Many drugs are conjugated to PEG (PEGylated) to increase half-life and efficacy [111]. It has been shown that PEGylated drugs can lead to hypersensitivity reactions, especially in case of high molecular weight PEG administered intravenously [112]. An example of a PEGylated drug is doxil, PEGylated doxorubicine, a chemotherapeutic for leukemia and lymphomas, mamma carcinoma and other forms of cancer. Doxil can lead to hypersensitivity reactions in up to 40 percent of patients [113]. Anaphylactic reactions can occur in patients who have been treated with high doses of PEG3350 or 4000 to empty the colon before a colonoscopy [108]. In above patient categories who could be sensitized to PEG, a skin prick test with PEG can yield a positive result. Remarkably, Sellaturay et al. describe a patient, with a known hypersensitivity to PEG, who developed a severe allergic reaction after being vaccinated with the Pfizer mRNA vaccine. Skin prick testing was done with the vaccine and a range of PEG with increasing MW. Only PEG4000, but not lower or higher MW PEGs, nor the vaccine, gave a clear positive response [114]. Other patients with an allergic reaction to the Pfizer mRNA vaccine may show negative skin prick tests to a wide range of PEGs, underscoring the need but also the difficulty of allergy testing for PEGs and mRNA vaccines [114, 115].

In athymic nude mice, IgM anti-PEG antibodies can be induced by intravenous immunization with PEGylated liposomes [116]. On the other hand, the induction of IgM anti-PEG by Pegfilgastrim, PEGylated-G-CSF or by PEGylated ovalbumin have been shown to be T cell dependent process [117, 118]. It should be kept in mind that these studies in mice are restricted to IgM antibodies. IgG anti-PEG antibodies are not formed in mice, and IgE has not been investigated.

### 3.3.1. Antigen presentation of PEG

PEG, obviously not a peptide, would have to be presented through an alternative, non-MHC, pathway. A possible pathway would be CD1 antigen presenting. CD1 is a protein (family) related to the non-polymorphic and MHC class I molecules. CD1 proteins are able to present (self and foreign lipid antigens) to T lymphocytes. Group 1 (CD1a-c) and 2 (CD1d) CD1 proteins are expressed on the cell surface and function as antigen presenting molecules. Group 3 (CD1e) is only expressed extracellularly and is involved with processing and editing of lipids for presentation by the other CD1 isoforms [119]. Although structurally related (see Figure 1), a difference between MHC class I and CD1 is that the inner surface of CD1 is covered with hydrophobic residues and the  $\alpha$  helices differ as well, there is a deeper antigen-binding groove in CD1 (which differs per CD1 isoform). CD1 molecules are expressed by monocytes, B lymphocytes and dendritic cells. CD1a, b, c and d are assembled and folded in the ER where they associate with  $\beta$ 2 microglobulin, capture endogenous self-lipids and move to the cell surface [120]. The different CD1 isoforms take different intracellular paths, which enables them to sample lipid antigens from different intracellular compartments. Exogenous lipid antigens, which are taken up by endocytosis, can associate with CD1 in endosomes and re-exposed on the cell surface of the antigen presenting cell [119]. For example, CD1b c and d use tyrosine residues in their cytoplasmic tails to navigate through the endosomes. CD1b and c bind to  $\mu$ -subunits of adaptor protein 2 complexes [121]. These AP-2 complexes are used to internalize the contents in clathrin-mediated endocytosis. The several CD1 isoforms thus allow for a wide range of non-protein antigens to be presented and recognized by T lymphocytes.

Very limited data are available about the potential role of BTN3A in presentation of SARS-CoV-2 antigens to the immune system. A search on PubMed (June 4, 2021) with BTN3A and SARS-CoV-2 yielded zero hits. Also for presentation of SARS-CoV-2 antigens by MHC class I related genes A and B (MICA and MICB), scarce data are available at the moment. In a study from Brazil, not yet published in a peer reviewed journal, Castelli et al analyzed 86 discordant Brazilian couples where one partner was infected and symptomatic while the partner remained asymptomatic and seronegative despite sharing the same bedroom during the infection. The authors found only a minor impact of classical MHC class I and class II genes associated with resistance. However, individuals producing higher amounts of MICA and low amounts of MICB were more susceptible to SARS-CoV-2 infection [122]. While these are preliminary data, which need to be confirmed, it is interesting from the perspective that MICB can serve as an antigen presenting molecule for  $\gamma\delta$  T cells.

### 3.4. Thrombosis and (V) ITP

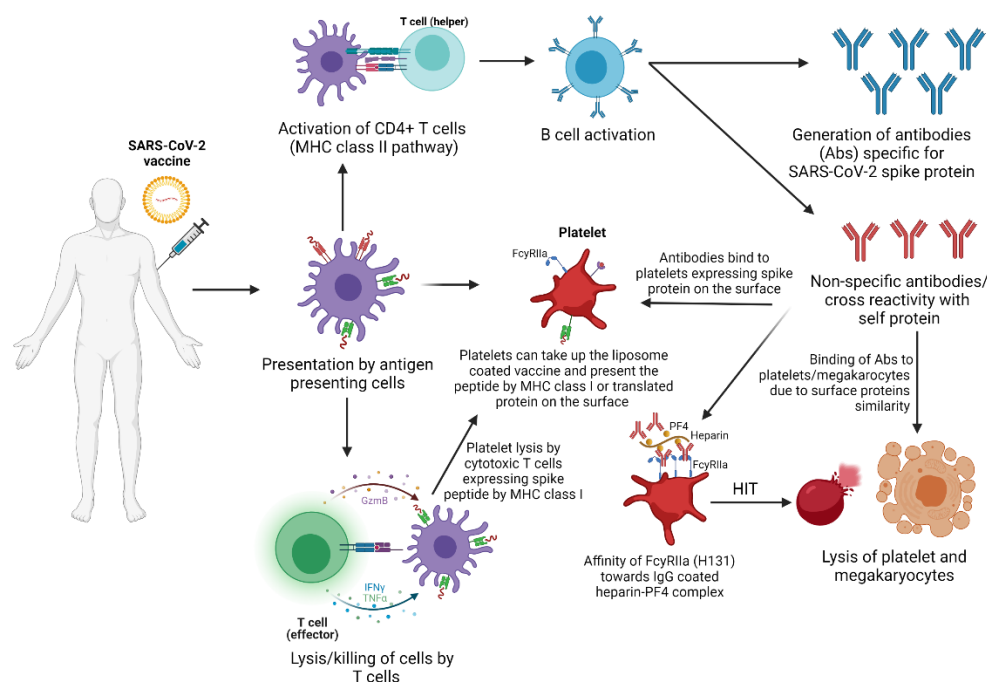
Vaccines have some mild to moderate side effects, some seen in clinical trials of COVID-19 vaccine development, in which thousands of volunteers have participated, and some rare cases of anaphylaxis occurred [1]. The thrombotic side effects were initially reported for recombinant genetic vaccines and have raised concerns about the safe use and development of vaccines. One of the severe events in COVID-19 infection is coagulopathy leading to various

thrombotic complications and even death [123]. Similar to COVID-19/SARS-CoV-2 infection-induced immune thrombocytopenia (ITP) [124], ITP post-vaccination is a possible adverse effect. ITP has also been previously reported with a number of other vaccines, such as flu, poliomyelitis, pneumococcal, hepatitis, MMR, and rabies vaccines [125]. ITP – is an autoimmune condition characterized by low platelet counts, spontaneous purpura, hematomas, or fatal sub-arachnoid, and internal bleeding [124].

The first reports of abnormal blood clot/thrombosis/thrombocytopenia/ITP post-vaccination were described after administering the ChAdOx1 nCoV-19 (AstraZeneca) and Ad26.CoV2.S (Janssen/Johnson and Johnson), followed by mRNA-1273 (Moderna) and BNT162b2 (Pfizer–BioNTech) vaccines [126]. An extensive evaluation/assessment of the adverse effects of thromboembolism associated with vaccines against SARS-CoV-2 was conducted and documented by different health organizations worldwide. Of thirty-one cases of post-vaccination thrombocytopenia, 17 cases were not associated with pre-existing thrombocytopenia. Fourteen cases with bleeding signs were reported from the Centers for Disease Control and Prevention (CDC). The vaccine adverse event reporting system (VAERS) has documented over 160 cases (June 2021) of thrombosis or thrombocytopenia as an adverse effect of mRNA vaccines [127]. Since the onset of VAERS, no cases of CVST (cerebral venous sinus thrombosis) with thrombocytopenia have been reported amongst the recipients of mRNA-based vaccines (mRNA-1273 and BNT162b2). However, some cases of cerebral venous sinus thrombosis (CVST) with thrombocytopenia have been reported in recipients of Ad26.CoV2.S vaccine in the US. These reports are similar to the cases reported post-vaccination with ChAdOx1 nCoV-19 in the UK, as both vaccines are adenoviral vector vaccines that encode the Spike glycoprotein of SARS-CoV-2 [128]. The European Medicines Agency (EMA) has reported at least 169 cases (June 4, 2021) of cerebral venous sinus thrombosis (CVST) and 53 cases of splanchnic vein thrombosis (SVT) among 34 million recipients of the ChAdOx1 nCoV-19 vaccine. Thirty-five cases of central nervous system thrombosis among 54 million recipients of the BNT162b2 mRNA vaccine, and 5 cases of cerebral venous sinus thrombosis among 4 million recipients of the mRNA-1273 mRNA vaccine, 6 cases of cerebral venous sinus thrombosis (with or without splanchnic vein thrombosis) have been reported amid the 6.85 million recipients of the Ad26.COVS adenoviral vector vaccine [1, 107].

According to a study from Oxford University, there is a five in a million chance of abnormal blood clotting for recipients of the ChAdOx1 nCoV-19 vaccine, followed by four per million for recipients of mRNA-1273 and BNT162b2 vaccines, while the chances of an abnormal blood clot are way higher in individuals infected with SARS-CoV-2 that is 39 per million [129]. The risk of blood clots through ITP related to the COVID-19 vaccines is now well known. The increasing numbers of rare adverse events are not surprising as vaccination numbers are constantly increasing. However, the pathogenesis/etiology of these adverse effects is not completely clear. We therefore present some possible mechanisms of vaccine-induced ITP, partly based on abnormal antigen processing and/or presentation.





**Figure 3:** Possible etiological scenarios of vaccine induced immune thrombocytopenia

#### 3.4.1. Possible mechanisms/etiology of vaccine-induced ITP

##### 3.4.1.1. Molecular mimicry

Vaccines may induce ITP by several mechanisms; however, molecular mimicry has been considered the classic mechanism for vaccine-induced ITP [130]. It has been suggested that the antibodies produced can potentially cross-react with surface antigens of platelets or megakaryocytes of the host instead of the virus [125]. These autoantibody-bound platelets or megakaryocytes undergo reticuloendothelial phagocytosis or opsonization or apoptosis or direct lysis by cytotoxic T-cells, all of these mechanisms leading to thrombocytopenia. Several pathogens can induce ITP besides SARS-CoV-2, especially *Helicobacter pylori*, influenza virus, dengue virus, among others.

##### 3.4.1.2. Molecular mimicry between SARS-CoV-2 Spike protein and angiotensin-I

Another possible mechanism of vaccine-induced ITP is from molecular mimicry between viruses and human peptides [131], as there are similarities (~45%) between SARS-CoV-2 Spike glycoprotein and the human protein angiotensin-I. The rationale behind this is that post-infection, immune responses raised against the pathogen can cross-react with human proteins, which share peptide sequences with the pathogen, leading to autoimmune responses or ITP [132, 133]. The data relating to SARS-CoV-2 associated diseases favor molecular mimicry between hexa- and -heptapeptide of SARS-CoV-2 Spike glycoprotein and human peptides [134].

##### 3.4.1.3. Translation and antigen presentation by MHC class I by platelets

Platelets have the potential for mRNA translation and protein synthesis intracellularly [135]. Platelets also present peptides via MHC class I. If SARS-CoV-2 infects platelets, mRNA translation is possible, and subsequent synthesis of Spike protein may arise. Alternatively, platelets might present SARS-CoV-2 Spike peptides in the context of MHC class I. These

mechanisms can lead to direct lysis of platelets by cytotoxic T cells and/or natural killer cells, causing a decrease in platelet count and thrombocytopenia.

#### 3.4.1.4. PF4 complex

In most of the thrombotic events reported post-vaccination, findings were consistent with antibodies against platelet factor 4 (PF4) complex. In these cases, higher platelet-activating antibodies were identified by enzyme-linked immunosorbent assay (ELISA). PF4 complex is the antigen in heparin-induced thrombocytopenia (HIT), an unusual autoimmune reaction seen after administering the anticoagulant heparin. Thus in thrombotic events, post-vaccination, platelet-activating antibodies against PF4 clinically mimic heparin-induced thrombocytopenia [136]. In addition, a detailed study found that anti-PF4 antibodies form a complex with the CXCL4 platelet factor and bind to the Fc $\gamma$ -receptor IIa on thrombocytes. This platelet consumption leads to thrombocytopenia/ITP [8, 137] (Figure3).

#### 3.4.1.5. Fc $\gamma$ RIIa polymorphism

Fc receptors for IgG are expressed by many immune cell types and are involved in executing and regulating antibody-mediated immune responses. Human platelets specifically express Fc $\gamma$ RIIa, known as CD32a which is an activating receptor with low affinity for monomeric IgG but a high affinity for IgG opsonized immune complexes (ICs). Fc $\gamma$ RIIa is polymorphic and expresses two different allelotypes Fc $\gamma$ RIIa-H131 (Histidine), with a higher affinity for human IgG2 and IgG3, while Fc $\gamma$ RIIa-R131 (arginine) has a lower affinity towards ICs. This polymorphism is due to a single base substitution of adenine to guanine at nucleotide position 494 [138, 139]. Thus, individuals expressing H131 allelic form of Fc $\gamma$ RIIa receptors on their platelets could be more susceptible to ITP via HIT, autoantibodies, or IC-mediated activation. It should be noted that the association of Fc $\gamma$ RIIa H131 with platelet activation was found in SLE [140], but not in HIT[141].

#### 3.4.1.6. Tissue plasminogen activator (tPA)

tPA is a serine protease found on endothelial cells, catalyzing the plasminogen to plasmin reaction and helps break down blood clots. ChAdOx1 nCoV-19 vaccine contains a leader sequence for tPA to create a boosting effect on Spike protein production, tailored to induce a more robust immune response [142]. However, the ChAdOx1 nCoV-19 vaccine has been shown to induce more blood clot-related issues, as mentioned before. We hypothesize that this could be partly due to the enhanced production of tPA, which can lead to hyperfibrinolysis and cause an increase in bleeding or vascular permeability.

#### 3.4.1.7. Human proteins/peptides present in vaccines

Post-vaccination ITP can also be elicited by other elements like trace amounts of proteins from the culture media, adjuvants, preservatives, and formulation carriers. Accordingly, as reported previously, it can be presumed that antibodies against those other elements can attach many platelets and/or other immune cells and trigger a cross-reaction like a natural infection can [143, 144].

#### 3.4.2. History of autoimmune disorders and (v)ITP

Autoimmune disorders cause abnormal activity of the immune system, rather low or high. In highly active or overactive autoimmune disorders the body attacks self tissues or cells [145]. VAERS and EMA were also reported from case studies, many of those with severe vaccine effects have a history of autoimmune disorders, including Type-1 diabetes, rheumatoid arthritis, hyper or hypo thyroidism.

Individuals having chronic autoimmune inflammatory diseases (AIIDs) have concerns about vaccination. However, COVID-19 could trigger AIID flares and is linked to autoimmune disorders and their consequences [146]. In genetically susceptible individuals, autoinflammatory dysregulation and other autoimmune mechanisms such as epitope spreading, antigen presentation, cytokine production, polyclonal activation of B cells, and bystander activation might also contribute to severity linked to COVID-19 vaccines [147]. According to some cases reported in VAERS, severe thrombocytopenia in some patients may have been induced by enhancing macrophage-mediated clearance or impaired platelet production as part of a systemic inflammatory response to COVID-19 vaccination [126, 130].

The benefits of vaccination in preventing COVID-19 must be emphasized, and WHO, EMA and MHRA assured that the benefits of the vaccine far outweigh the thrombotic risks [148]. However, we cannot deny the possibility that COVID-19 vaccines can trigger ITP, though reported rarely. Furthermore, it is hard to distinguish between the events of accidental ITP and post vaccination ITP. Thus, concentrated monitoring will be needed to identify the true incidence of ITP post-vaccination [126], and the primary emphasis should be to study patients with severe thrombotic events. A better understanding of how the vaccines induce these abnormal autoimmune responses may provide insight into vaccine duration and risk of reoccurrence of thrombosis, thereby improving vaccine design.

#### 4. Conclusions

Both mRNA based as well as viral vector vaccines with the genetic information for the SARS-CoV-2 Spike protein have turned out to induce an efficient humoral and cellular immune response. The design of these vaccines ensures that the antigens are presented to CD4+ T cells in MHC class II and to CD8+ T cells in MHC class I. The role of unconventional T cells, and presentation of vaccine antigens to these unconventional T cells is incompletely understood at the moment. In rare cases, immune mediated side-effects are observed, in particular hypersensitivity reactions, including anaphylaxis, and the combination of thrombosis and thrombocytopenia. Delineation of the molecular mechanisms underlying these adverse effects will be required to reduce the incidence and to develop adequate testing and treatment modalities.

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