# Uncharted Territory

## Early Events After Adenovirus 26-Based Vaccination

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# Uncharted Territory: Early Events After Adenovirus 26-Based Vaccination

Onbekend Terrein: De Initiële Gebeurtenissen Na Vaccinatie Met Op Adenovirus 26-Gebaseerde Vaccins

(met een samenvatting in het Nederlands)

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*A mis padres*

CHAPTER 1

# General introduction

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# 1. Vaccines



Since the development of the smallpox vaccine in 1798, vaccines have played a crucial role in improving human health [1]. Routine mass immunization programs in developed countries have led to an almost complete reduction in the incidence and mortality associated with vaccine-preventable diseases, such as diphtheria, smallpox, polio, measles, mumps, and rubella [2-4]. The impact of vaccination has become even more evident to the public during the COVID-19 pandemic, with an estimated 14.4 million deaths prevented through vaccination between 8th December 2020 and 8th December 2021 alone [5]. In addition to reducing the mortality associated with vaccine-preventable diseases, the efforts to prevent and eradicate diseases have a socio-economic impact, with an estimated 280 billion US dollars in social and economic benefits from vaccination between 2001 and 2020 worldwide [6,7].

Vaccines confer protection against specific pathogens by triggering an immune response in the host. The first vaccines following the smallpox vaccine were produced in the late 19th century through the inactivation or weakening of pathogens or toxins. Prophylactic and therapeutic vaccines have evolved toward new approaches in the second half of the 20th century, such as viral vectors (adenovirus, modified vaccinia virus Ankara (MVA), vesicular stomatitis virus, etc.), subunit vaccines, and genetic vaccines (e.g., mRNA vaccines) [1,8-10].

Replication-incompetent adenoviral vectors have been broadly used in the field of gene therapy, and the knowledge gained from these studies has led to a good understanding of the structure of the genome and its manipulation to insert foreign genes to induce immune protection against disease [11]. More than 400 gene therapy clinical trials with human adenoviral vectors have been initiated, most of them for cancer treatment [12]. Moreover, an oral live adenovirus vaccine (Ad4 and Ad7) has been broadly used by the US military against acute respiratory disease since 2011 [13]. The first prophylactic vaccine based on a replication-incompetent adenoviral vector (Ad26.ZEBOV), in combination with an MVA component in a two-dose regimen, was approved by the European Medicine Agency (EMA) in 2020 for use against Ebola virus disease [14]. More recently, adenovirus-based vaccines (Ad26, Ad5, and ChAdOx1) played a crucial role in the COVID-19 pandemic [15-17].

Adenoviral vectors present certain characteristics that make them excellent vaccine platforms, such as the induction of transgene-specific immunity, large packaging capacity (up to 35 kbp of transgenic sequence), and broad tropism in dividing and non-dividing cells [18-23]. Additionally, adenovirus-based vaccines are more cost effective and can be formulated with a higher thermostability than mRNA vaccines [24].

However, there is a gap in the knowledge regarding early events in the host following adenovirus-based vaccination. Early events, such as transgene expression or innate immune responses, play an important role in shaping adaptive immune responses and the development of adverse effects [25,26]. Therefore, further investigation of early events after vaccination is of great importance to understand the mode of action of adenovirus-based vaccines.

## 2. Adenovirus

The origin of the family name *Adenoviridae* derives from the human adenoid tissue culture from which the cytopathogenic agent was first isolated by Rowe et al. in 1953 [27,28]. Shortly thereafter, Hilleman and Werner isolated the same viral agent from adenoids of patients with acute respiratory disease in 1954. The family *Adenoviridae* can be subdivided into 5 genera, depending on the natural host of infection: *Mastadenoviridae*, comprising viruses that only infect mammals; *Aviadenoviridae*, comprising only bird adenoviruses; *Ichtadenoviridae*, which includes viruses that can only infect fish; *Atadenoviridae*, comprising viruses that present a broad host range (birds, ruminants, reptiles, and opossums); and *Siadenoviridae*, comprising viruses that can infect birds, reptiles, and amphibians [29,30].

There are 114 known types of human adenoviruses, classified into 7 species (A to G) (**Figure 1**, species classification up to human Ad55) [31]. Up to human Ad51, adenoviruses have been classified into serotypes by cross-neutralization, whereas for the newer types, the classification is mainly based on genomic analysis (new sequences or recombinant phylogeny in genes encoding major capsid proteins). Based on these classification criteria, several non-human primate (NHP) adenoviruses have been included in human adenovirus species due to similarities in genetic analysis, suggesting cross-species transfer [32,33]. Most human adenoviruses belong to species D, and the main factor contributing to the diversity within this group is the recombination of genes encoding capsid proteins (hexon, penton, and fiber) [31].

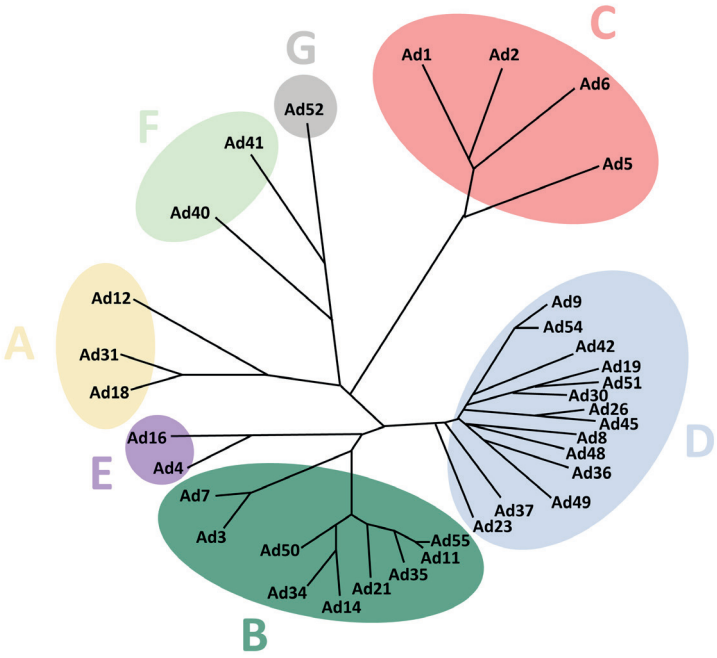


**Figure 1. Phylogenetic tree of human adenoviruses (adapted from Geisbert et al. [34]).** The tree was constructed using the neighbor-joining method (Clustal X package) on the adenovirus hexon sequences.

The most prevalent adenovirus types in humans are 1-5, 7, 21, and 41, but their prevalence differs across geographic regions and changes over time [35]. Human adenoviruses are commonly associated with upper or lower respiratory tract infections, although they may also be associated with gastrointestinal, urogenital, ocular, or neurological symptoms. Adenoviruses display different tissue tropisms and clinical manifestations [36-40]. Adenovirus infections are more common and often more severe in children, due to immature humoral immunity, and immunocompromised populations [35].

Both human and NHP adenoviruses have proven to be valuable tools in vaccine development and gene therapy strategies [41-46]. The wide variety of adenovirus types provides the opportunity to select those with advantageous characteristics in terms of vectorization and immunogenicity for human vaccination, such as their tropism (see **4.1** and **4.2**) or low preexisting immunity in human populations (see **3.2.2.**).

## 2.1. Viral structure

Adenoviruses are non-enveloped double-stranded DNA viruses that consist of an icosahedral 65-90 nm capsid, containing 3 major proteins (hexon, penton base, and

fiber) and 4 minor proteins (IIIa, VI, VIII, and IX) (**Figure 2**) [47,48]. Six core non-structural proteins are associated with the genome of the virus (V, VII, μ, Iva2, terminal protein (TP), and adenovirus protease (AVP)) [47,49]. The viral structure of adenoviruses has been mainly studied for Ad2 and Ad5. Although this viral structure is common to most adenovirus types, there can be variations in their amino acid sequences and structural features among different adenovirus types [50].

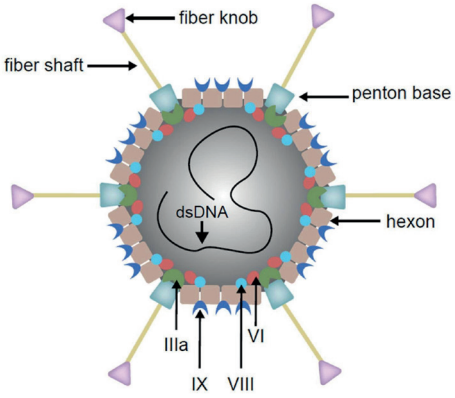


**Figure 2. Adenovirus virion structure with main capsid proteins [25].**

### *2.1.1. Genome*

The genome of adenoviruses is composed of linear double-stranded DNA molecules ~26-45 kb in size flanked by inverted terminal repeats (ITR) [48]. The adenovirus genome is organized into early and late transcription units (**Figure 3**). The transcription of adenoviruses occurs in the nucleoplasm and can be divided into early phase and late phase [51].

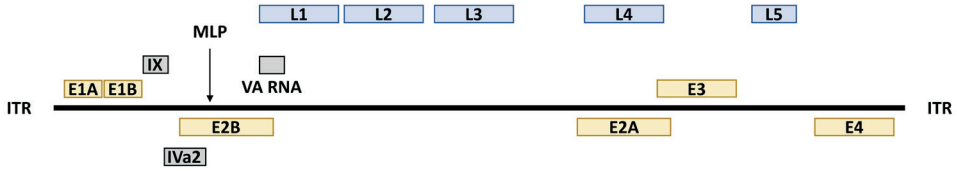


**Figure 3. Representation of a prototype human adenoviral genome with transcriptional units.** Transcriptional units of the early phase are denoted with an "E" and those of the late phase are denoted with an "L". Major late promoter (MLP) of late proteins is indicated by an arrow. Proteins IX and IVa2 are transcribed immediately after early genes, whereas VA RNAs are transcribed in the intermediate/late phase. The adenoviral genome is flanked by inverted terminal repeats (ITR). Transcriptional units located above the genome line are encoded on the positive DNA strand, and transcriptional units below the genome line are encoded on the minus DNA strand.

In the early phase, the proteins encoded in the early transcription units regulate the switch to the S phase (necessary for DNA synthesis), prevent antiviral responses, and produce the proteins required for viral replication [52].

The first transcription unit to be expressed is E1A. Proteins derived from the transcription of E1A are key for the transcription of other early factors (E1B, E2A, E2B, E3, and E4) [53,54]. These factors have many functions, including hijacking cellular processes such as apoptosis (through E1B and the prevention of p53 function) or the recognition of infected cells by cytotoxic T lymphocytes (blockage of histocompatibility complex class I through E3gp19K) [55,56]. Moreover, early factors influence viral mRNA transport, splicing and translation [57-61].



The intermediate units IVa2 and IX are transcribed at the beginning of the DNA synthesis [62]. IVa2 is involved in capsid assembly, viral genome encapsulation, and enhancement of transcription of the major late transcriptional unit MLTU [63-67]. Protein IX is a structural protein of the capsid that has diverse regulatory functions despite being dispensable in the production of virions *in vitro* [68,69].

In the late phase, high levels of silenced adenovirus genomes are produced and packed into new virion particles. Replication of the viral genome (late phase) is initiated through the action of the viral precursor to the terminal protein pTP, adenoviral DNA polymerase and DNA-binding protein DBP, nuclear factor I NFI and CCAAT box transcription factor CTF, nuclear factor III NFIII and octamer-binding transcription factor 1 Oct-1, and cellular type I DNA topoisomerase NFII [70-74].

The major late promoter (MLP) is transcribed, processed, and enhanced by IVa2; and drives the transcription of a primary transcript [66,67]. The primary RNA molecule is processed through splicing and polyadenylation, producing 5 different families of late mRNA (L1-L5) [75]. This is necessary for viral replication because the L4 protein 100 K blocks host cell translation by inhibiting the function of cap-initiation complexes [76,77]. Late mRNA transcripts encode structural proteins and proteins involved in viral assembly and other regulatory functions [52]. Virus assembly takes place in the nucleus [78]. Cell lysis and subsequent virion release are driven by the adenovirus death protein ADP (encoded in E3) at approximately 30 hours post-infection [79,80].

Human adenoviruses express a diverse array of non-coding RNA species, including virus-associated RNAs, microRNAs, and other non-coding transcripts [81,82]. They play a role in viral replication ([83]), gene expression regulation ([84]), and the prevention of early innate immune responses (e.g., by inhibiting dsRNA-activated kinase and interacting with host proteins RIG-I and OAS1 [81,85-87]).

#### *2.1.2. Major and minor capsid proteins*

Hexon monomers are the most abundant protein in the capsid. Hexon monomers self-associate into trimeric hexagons that form a 12 homotrimer structure that comprises each face of the icosahedron. Hexon monomers have a conserved base and a hypervariable region that faces the exposed surface of the icosahedron. This region is composed of 7 flexible, serotype-specific loops (HVR1-7), and their location facilitates interactions with neutralizing antibodies, receptors, proteins, and cells [88,89]. Hexon trimers directly associate with 5 penton monomers at the vertices of the structure. These 5 penton monomers form a ring-like pore in the center of the structure, where the fiber trimers bind through the N-terminal region of each fiber. The main role of the fiber protein is to interact with cellular receptors, leading to attachment of the virion to the cell surface [90]. Penton proteins are highly conserved among adenovirus types, except for the Arg-Gly-Asp (RGD) motif region, which is a protein loop involved in cell interaction and transduction. The RGD region is surrounded by linker peptides in all human adenoviruses, except for Ad40 and Ad41 (species F) [91-94].

Minor capsid proteins are highly conserved and are essential for the assembly and structural stability of the capsid [95,96]. For example, protein IX is a flexible protein exclusive to the *Mastadenovirus* genus and is key to the stabilization of hexon subunit interactions, capsid binding, virus stability properties, and full-length genome packaging, although viral particles can be produced without it [97-99].

## 3. Adenoviral vectors as prophylactic vaccines

### 3.1. The development of adenovirus-based vaccines

The molecular and biological characteristics of adenoviruses make them effective tools for gene delivery. Adenoviruses present a small, well-characterized genome, broad cell tropism in dividing and non-dividing cells, mild disease in humans, and high-yield production in cell culture [100-102]. Adenoviral vectors are currently used as prophylactic and therapeutic vaccines as well as for gene therapy in humans [14-16,41,42].

There are 2 types of adenoviral vectors: non-replicating and replication-competent [41]. In the field of prophylactic vaccines, the use of adenoviral vectors is limited to non-replicating vectors. These vectors are engineered through genetic modifications of the virus to produce a non-replicating virion that can carry a transgene of interest. Adenoviral vectors used as vaccines present modifications that include the deletion of the E1A and E1B regions, which are essential for replication, the insertion of a transgene of interest that will drive the protective immune response, and the insertion of a high-activity promoter, such as the cytomegalovirus immediate early promoter, to induce

high levels of transgene expression [41,103]. Most adenoviral vectors used as vaccines also present deletions of E3 genes to prevent the elimination of transduced cells by the immune system and provide additional packaging space [41,104].

To produce this type of vector, the E1 genes necessary for viral replication need to be provided by the cell line in which they are produced (e.g., HEK293, 911, or PER.C6) [105-107]. E3 genes are not essential for replication and do not need to be complemented by the production cell line [104]. During viral propagation in the cell line, E1-positive replication-competent adenoviral vectors may arise at very low frequencies due to homology sequences between the cell line and viral genome, allowing for a double crossover recombination event to occur, an issue that is reduced in frequency in newer E1-complementing cell lines [108].



The development of adenovirus-based vaccines was originally focused on species C Ad5, which has proven to induce potent immune responses against different transgenes in preclinical models and Ad5-seronegative humans [109,110]. However, the results from the HIV Ad5 vaccine candidate in phase IIb of the STEP clinical trial revealed reduced immunogenicity in Ad5 pre-exposed vaccinees [111-113]. Consequently, research on new adenovirus-based vaccines has focused on alternative adenovirus types with lower or no prevalence in humans, such as NHP adenoviruses or less prevalent human adenoviruses. Vectors derived from NHPs, such as chimpanzee or gorilla adenoviruses, can transduce human cells and induce potent cellular and humoral responses in preclinical models and humans.

## 3.2. Immunogenicity

### *3.2.1. Transgene-specific immune responses*

Adenovirus-based vaccines induce potent immune responses against the transgene of interest. Adenovirus-based vaccines stimulate transgene-specific CD8+ T-cell responses in preclinical models and humans against different pathogens, including HIV, SARS-CoV-2, and Ebola virus, and have proven superior in inducing cellular immunity compared with other vaccine platforms, such as DNA vaccines or other viral vectors [43,45,46,114-125].

The development of T-cell responses after vaccination is usually divided into priming and expansion of CD8+ T-cells, contraction of the primary effector population, and maintenance of a memory population [126-129]. Interestingly, Ad5 vectors induce memory inflation in mice, a phenomenon in which the primary effector response does not contract, and the expanded CD8+ T-cell populations are maintained long-term [130-134]. One of the key drivers of memory inflation is low-level persistence of the

transgene [130,135]. While memory inflation has not been extensively characterized in models other than mice for adenoviral vectors, durable cellular responses have been described in humans after adenovirus-based vaccination, including Ad26 and ChAdOx1 [136,137].

Historically, adenoviral vectors were mainly used to induce T-cell responses but, due to the surge of this vector platform in the context of prophylactic vaccines, further characterization of the humoral responses triggered by adenoviral vectors has been performed [130]. Clinical trials of Ad5, Ad26, and ChAdOx1-based vaccines have shown persistence of transgene-specific antibodies up to 6 months after one-dose vaccination and induction of neutralizing antibodies [130,136,138-142].

Few studies have investigated the early events leading to the induction of transgene-specific cellular and humoral responses after adenovirus-based vaccination, although some have suggested the role of specific cell populations. Depletion of macrophages has been shown to impair transgene-specific antibody and T-cell responses after Ad5 administration in mice, suggesting a role for this cell type in B-cell priming, CD4+ T-cell priming, or both [143]. Natural killer (NK) cell activation has been associated with stronger neutralizing responses after ChAdOx1 vaccination in humans and rhesus macaques; however, the mechanism underlying the possible involvement of NK-cells in the development of adenoviral vector-induced antibody responses remains unknown [144,145].

***3.2.2. Anti-vector immune responses***

Adenovirus-based vaccines also induce immune responses against the adenoviral particles. Anti-vector immune responses consist of both anti-vector antibodies and cellular responses. High levels of preexisting anti-vector responses have been reported to impair immunogenicity against the transgene of interest in Ad5 vaccinees [111,146]. Preexisting Ad5 anti-vector immunity can lower vaccine effectiveness by blocking transduction and transgene expression [147]. Preexisting anti-vector immunity can derive from a past adenovirus infection or a previous adenovirus-based vaccination. The high prevalence of Ad5 infections in humans makes this vector less attractive for use as a prophylactic or therapeutic vaccine. Preexisting immunity to other adenovirus types, such as Ad26 or ChAdOx1, due to natural infection is rare, and if so, lower anti-vector antibody titers are detected [45,148-150]. However, preexisting immunity to these vectors has significantly increased due to the extensive use of these vaccine platforms as prophylactic vaccines against COVID-19 in humans. A recent study assessed the influence of subsequent Ad26-based vaccination on transgene-specific immune responses in NHPs [151]. No clear consistent effect of preexisting immunity was observed, aligning with clinical data from homologous Ad26 or ChAdOx1 regimens

showing consistent boosting of transgene-specific immune responses after the second dose [43,151-157]. The effect of preexisting immunity on transgene-specific immune responses differs across adenovirus types; however, the mechanisms underlying these disparities remain unexplained. Adenoviruses present differences in cellular receptors, intracellular trafficking, and innate and antiviral responses [25,158] that may play a role in their sensitivity to preexisting immunity.

### 3.3. Adenovirus-based prophylactic vaccines in the clinic



The first adenovirus-based prophylactic vaccine was approved in 2020 (Ad26.ZEBOV-GP, or Zabdeno) [14]. Ad26.ZEBOV-GP is a vaccine approved for the use in adults and children against Ebola virus disease caused by Zaire ebolavirus. Ad26.ZEBOV-GP is used in combination with an MVA component (MVA-BN-Filo, or Mvabea). The vaccination regimen was well tolerated and immunogenic, and the optimal interval between vaccinations in terms of the magnitude of humoral responses was 56 days. Antibody levels persisted for at least one year after the first vaccination in most participants [159].

Since 2020, 6 other adenovirus-based vaccines have been approved for use in humans against COVID-19 disease. Four are administered intramuscularly: Convidecia (Ad5) [146], Sputnik V (Ad26 and Ad5) [17,160], Jcovden (Ad26) [15,152,161], and Vaxzevria (ChAdOx1) [16,157]; and 2 are administered intranasally: iNCOVACC (ChAd36) [162], Convidecia Air (Ad5) [163]. All these vaccines elicited potent immune responses, including the induction of anti-spike protein antibodies and spike-specific CD8+ T-cell responses.

## 4. Mode of action of adenovirus-based vaccines

Adenoviral vectors retain some characteristics of the adenovirus in terms of cellular entry, intracellular trafficking, and triggering of antiviral pathways (for some serotypes). The vector itself acts as an adjuvant, eliciting innate immune responses that can increase or diminish the immunogenicity against the transgene they encode.

### 4.1. Cell entry

Adenoviral vectors can efficiently transduce a wide range of dividing and non-dividing cells. Adenoviral vectors attach to the cell via the interaction of the fiber protein with its primary cellular receptor (coxsackie adenovirus receptor CAR for Ad5) (**Figure 4**). This leads to the anchoring of the viral particles on the cell surface and the interaction of the penton protein with cellular integrin molecules, initiating clathrin-dependent endocytosis [164,165]. The interaction between adenoviral particle and integrin is primarily facilitated by the penton RGD motif, which binds integrins, such as αvβ3 and

αvβ5 [166]. The interactions with CAR and αv integrins cause mechanical stress, leading to the tearing of fiber proteins from the virion. This tearing exposes protein VI and initiates the disassembly of the virion [158,167,168]. The remaining virion escapes the endosome and is trafficked along the cellular microtubules through the cytoplasm into the nucleus [169]. Once the viral particle reaches the nuclear pore complex, the capsid is disassembled, and the viral DNA enters the nucleus but does not integrate into the host cell DNA [170,171].

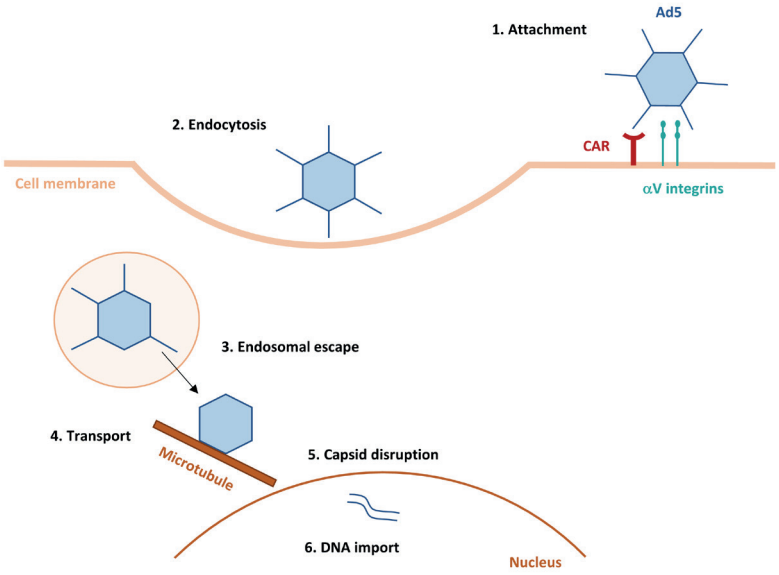


**Figure 4. Human Ad5 cell entry. Schematic outline of the cellular entry mechanism.**

The cell entry process has been mainly described for human C-type Ad5; however, key differences have been identified among adenovirus types, including primary receptors, internalization process, and endosomal escape [25]. These differences can have an important impact on vector tropism, innate immune recognition, transgene expression, and adaptive immune responses. The adenovirus-type differences in this process and their impact are extensively described in **Chapter 2**.

## 4.2. Cellular interactions

To understand how adenovirus-based vaccines prime the host to induce an immune response against the transgene, it is important to elucidate the interactions of adenoviral particles with host cells. Adenovirus-based vaccination leads to the transduction of host cells and the subsequent recruitment of immune cells to the site of immunization.

The tropism of adenoviral vectors is dependent on their interaction with a cellular receptor and the consequent cell entry process, which differs across adenovirus species [40,158]. The primary cellular receptor for species C adenoviruses, such as Ad5, is CAR, whereas other adenovirus species, such as B and D, utilize other receptors (CD46, DSG-2, etc.) [172-174]. Some proteins on the virion surface, such as hexon HVRs or the fiber knob protein, can be modified to alter the tropism and transgene expression of adenoviral vectors [175-181].



Target cells and infiltrating cells after adenovirus-based vaccination in preclinical models have been reported for chimpanzee type-C adenovirus 155 (ChAd155) [182]. Transgene mRNA expression was detectable in the mouse muscle (site of immunization) from 6 hours post-administration, which led to the release of cytokines that promoted monocyte/macrophage chemotaxis to the muscle. At 6 hours post-administration, transgene expression was also detectable in draining lymph nodes of mice, indicating the potential transport of free adenoviral particles, free antigen, or migration of antigen-loaded phagocytic or infected cells. At 24 hours after vaccination, hematopoietic and non-hematopoietic cells (likely muscle-resident non-immune cells) were positive for the transgene protein (GFP) in the muscle, and dendritic cells, monocytes, and B-cells were positive in the draining lymph nodes [182]. Other studies have demonstrated that type-C Ad5 can transduce monocytes, low levels of dendritic cells, myoblasts, and endothelial cells *in vitro* [183-186]. A comprehensive characterization of the target and infiltrating cells *in vivo* is lacking for type-C and other adenovirus species. Understanding the target cell populations could allow retargeting of adenoviral vectors to more specific populations of antigen-presenting cells, leading to increased or modulated immunogenicity against the transgene product.

### 4.3. Innate immune responses

The development of transgene-specific immune responses after adenovirus-based vaccination requires the activation of innate immune signaling pathways [187]. The interplay between innate immune responses, such as the activation of pattern recognition receptors, cytokine production, and immune cell recruitment, shapes the adaptive immune responses after adenovirus-based vaccination. Innate immune recognition is a necessary adjuvating response, but an excess of proinflammatory signals can dampen adaptive immune responses either directly by clearing transduced cells or indirectly through cytokine signaling [116,188,189].

The balance between innate immune suppression and stimulation is essential for the development of potent adaptive immune responses after adenovirus-based vaccination [25]. The quality and magnitude of innate immune responses depend on the adenovirus

type [190]. Innate immune responses, adenovirus-type differences, and their effects are extensively described in **Chapter 2**.

## 5. Adverse effects after vaccination

Any health problem after vaccination is considered an adverse event. An adverse event is considered a true adverse reaction, also known as a side effect, when it is related to the vaccine [191]. Any licensed vaccine can cause side effects, but most of these are mild or moderate and disappear within a few days after vaccination. Intramuscular vaccination is commonly associated with local reactions (such as tenderness, pain, and bruising at the injection site) and systemic reactions (such as fatigue, nausea, headaches, muscle pain, and fever) [192]. Licensed prophylactic vaccines are generally well tolerated but can cause rare severe systemic adverse effects, the prevalence of which may vary among vaccine platforms [193,194].

The global COVID-19 pandemic and the high morbidity and mortality associated with these infections led to the rapid development of a variety of vaccines. Aside from the adenovirus-based vaccine against Ebola (Zabdeno), some of these vaccine platforms (adenovirus-based and mRNA) had not been previously authorized, and the post-marketing safety profiles in large populations were not known [14,195].

The mass COVID-19 vaccination campaigns led to the detection of rare adverse effects across vaccine platforms that had not been detected in clinical trials due to their low incidence. Guillain-Barré syndrome (GBS) is a neurological disorder in which the body's immune system damages the nerve cells [196]. Although most patients fully recover from GBS, some may experience permanent nerve damage. GBS is mainly associated with bacterial and viral infections; however, it can be associated with vaccination on very rare occasions. GBS has been associated with influenza vaccination and adenovirus-based COVID-19 vaccination (Ad26.COV2.S (Jcovden) and ChAdOx1 (Vaxzevria)) [15,16,197]. The incidence of GBS after adenovirus-based COVID-19 vaccination is less than 1 in 10.000 vaccinees. Inflammatory diseases of the heart, such as myocarditis (inflammation of the heart muscle) and pericarditis (inflammation of the membrane around the heart), have been reported in up to 1 case in 10.000 people after COVID-19 mRNA vaccination and at a lower incidence after adenovirus-based COVID-19 vaccination [15,16,198,199]. Patients with these inflammatory conditions usually respond well to treatment and achieve complete recovery. Additionally, adenovirus-based COVID-19 vaccination (Ad26.COV2.S (Jcovden) and ChAdOx1 (Vaxzevria)) has been associated with rare cases of vaccine-induced immune thrombotic thrombocytopenia (VITT) [15,16]. The incidence

of VITT after vaccination is very low (<1 in 10.000 vaccinees); however, the mortality rate is high, although immediate treatment can significantly reduce it [200,201].

## 5.1. Vaccine-induced immune thrombotic thrombocytopenia

VITT is a rare adverse effect induced by adenovirus-based COVID-19 vaccination, characterized by thrombocytopenia and thrombosis, often in atypical anatomical locations, and the presence of antibodies against platelet factor 4 (PF4) [202]. VITT has been reported in approximately 2.3 to 5.5 cases per 1 million vaccinees after Ad26.COV2.S dosing, depending on the definition of the syndrome [203] (Centers for Disease Control, USA [204,205] and Prevention of Pharmacovigilance Risk Assessment Committee, EMA [206]). The estimated incidence of VITT is 8.1 per 1 million vaccinees after the first dose of ChAdOx1 and 2.3 per 1 million vaccinees after the second dose [207]. VITT occurs in the period from 5 to 43 days [204,208,209] after the first dose of ChAdOx1 nCoV-19 [210] or Ad26.COV2.S [211]. It has sporadically been reported after COVID-19 vaccination with mRNA-1273 [212], inactivated COVID-19 [213,214], or Gam-COVID-vac vaccines [215].



### *5.1.1. Mechanism of induction of vaccine-induced immune thrombotic thrombocytopenia*

VITT is characterized by the induction of IgG antibodies against PF4, which can activate platelets in the absence of heparin. IgG antibodies bind to a site on the PF4 molecule that overlaps with the binding site for heparin [210,216-218]. VITT was originally compared to heparin-induced thrombocytopenia (HIT), a syndrome in which heparin treatment leads to an anti-PF4 response; however, the sites of thrombosis are remarkedly different [217,219]. Moreover, in HIT, IgG antibodies bind to PF4-heparin complexes to form immune complexes that can bind and activate platelets, leading to platelet aggregation and thrombotic events [220-222]. In this regard, VITT is more similar to autoimmune HIT (aHIT), where antibodies can form and activate platelets in the absence of heparin [223,224].

The mechanism behind the induction of anti-PF4 antibodies in VITT remains unclear. It has been hypothesized that PF4 binds to polyanions such as heparin, hypersulfated chondroitin sulfate, DNA, RNA, polyphosphate, or bacterial-wall components [225]. IgM could bind to the PF4/polyanion complex and activate complement, similar to what has been described for HIT [226]. This would result in the binding of the PF4/polyanion complex to cognate immunoglobulin receptors for PF4 on B-cells [200,227,228]. Cross-linking of B-cell receptors along with a proinflammatory co-signal would trigger the release of anti-PF4 antibodies into the circulation [229,230]. Anti-PF4 antibodies are induced by day 5 after ChAdOx1 vaccination, suggesting the activation of preexisting PF4-reactive memory B-cells [229,231]. Binding of the polyanion to PF4 can cause a conformational change that exposes the binding site for anti-PF4 antibodies [200,232].

PF4/anti-PF4 clusters can then activate platelets, induce platelet/neutrophil aggregates, and stimulate NETosis by neutrophils [229,230,233,234].

Some of the factors that might contribute to the development of anti-PF4 antibodies (through the induction of PF4 release or formation of a PF4/polyanion complex) are the vaccine-encoded spike protein and/or adenoviral vaccine components following adenovirus-based COVID-19 vaccination (**Figure 5**).

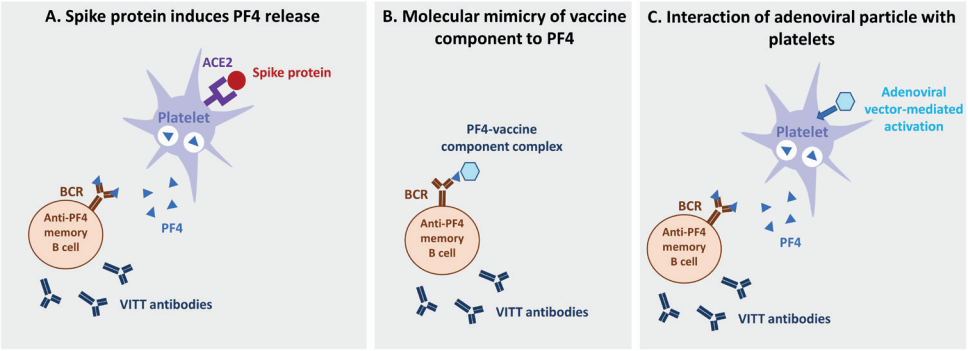


**Figure 5. Potential mechanisms underlying the induction of VITT antibodies. A**) The spike protein may trigger PF4 release by binding to platelets via the ACE2 receptor. **B**) The formation of a complex PF4-adenoviral vector may trigger the production of anti-PF4 antibodies by PF4-reactive memory B-cells. **C**) Adenoviral particles might interact with and activate platelets, leading to PF4 release and anti-PF4 antibody production. ACE2: Angiotensin-converting enzyme 2; BCR: B-cell receptor; VITT: vaccine-induced immune thrombotic thrombocytopenia.

#### *5.1.1.1. Spike protein*

Adenovirus-based COVID-19 vaccines are based on recombinant non-replicating vectors that encode the full-length spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), based on the Wuhan strain [121,152]. The spike protein is a large molecule (180–200 kDa) and contains 2 subunits: S1, which serves as the receptor-binding domain, and S2, which mediates the membrane fusion of the protein and anchors the protein in the cell membrane. S1 comprises an N-terminal signal peptide (SP), an N-terminal domain (NTD), and a receptor-binding domain (RBD). S2 contains a fusion peptide (FP) domain, an internal fusion peptide (IFP), 2 heptad repeat domains (HR1 and HR2), a transmembrane domain, and a C-terminal domain [235-240]. Cleavage of the spike protein of SARS-CoV-2 into S1 and S2 is a crucial step in viral entry into the host cell and must occur prior to viral fusion with the host cell membrane [238]. To stabilize the prefusion conformation and facilitate vaccine design and structure determination, proline substitutions can be added to the S2 domain [241-246]. Additionally, the furin site can be mutated to prevent cleavage.

Ad26.COV2.S expresses a membrane-bound spike protein that contains 2 stabilizing proline substitutions and mutations in the furin cleavage site that preserve the prefusion conformation of the protein [121]. All other COVID-19 vaccines licensed in Europe and North America, aside from NVX-CoV2373, contain a wild-type furin cleavage site, which has been shown to result in shedding of the S1 portion of the spike protein in the plasma of mRNA-1273 vaccinees [121,247].

While there are no studies to date elucidating the exact mechanism of VITT, there are several reports pointing toward the potential contribution of the spike protein in the development of this multifactorial syndrome. The spike protein has been shown to cause vascular damage in hamsters [248] and has been detected within the thrombus and in the adjacent vessel wall in patients with VITT-induced cerebral venous thrombosis [249]. Endothelial cells may be activated through the binding of the spike protein to the angiotensin-converting enzyme 2 (ACE2) receptor, thereby recruiting and activating platelets [250]. At the same time, platelets may be directly activated by the circulating spike protein via the ACE2 receptor (**Figure 5A**). Platelets would then release PF4 molecules and drive the activation of memory PF4 B-cells. The activation of the B-cell along with an inflammatory coestimulus could then lead to an increased anti-PF4 antibody production [229,230]. The presence of pre-primed B-cell could be due to a previous exposure to polyanionic components of bacteria or viruses.



The spike protein has also been linked to the potential activation of inflammatory responses or coagulation pathways that might be linked to VITT. The spike protein may activate coagulation pathways through the binding of ACE2 on platelets and/or endothelial cells [251], and it has been shown to promote inflammation [252] and the formation of blood clots with proinflammatory activity [253].

#### *5.1.1.2. Adenoviral vector*

The incidence of VITT is higher in adenovirus-based vaccinees (Ad26.COV2.S and ChAdOx1) compared with mRNA-1273, inactivated COVID-19, or Gam-COVID-vac vaccines [210-215], indicating the possible involvement of adenoviral particles in the development of VITT.

One hypothesis focuses on the ability of the adenoviral vector to cluster PF4, which could form complexes that stimulate memory B-cells to produce anti-PF4 antibodies (**Figure 5B**). However, the formation of complexes has only been observed between ChAdOx1 and PF4 and not with Ad26.COV2.S, even though both vaccines are associated with VITT [254,255].

Additionally, accidental systemic exposure of adenoviral vectors (due to either accidental intravenous injection or leakage from the muscle injection site into the blood) may lead to the interaction of the vectors with platelets. It has been proposed that the binding of the adenoviral particles to platelets might trigger platelet activation and the subsequent release of PF4 and induction of anti-PF4 antibodies (**Figure 5C**). The ChAdOx1 adenoviral particle has been shown to directly bind platelets *in vitro*, and a recent study reported that intravenous, but not intramuscular, injection of a high dose of ChAdOx1 in mice resulted in platelet-adenoviral vector aggregate formation and platelet activation [256-258]. Thrombocytopenia was not observed after intravenous administration of another adenovirus type, ADV-004, suggesting that ChAdOx1-induced thrombocytopenia may be dependent on the adenoviral particle and adenovirus-type differences might be key in the development of VITT [258].

## 6. Scope of thesis and outline

The aim of the work described in this thesis is to understand the influence of early events on immunogenicity and adverse effects after Ad26 immunization in preclinical models.

In **Chapter 2**, early events (cellular entry, transgene expression, and innate immune responses) following adenovirus-based vaccination are reviewed. This chapter focuses on adenovirus-type differences and the effects of early events on transgene-specific adaptive immune responses.

In **Chapter 3**, we investigated the effect of transgene expression on adaptive immune responses after intramuscular administration of Ad26 in mice. We characterized the magnitude and duration of transgene expression after a single intramuscular administration of Ad26 in mice and evaluated the differences to Ad5.

In **Chapter 4**, the contribution of the transgene protein (spike) to the development of VITT after Ad26.COV2.S dosing was evaluated. We investigated the biodistribution, kinetic, and composition of the spike protein after intramuscular dosing with Ad26.COV2.S in preclinical models and clinical samples as potential drivers of VITT.

In **Chapter 5**, we studied whether intravenous dosing (as a model for accidental systemic exposure) of Ad26.COV2.S was associated with the development of VITT in preclinical models by evaluating clinical pathology parameters, histopathology findings, and systemic spike protein exposure compared with intramuscular dosing in rabbits.

**Chapter 6** comprises a summarizing discussion of the results described in this thesis, the introduction of a new mouse model to study target cells after vaccination, and the future perspectives of adenovirus-based vaccines.

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**CHAPTER 2**

# Cell entry and innate sensing shape adaptive immune responses to adenovirus-based vaccines

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## Abstract

Non-replicating adenovirus-based vectors have been successfully implemented as prophylactic vaccines against infectious viral diseases and induce protective cellular and humoral responses. Differences in the mechanisms of cellular entry or endosomal escape of these vectors contribute to differences in innate immune sensing between adenovirus species. Innate immune responses to adenovirus-based vaccines, such as IFN signaling, have been reported to affect the development of adaptive responses in preclinical studies, although limited data is available in humans. Understanding the mechanisms of these early events is critical for the development of vaccines that elicit effective and durable adaptive immune responses while maintaining an acceptable reactogenicity profile.

## Introduction

Adenovirus-based vaccines are engineered by modifying the adenoviral genome to be able to produce non-replicating virion particles capable of carrying a transgene of interest with the aim to induce a protective immune response against the transgene product. Licensed vaccines against COVID-19 disease (Ad26, Ad5, and ChAdOx1-based vector vaccines) and the disease caused by Ebola virus infection (Ad26 in combination with a Modified Vaccinia Ankara component) use this adenovirus-based vaccine technology [1-3]. Seminal studies have been conducted to understand the structure, tropism, and host response to adenoviruses; and these studies have also brought insights into the mode of action of adenovirus-based vectors. Here we describe the latest findings on cellular entry and innate immune responses to adenovirus, highlighting those adenovirus-based vectors authorized for human use as prophylactic vaccines. Moreover, the effect of cell entry and innate sensing mechanisms on adaptive immune responses is discussed.



## Adenoviruses

Adenoviruses are non-enveloped double-stranded DNA viruses that present an icosahedral capsid, containing 3 major proteins (hexon, penton base, and fiber) and 4 minor proteins (IIIa, VI, VIII, and IX) (**Figure 1**) [4,5]. The hexon is the most abundant protein. Hexon monomers consist of a conserved base and a hypervariable region (HVR), which is composed of 7 flexible, adenovirus type-specific loops (HVR1-7), whose location allows them to interact with different receptors, proteins, and cells [6,7]. Penton base proteins are highly conserved among adenovirus types except for the Arg-Gly-Asp (RGD) motif region, a type-specific protein loop involved in cell interaction and transduction [8-10]. Each vertex of the virion is composed of a pentamer of penton base protein in combination with a trimer of the fiber protein [9]. The main role of the fiber protein is to interact with cellular receptors leading to the attachment of the virion to the cell surface [11].

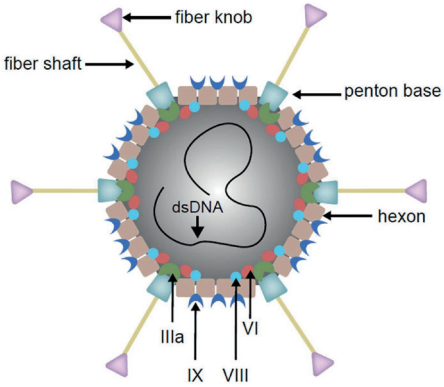


**Figure 1. Adenovirus virion structure with main capsid proteins.**

The adenovirus genome is organized into 5 early transcription units (E1A, E1B, E2, E3, E4), intermediate transcription units (including IX, IVa2, and E2 late), and a single late transcription unit that produces 5 mRNAs families (L1-L5) [12]. The replication process can be divided into an early phase and a late phase, separated by the initiation of viral DNA replication. In the early phase, low levels of transcriptionally active genomes express proteins that regulate gene expression for the cell to enter S phase (necessary for DNA synthesis), suppress antiviral responses and enable viral replication [13-16]. The goal of the late phase is the production of new virions by generating a high number of adenoviral genomes and structural and regulatory proteins involved in viral assembly and release [12,13].

There are over 70 different human adenoviruses described, which are classified into 7 species (A to G) according to cross-neutralization or, more recently, genetic homology. Non-human primate (NHP)-derived adenoviruses isolated from gorillas, bonobos, and chimpanzees have been classified into one of the 7 human adenovirus species based on genetic homology [17,18] as several have been vectorized for human use (**Table 1**) [19-21].

## Adenovirus-based vectors

Non-replicating adenovirus-based vectors are engineered through genetic modifications including deletions of the E1 region that encodes for proteins essential for viral replication, thereby creating space to insert a transgene of interest. Other modifications in the E3 and E4 regions, included in current vector generations, alter immunogenicity

toward the vector, facilitate manufacturing, and create additional space in the vector genome [22,23]

Adenovirus-based vectors are also used for gene therapy and therapeutic vaccination [24,25], but this review focuses on their use as prophylactic vaccines (**Table 1**), and these have a genome that mainly contains E1 and E3 deletions. Non-replicating adenovirus-based vaccines induce strong adaptive responses against the transgene product both in humans and preclinical models [26-29]. Some adenovirus-based vaccines are also strong inducers of innate immunity, characterized by signaling cascades mediated by type I interferons (IFNs) and nuclear factor kappa B (NF–κB) driven proinflammatory cytokines and chemokines [30].

## Mechanisms of cellular entry of adenovirus-based vectors



Extensive research *in vitro* and in preclinical models has led to a better understanding of cellular entry and internalization of adenovirus-based vectors when administered intramuscularly, the main route of administration of these vectors in humans [26-31].

The mechanism of cellular entry of adenoviruses and adenovirus-based vectors has been described for 'prototype adenovirus' Ad5 (species C) which employs a two-step mechanism: they attach to their primary receptor through the fiber that holds the virion on the cell surface which allows the interaction with an integrin molecule initiating endocytosis [32,33]. The primary receptor for species C adenoviruses is the coxsackievirus and adenovirus receptor (CAR), whereas adenovirus species such as in species B and D utilize other receptors (CD46, DSG-2, and others) (**Table 1**) [34-36]. The receptor interaction (**Table 1**) and consequent cell entry process is dependent on the adenovirus species, and these interactions shape their cellular tropism, distribution, and recognition; as previously described [30,31].

Some locations on the virion surface, such as the hexon HVRs [41,65,66] or the fiber knob protein [67-70] can be modified in order to alter the tropism and transgene expression of adenovirus-based vectors. This could allow retargeting of adenovirus-based vectors to more specific populations of antigen-presenting cells leading to increased immunogenicity against the transgene product. However, the promiscuity of receptor usage by adenoviruses could complicate this application.

**Table 1. Classification of adenovirus types and receptor usage.**

| Species | Type | Cellular receptors | References |
|---|---|---|---|
| A | 12, 18, 31 | CAR | [37] |
| B | 3, ***7***, 11, 14, 16, 21, 34, ***35***, 50 | CD46, DSG2, MARCO, CD80, CD86 | [34,36,38-42] |
| C | 1, 2, 5, 6, 57, ChAd155, ***ChAd3*** | CAR, αvβ3, αvβ5, VCAM, HSPG, MHC1-α2, SR, CRIg, MARCO, SREC-1, LRP, CD36, DC-SIGN | [35,43-57] |
| D | 8–10, 13, 15, 17, 19, 20, 22-25, ***26***, 27-30, 32, 33, 36–39, 42–49, 51, 53, 54 | CD46, DSG-2, αvβ3 integrin, sialic acids, CD80, CD86, CAR, HSPG, MARCO | [35,41,42,58-60] |
| E | ***4***, ***ChAdOx1***, ***ChAd63***, ChAd68, ChAd7 | CAR | [35,45,46,57,61-63] |
| F | 40, 41 | CAR | [35,61] |
| G | 52 | CAR, sialic acids | [64] |

**Adenoviruses vectorized or used for the development of prophylactic vaccines are in bold *italics*.** αvβ3 and αvβ5; receptors for vitronectin; CAR, coxsackievirus and adenovirus receptor; CRIg, complement receptor of the Immunoglobulin superfamily; DC-SIGN, C-type lectin receptor; DSG2, desmoglein; HSPG, heparan sulfate proteoglycan; LRP, LDL receptor–related protein; MARCO, macrophage receptor with collagenous structure; MHC1-α2, major histocompatibility complex class 1, alpha 2; SR, scavenger receptor; SREC-1, scavenger receptor expressed by endothelial cell-I; VCAM, vascular cell adhesion protein.

Adenoviruses use different primary receptors depending on the route of administration or infection. Ad26 (species D) is described to use CD46 as a primary receptor in certain situations, but additional receptors such as αvβ3 integrin and sialic acids also play a role in transduction [58,60,71]. Although mice do not express the primary receptor for D species vectors (CD46), a recent study in transgenic mice expressing human CD46 shows that this receptor improves Ad26 vector transduction to a certain extent after intramuscular administration, but not intranasal administration, compared to wild-type mice [71]. These results may be due to the presence of sialic acids in certain cells in the mouse respiratory tract [72] that have been shown to interact with the fiber knob of Ad26 [58], allowing vector transduction in the absence of the CD46 receptor. A similar observation was described for species B Ad35, which also utilizes CD46 to transduce cells, showing increased transgene expression upon intramuscular administration in mice expressing human CD46 compared to wild-type mice [73].

For Ad5 vectors, the initial interaction with CAR is followed by the internalization through clathrin-mediated endocytosis [32], although other adenoviruses, such as Ad3 [74], can be internalized through pinocytosis. The immobilization of CAR on the cell surface leads to tearing of the fiber proteins from the virion due to mechanical stress, resulting in exposure of protein VI and initialization of virion disassembly [31,75,76]. The remaining virion escapes the endosome and is then transported through the cytoplasm to the nuclear pore complex, the capsid is disassembled, and the viral DNA enters the nucleus but does not integrate into the host cell DNA [77,78]. Other adenovirus species (Ad26,

species D; Ad35, species B), accumulate in late endosomes after cellular entry instead of undergoing early endosomal escape, and the interaction with sensors, such as toll-like receptors (TLRs), can trigger antiviral innate immune responses [79], dependent on endosomal acidification, which is not the case for Ad5.

The mechanism of endosomal escape by adenoviral vectors is influenced by other factors, such as human α-defensins. These peptides have been shown to decrease transgene expression of adenovirus species C *in vivo*, which is likely explained by the inhibition of endosomal escape, accumulation in late endosomes eventually leading to destruction of the virus in a lysosome and triggering antiviral immune responses. At the same time, human α-defensins increase transgene expression for species D, but the mechanism behind this effect remains unclear [80].



Overall, the cellular entry of most adenoviruses does not rely on one unique receptor but on multiple receptors with different efficiency that are influenced by the adenovirus type, host organism, and route of administration. Differences in the initial cellular interaction triggers different antiviral sensing mechanisms that might ultimately influence adaptive immune responses against the transgene.

## Innate immune sensing of adenovirus-based vaccines

The mechanism of cellular entry as well as the cellular trafficking of vectors triggers sensing mechanisms against the vectors that lead to the production of cytokines and chemokines. These molecules will attract certain immune cell populations to the site of immunization and might ultimately influence adaptive responses. However, limited human data describing the specific cytokine response are currently available, and most information is based on *in vitro* assays and preclinical models [30,81].

### Innate immune sensing of adenovirus

Upon cellular transduction, adenoviral DNA or RNA transcripts can trigger innate responses through the activation of pattern recognition receptor like TLR. *In vitro*, adenoviruses trigger type I IFN, cytokines and chemokines, mainly driven by IRF3, IRF7, and NF–kB transcription factors [30,82]. Type I IFN genes are regulated by IRF7 through a signaling cascade involving adenoviral DNA recognition by TLR9 in the endosomes, or through the activation of the SAPK/JNK axis in a TLR-independent manner. Type I IFN genes can also be activated through the recognition of viral DNA in the cytoplasm with activation of the cGAS/STING/TBK1/IRF3 axis [83]. The transcription of proinflammatory cytokine genes is triggered through NF–kB in a MyD88-dependent manner or through

inflammasome activation and cleavage of IL-1β in mice after intravenous immunization [30].

Adenoviruses have developed mechanisms to evade antiviral responses that interfere with efficient virus production. They produce small, non-coding RNAs that suppress protein kinase R and inflammasome activation (species C) [84] and can inhibit cytokines and chemokines downstream of NF–kB through E3-encoded protein RID1α (species C) [85]. The immunomodulatory proteins encoded in the E3 and E4 regions have only been partially described and their effect may differ between adenovirus types.

## Innate immune sensing of adenovirus-based vectors

Adenovirus-based vaccines approved for human use employ several genetic modifications, including E3 deletions, and lack some of the immunomodulatory properties of adenoviruses that lead to the evasion of innate immune sensing mechanisms [1-3,86,87].

The proinflammatory environment triggered by adenovirus-based vectors has been described for ChAd55 in preclinical models and is characterized by type I IFN release (IFN-α), cytokines and chemokines (CXCL9, CXCL10, CCL2). The release of proinflammatory signals coincides with the recruitment of immune cells to the site of immunization (monocytes and macrophages), and subsequently to the draining lymph nodes (natural killer [NK] cells, dendritic cells [DCs], monocytes) [88]. These proinflammatory signals are detected after vaccination with human adenovirus-based vectors (Ad35, Ad26 and Ad48) in the serum of rhesus monkeys, but not after Ad5-based vector immunization [89]. Capsid components involved in the initial cellular interaction seem to play a role in the activation of proinflammatory signals. Teigler et al. demonstrated that the exchange of the hexon HVRs of Ad5 with those of Ad48 (Ad5HVR48) led to a partial recovery of the early innate profile of Ad48 [89], indicating that differences in cytokine profiles may be explained by differences in virus cellular entry, trafficking and activation of antiviral sensing pathways, with Ad5 circumventing sensing mechanisms through endosomal escape.

Human PBMC exposed to Ad5, Ad26, Ad35 or Ad48 elicited type I and type-II IFN responses and the induction of cytokines and chemokines, with certain immune populations contributing differently to the release of these proinflammatory signals [89]. For example, IFN-γ secretion was abrogated in T cell depleted PBMC stimulated with Ad26 or Ad35.

In humans, adenovirus-based vectors also trigger a proinflammatory environment characterized by type I IFN responses and the induction of cytokines and chemokines

[90,91]. Ad5 induces CXCL10, IL1-Ra and IFN responses in humans, which is opposite to what was observed in preclinical models. This disparity highlights the need for further investigation on innate immune sensing to adenovirus-based vectors in humans, as the vectors are based on adenoviruses that have evolved in human hosts.

Overall, innate immune sensing of adenoviruses and adenoviral-based vaccines is characterized by type I IFN production and the release of cytokines/chemokines, and it is adenovirus-type dependent. Further studies are needed to understand the innate responses in humans, which will also bring insights into the translation of data from preclinical models.

## Implications of innate immune sensing of adenovirus-based vectors for adaptive immune responses against the transgene



Adenovirus-based vaccines elicit strong antigen-specific T-cell responses and antibody titers in preclinical models and humans [26-29]. The development of adaptive immune responses is assumed to depend on early events upon vaccination, such as route of immunization, tropism, transgene expression and innate immune responses; however, studies that systematically address this question are scarce, especially in humans.

### Adenovirus-based vaccines in preclinical models

Type I IFN responses have been reported early after vaccination in preclinical models with a wide range of adenovirus vectors, with higher levels linked to decreased transgene expression, decreased antigen-specific antibody responses and lower CD8+ T-cell responses [89,92,93] as highlighted in the recent review by McCann et al. [94]. Quinn et al., [93] demonstrated that type I IFN and stimulator of interferon genes (STING) induction decreased transgene expression upon adenovirus-based vaccination in mice, and that the amount and duration of transgene expression was the best predictor of CD8 T-cell responses. This study examined a wide range of adenovirus vectors of human (Ad5, Ad28, Ad35), chimpanzee (ChAd3, ChAd63), and simian (sAd11, sAd16) origin. The vectors that induced the mildest innate responses upon vaccination, such as Ad5, induced stronger antigen-specific T-cell responses than those with a stronger innate profile, such as Ad35. Johnson et al., [95] reported that IFN-α and -β receptor knockout mice elicited stronger antigen-specific T-cell responses than wild-type mice when immunized with Ad28 and Ad35, but not Ad5. However, the proinflammatory responses triggered by Ad5 in humans seem to differ from those observed in preclinical models [89,91], so their effect on antigen-specific T-cell responses might not be translatable to humans.

Another study suggests that the activation of certain innate responses seem to play a positive role in the development of CD8 T-cell responses to some extent. Rhee et al., [96] showed no changes in antigen-specific CD8 T-cell responses in TRIF(-/-) or TLR3(-/-) mice, but observed a decrease in MyD88(-/-) mice immunized with Ad5, Ad26 and Ad35. CD8 T-cell responses were also not decreased in mice lacking other individual TLRs (TLR2, TLR4, TLR5, TLR6, TLR7, or TLR9), IL-1R or IL-18R, indicating that a single sensing mechanism is not responsible for the activation of MyD88, but it is rather the integration of multiple mechanisms.

Overall, these studies suggest that the fundamental activation of MyD88 is conserved across adenoviral species and minimal innate sensing is required to develop adaptive responses, but that an excess of proinflammatory signals dampens these responses. However, these observations might not be directly translatable to humans.

## Adenovirus-based vaccines in humans

Despite the limited clinical data characterizing the innate immune profile after adenovirus-based vaccination, some of the target cells mediating innate signaling upon vaccination in humans with ChAdOx1nCoV-19 have been recently characterized [97]. This study shows the correlation between TLR-induced B-cell activation, NK and monocyte activation with SARS-CoV-2 neutralizing antibody titers in vaccinated individuals. These responses are diminished in older individuals compared to a younger population. Age-associated decrease in TLR function has been previously reported to affect vaccine immunogenicity [98], and it may explain the decrease in neutralizing antibodies in older individuals after ChAdOx1nCoV-19 vaccination.

Another key player in the development of antigen-specific adaptive immune responses after ChAdOx1 vaccination in humans are mucosal-associated invariant T (MAIT) cells, which are innate sensors that are activated through plasmacytoid dendritic cell IFN-α and monocyte-derived IL18 and TNF [99]. In this study they showed that MAIT-cell deficient mice had decreased CD8 T-cell responses after ChAdOx1 and ChAd63 vaccination, and that the activation of MAIT cells correlated with T-cell responses in ChAdOx1 vaccinated humans.

NK cell activation seems to play a role in the development of antigen-specific adaptive immune responses [97,100,101] and it is likely adenovirus-type dependent, with Ad5 requiring the presence of T-cells for the activation of NK-cells and Ad35 relying on the activation through plasmacytoid DCs and TLR9 signaling [102], although this hypothesis needs further investigation.

The recruitment and activation of these immune cell populations at the site of administration may also lead to the activation of pattern recognition receptors that can induce proinflammatory signals, likely causing specific local and/or systemic reactogenicity profiles in humans [103]. However, the correlation between specific proinflammatory signals and reactogenicity profiles in humans remains to be addressed, although systemic adverse effects after ChAdOx1 nCoV-19 vaccination seem to be associated with cytokine responses and are not associated with humoral immune responses [104]. Due to the advances in omics technologies, further clinical data on innate responses and association to adaptive immune responses can be studied more easily, as it has already been done for other vaccine platforms such as mRNA [105], and these questions may be addressed in detail.

## Conclusions



Adenovirus-based vectors use a repertoire of cellular receptors to transduce cells, and receptor use depends on adenovirus type and route of administration. Further mechanistic insights of cellular entry after adenovirus-based vaccination remains to be elucidated and will provide insights into the tropism and innate immune sensing of these vectors.

With the use of adenovirus-based vaccines in humans, more studies have been focusing on the effect of early events post-vaccination on adaptive immune responses. The innate response triggered by adenovirus vectors is characterized by type I IFN and NF–κB driven proinflammatory signals that needs to be tightly balanced for the development of optimal adaptive responses, with stronger innate responses associated with a reduced adaptive response. The activation of certain innate immune sensing mechanisms (such as activation of MyD88), seems to be necessary to develop robust adaptive responses. The data collected from preclinical models may not be directly translatable to humans, and further research will shed light on the mode of action of adenovirus-based vaccines. Understanding the mechanisms of these early events is critical for the development of vaccines that elicit effective and durable adaptive immune responses.

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***Conflict of interest statement***

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***Author contributions***

Sonia Márquez Martínez: conceptualization, writing – original draft preparation; Aneesh Vijayan: reviewing and editing; Selina Khan: conceptualization, reviewing and editing; Roland Zahn: conceptualization, reviewing and editing.

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**CHAPTER 3**

# Peak transgene expression after intramuscular immunization of mice with adenovirus 26-based vector vaccines correlates with transgene-specific adaptive immune responses

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## Abstract

Non-replicating adenovirus-based vectors have been broadly used for the development of prophylactic vaccines in humans and are licensed for COVID-19 and Ebola virus disease prevention. Adenovirus-based vectored vaccines encode for one or more disease-specific transgenes with the aim to induce protective immunity against the target disease. The magnitude and duration of transgene expression of adenovirus 5- based vectors (human type C) in the host are key factors influencing antigen presentation and adaptive immune responses.

Here we characterize the magnitude, duration, and organ biodistribution of transgene expression after single intramuscular administration of adenovirus 26-based vector vaccines in mice and evaluate the differences with adenovirus 5-based vector vaccine to understand if this is universally applicable across serotypes. We demonstrate a correlation between peak transgene expression early after adenovirus 26-based vaccination and transgene-specific cellular and humoral immune responses for a model antigen and SARS-CoV-2 spike protein, independent of innate immune activation. Notably, the memory immune response was similar in mice immunized with adenovirus 26-based vaccine and adenovirus 5-based vaccine, despite the latter inducing a higher peak of transgene expression early after immunization and a longer duration of transgene expression. Together these results provide further insights into the mode of action of adenovirus 26-based vector vaccines.

## 1. Introduction

Non-replicating adenovirus-based vectors (AdV) have been extensively used for gene therapy and therapeutic vaccination, as well as prophylactic vaccines against infectious diseases that led to the licensed vaccines against COVID-19 disease (non-replicating adenovirus 26, adenovirus 5, and Y25-based vectors; Ad26, Ad5, ChAdOx1, respectively) and Ebola virus disease (Ad5 and Ad26 in combination with a Modified Vaccinia Ankara component) [1-5]. Adenoviruses are non-enveloped, double-stranded DNA viruses [6], and AdV vaccines have been engineered through genetic modifications that prevent viral replication, including deletions of the E1/3 regions of the adenoviral genome, creating space to insert a transgene of interest to induce an immune response against the transgene.

The development of transgene-specific adaptive immune responses is thought to be dependent on early events after vaccination such as AdV tropism, transgene expression and innate immune responses [7-9]. For instance, studies with Ad5 and other non-replicating adenovirus-based vectors (Ad28, Ad35, ChAd3, ChAd63, sAd11, sAd16, ChAdC68) have shown that the level and duration of transgene expression influences the maintenance and phenotype of cellular and/or humoral immune responses in mice [8-14]. However, there are few studies that address the direct relationship between early events and transgene-specific adaptive immune responses for other serotypes than adenovirus 5. One of these studies demonstrated that early termination of transgene expression in Ad5 immunized mice led to impaired memory CD8+ T-cell responses [9]. At the same time, transgene expression is influenced by certain innate immune responses [8,15], although the individual effect of transgene expression and innate immune responses on adaptive immune responses independently of each other has not been characterized.



While Ad26 vaccines have demonstrated to induce strong cellular and / or humoral immune responses against the transgene both in humans and preclinical animal species [16-19] a comprehensive understanding of the magnitude and duration of transgene expression after Ad26 vaccine dosing is limited [20]. These insights could lead to development of more immunogenic vectors through the modifications of the adenoviral particles, aiming to increase the magnitude of transgene expression by circumventing antiviral innate sensing mechanisms [15] or retargeting transgene expression to more specific populations of antigen-presenting cells [21,22].

Here we characterized the magnitude and duration of transgene expression after a single intramuscular administration of Ad26 in mice and evaluated the differences to Ad5. We demonstrated higher peak transgene expression and duration of expression

in mice immunized with Ad5 compared to Ad26. We showed that the magnitude of transgene expression early after Ad26 immunization correlates with transgene-specific cellular and humoral responses, while the difference in duration of transgene expression between Ad26 and Ad5 did not translate into differences in the magnitude of transgene-specific cellular memory responses.

## 2. Results

### 2.1. Magnitude and kinetics of transgene expression after intramuscular administration with AdV vaccines in mice

To understand the differences in the magnitude and kinetics of transgene expression, mice were immunized intramuscularly (IM) with $10^{10}$ adenoviral particles (VP)/mouse of Ad26 or Ad5 encoding firefly luciferase (FLuc) under a cytomegalovirus (CMV) promotor (Ad26.FLuc and Ad5.FLuc), or Ad26 encoding a human papillomavirus transgene (HPV16 E6E7fus) under a CMV promotor (negative control), and *in vivo* bioluminescent imaging (BLI) was conducted (**Fig 1A**). Residual FLuc protein was not detected in the vector batches confirming that all the measured FLuc signal came only from the transgene expression of the vector (**Suppl Fig S1**). The FLuc signal was detected at 6h after dosing in Ad26.FLuc and Ad5.FLuc immunized mice and peaked within the first 24h after dosing. The highest signal was observed at the site of immunization (quadriceps) (**Fig 1B**), in all Ad5.FLuc and Ad26.FLuc dosed animals. The peak of FLuc signal was determined per animal (6h or 12h after dosing) and the magnitude of FLuc signal at the peak of expression was determined for the Ad26.FLuc ($7.73x10^5$ p/s/cm$^2$/sr $\pm$ $3.98x10^5$) and Ad5.FLuc ($2.31x10^7$ p/s/cm$^2$/sr $\pm$ $1.80x10^7$) groups, showing significantly higher magnitude in the Ad5.FLuc group ($p$=0.0003, two-sample t-test). Notably, the FLuc signal was maintained for a year in Ad5.FLuc immunized mice, whereas the FLuc signal in Ad26.FLuc immunized mice was detectable until day 77 (**Fig 1C, Suppl Fig S2**). The FLuc signal in the Ad26.FLuc group is considered positive until day 77 because there is detectable signal above lower limit of detection (LLOD) in at least one mouse in all timepoints until day 77 and all mice in the group present signal above LLOD at day 77. An area under the curve analysis showed a 32-fold difference in the FLuc expression between Ad5.FLuc and Ad26.FLuc dosed animals (**Fig 1D**). Longer duration of FLuc expression in the Ad5.FLuc group did not lead to a statistically significant difference in the number of FLuc-specific IFN-γ producing cells one year after dosing compared with Ad26.FLuc induced cellular responses ($p$=0.1019, ANOVA) (**Fig 1E**).

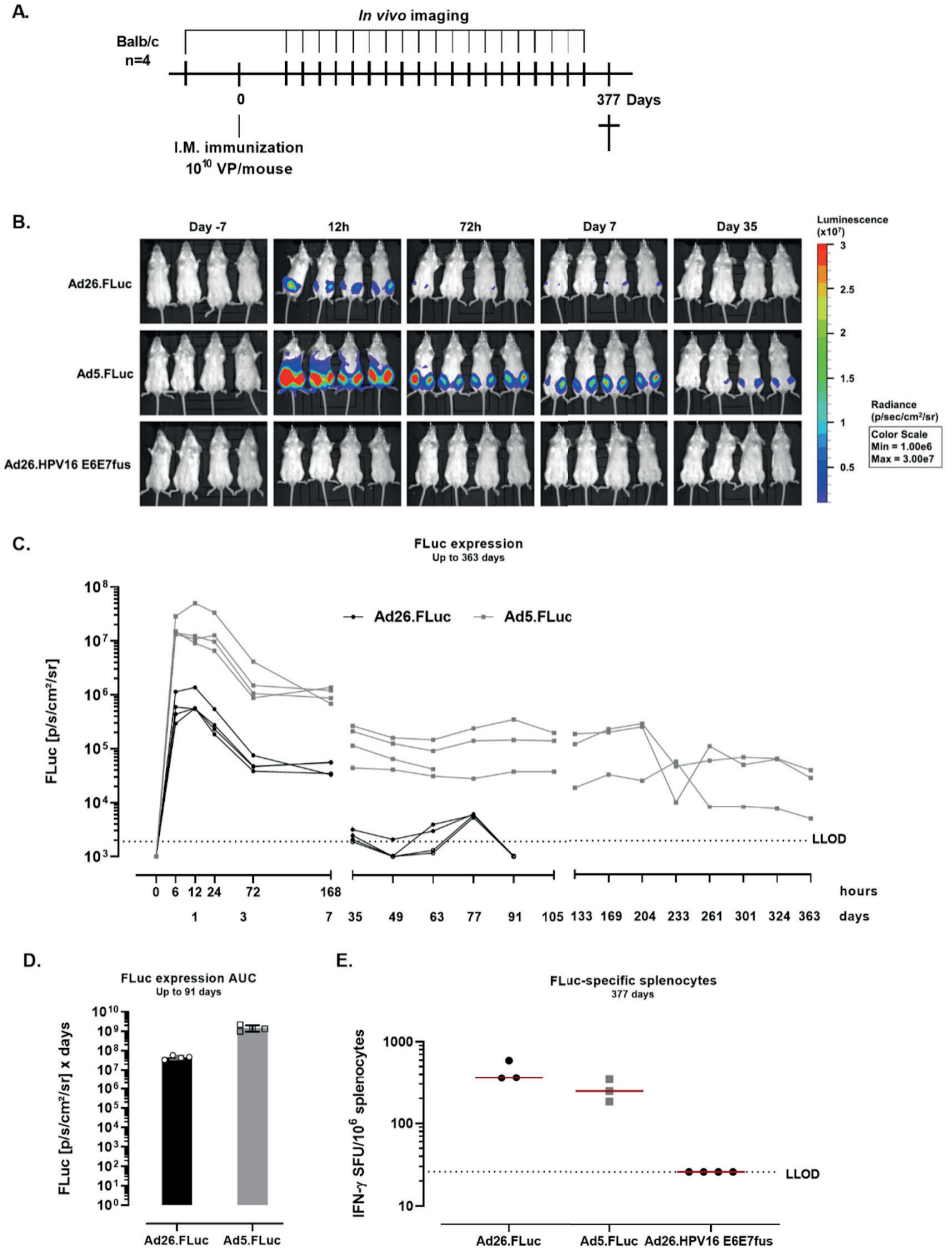


**Figure 1. *In vivo* whole-body FLuc expression after AdV immunization in mice and FLuc-specific cellular responses. A.** Experimental design. BALB/c mice (n=4 per group) were dosed IM with $10^{10}$ VP/mouse of Ad26.FLuc, Ad5.FLuc, or Ad26.HPV16 E6E7fus (19), and FLuc signal was measured through *in vivo* bioluminescence imaging at different timepoints. **B.** Representative images of FLuc signal at different timepoints. **C.** Quantification of FLuc expression (photons per second per square centimeter per steradian, p/s/cm$^2$/sr) after background subtraction (background = mean of signals measured in the Ad26.HPV16 E6E7fus group at the specific timepoints). The dashed line defines the lower limit of detection (LLOD) and corresponds to the average of the expression measured from the Ad26.HPV16 E6E7fus control group across timepoints + 3*STD D. Area under

the curve (AUC) of the background subtracted measurements, up to day 91 **E.** FLuc-specific IFN-γ producing splenocytes (Spot forming units, SFU) were measured at day 377 after dosing. Splenocytes were stimulated with a peptide pool spanning the FLuc protein as described in the material and methods section. The dotted line indicates the background level (95$^{th}$ percentile of the medium stimulation). Datapoint from one mouse in the Ad26.FLuc group was not included due to a technical error in the ELISpot assay. One animal in the group dosed with Ad5.FLuc died during the course of the study (at day 77, FLuc expression data of this mouse is included up to day 63). Data were analyzed using a one-way ANOVA.

To understand whether FLuc expression is limited to the site of immunization (hind legs, quadriceps), or it is distributed to other areas, mice were immunized with Ad26.FLuc or Ad5.FLuc at a dose of $10^{10}$ VP/mouse and quadriceps, draining lymph nodes (iliac and inguinal) and liver were removed directly after administration of luciferin to the mice at multiple timepoints after dosing (**Fig 2A**). The highest *ex vivo* FLuc signal was observed at the site of immunization (quadriceps) at all timepoints for Ad26.FLuc and Ad5.FLuc (**Fig 2B** and **Suppl Fig S3**). In addition, the FLuc signal was detected in the draining lymph nodes (dLNs - inguinal and iliac) for Ad5.FLuc at 24h and rapidly waned to undetectable levels at 72h, while no signal was detected in the dLNs of Ad26.FLuc dosed animals at any timepoint (**Fig 2D** and **Fig 2E**). The FLuc signal from Ad5.FLuc dosed animals was detectable in the liver with the highest expression observed at 24h in 4/4 mice (208155-fold above background) while a transient low signal was detected for Ad26.FLuc dosed animals at 24h in 2/4 mice (1.2-fold above background) (**Fig 2C**). At 72h after dosing, the signal was no longer detectable in the mice immunized with Ad26.FLuc, whereas a low signal was detected at 72h and 168h after immunization with Ad5.FLuc.

These *ex vivo* data confirm the *in vivo* biodistribution data showing that the FLuc expression for Ad26 and Ad5 peaks within the first 24h and wanes overtime and that Ad5 immunized mice present a higher FLuc signal.

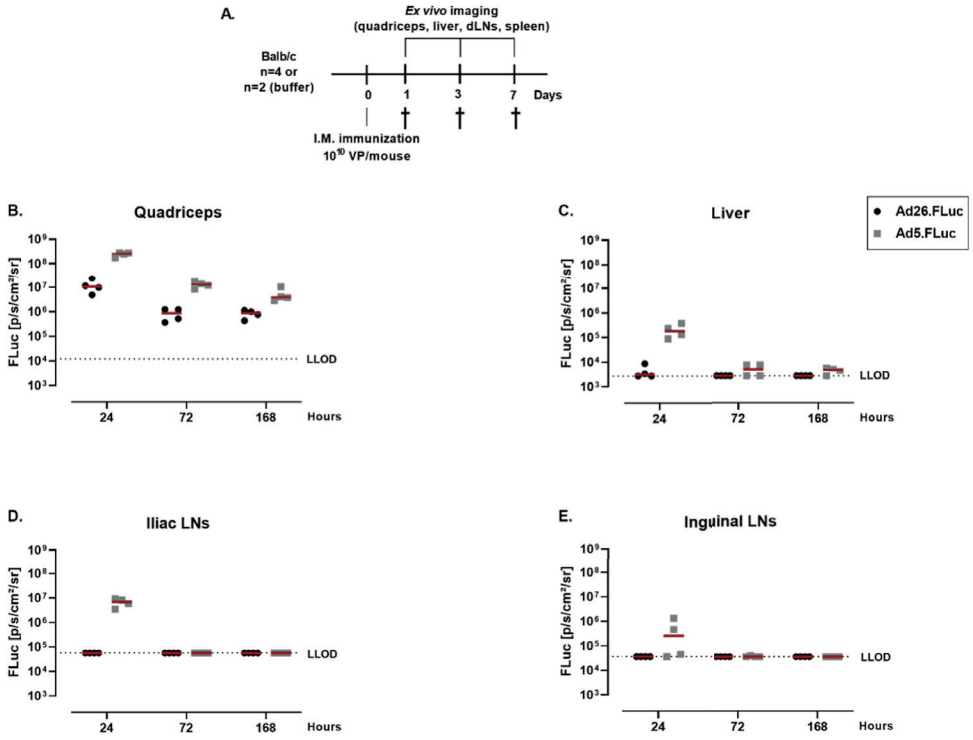


**Figure 2**. ***Ex vivo*** **imaging of luciferase expression in organs of immunized mice. A.** Experimental design. BALB/c mice (n=4 study groups; n=2 control group) were dosed IM with $10^{10}$ VP/mouse of Ad26.FLuc, Ad5.FLuc, or with saline buffer. Mice were sacrificed 24, 72 or 168hrs post dosing and **B.** Quadriceps **C.** Liver **D.** Iliac LNs **E.** Inguinal LNs were imaged *ex vivo*. Quantification of FLuc expression (p/s/cm$^2$/sr) after background subtraction (background = mean of signals measured in the buffer group at the specific timepoints). The LLOD is defined is defined for each specific organ and corresponds to the average of the values from the saline group across timepoints + 3*STD of all values from the negative control.

## 2.2. Correlation between peak transgene expression and transgene-specific immune responses after Ad26 intramuscular immunization

To understand whether transgene expression is a factor influencing adaptive immune responses after Ad26 vaccination, as has been described for Ad5 and AdC68 [9,14], the correlation between the transgene-specific adaptive immune responses and the peak transgene expression in mice was assessed for 2 different antigens, FLuc (intracellular antigen) and SARS-CoV-2 Spike (membrane bound antigen).

Mice were immunized IM with Ad26.FLuc at increasing doses ($10^8$, $10^9$, or $10^{10}$ VP/mouse) and the FLuc signal was measured in the timeframe of peak expression (at 6h or 24h after dosing) in two different groups of mice (**Fig 3A**). At 6h after dosing, FLuc signal showed a dose-response pattern across dose levels ($p$<0.0001, Tobit model) (**Fig**

**3B**). At 24h after dosing, there was no difference in FLuc expression between the groups immunized with $10^{10}$ VP/mouse and $10^{9}$ VP/mouse, while the group immunized with $10^{8}$ VP/mouse presented lower levels of FLuc expression compared with the higher dose groups ($p<0.0001$, Tobit model) (**Fig 3C**). In line with this, the number of of FLuc-specific IFN-γ producing splenocytes responses was significantly lower at a dose of $10^{8}$ VP/mouse compared with a dose of $10^{9}$ VP/mouse ($p<0.0001$, Tobit model), while the numbers were comparable at doses $10^{9}$ and $10^{10}$ VP/mouse (**Fig 3D**). There was a strong correlation between the FLuc expression (at 6h and 24h after dosing) and FLuc-specific IFN-γ producing splenocytes (R=0.72, $p<0.0001$, Spearman correlation) (**Fig 3E**). These results suggest a link between peak transgene expression and cellular responses after Ad26 vaccination in mice.

However, groups that presented higher FLuc expression also received a higher vaccine dose (VP/mouse) than the other groups, precluding a conclusion on whether the observed difference is due to the higher number of VP/mouse leading to increased innate immune responses and thereby enhancing the priming, or due to the higher level of transgene expression as a result of the higher number of VP/mouse used. To address this, mice were immunized IM with a total dose of $10^{10}$ VP/mouse with various ratios of Ad26.FLuc and Ad26.Empty (**Fig 4A**). The FLuc signal was measured 24h after dosing (**Fig 4B**) and FLuc-specific IFN-γ producing splenocytes were measured by IFN-γ ELISpot 2 weeks after dosing (**Fig 4C**). Lower doses of the Ad26.FLuc vector resulted in decreased expression of FLuc as well as FLuc-specific cellular responses.

In alignment with the data shown in **Fig 3**, a strong correlation (R=0.787, $p<0.0001$, Spearman correlation) was observed between transgene expression and transgene-specific IFN-γ producing splenocytes across all groups (**Fig 4D**), suggesting that transgene expression has a direct effect on transgene-specific cellular responses.

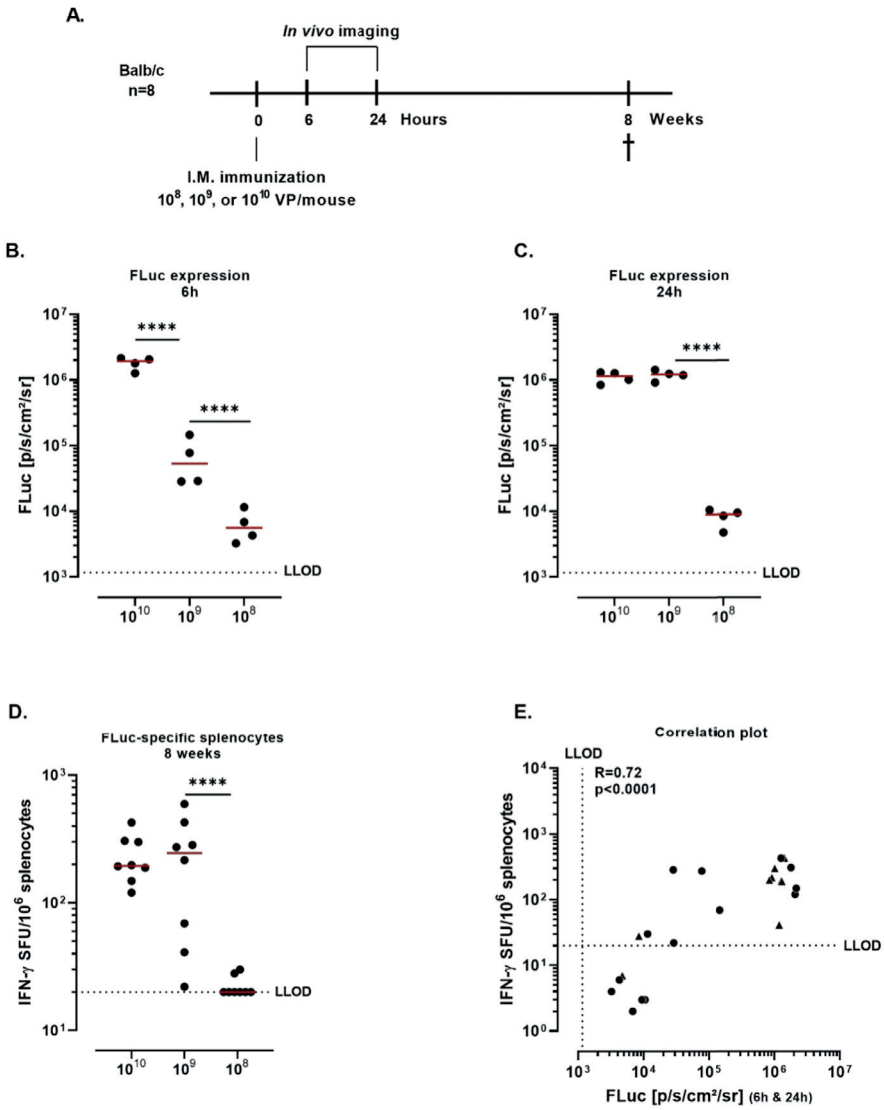


**Figure 3. FLuc expression and FLuc-specific cellular responses in Ad26 immunized mice. A.** Experimental design. BALB/c mice (n=8/group) were dosed IM with $10^8$ VP/mouse, $10^9$ VP/mouse, or $10^{10}$ VP/mouse of Ad26.FLuc. Mice were injected subcutaneously with D-Luciferin at 6hr and 24h, and FLuc signal was measured through *in vivo* imaging **B.** Quantification of FLuc expression (p/s/cm$^2$/sr) in half of the mice (n=4) at 6h **C.** Quantification of FLuc expression (p/s/cm$^2$/sr) (n=4, not the same mice that were measured at 6h) at 24h. Data were analyzed using the Tobit model (**** = $p$<0.0001) and a correction for multiple comparisons was applied (Bonferroni). **D.** FLuc-specific IFN-γ producing splenocytes (SFU) were measured at 8 weeks after dosing (n=8). Splenocytes were stimulated with a peptide pool spanning the FLuc protein. The dotted line indicates the background level (95$^{th}$ percentile of the medium stimulation). Data were analyzed using the Tobit model (**** = $p$<0.0001) and a correction for multiple comparisons was applied (Bonferroni). **E.** Correlation analysis of FLuc expression and FLuc-specific IFN-γ producing splenocytes. Circles correspond to group for which FLuc expression was measured at 6h, triangles correspond to group for which FLuc expression was measured at 24h. Spearman correlation coefficient (R) and p-value (p) were calculated for the analysis.

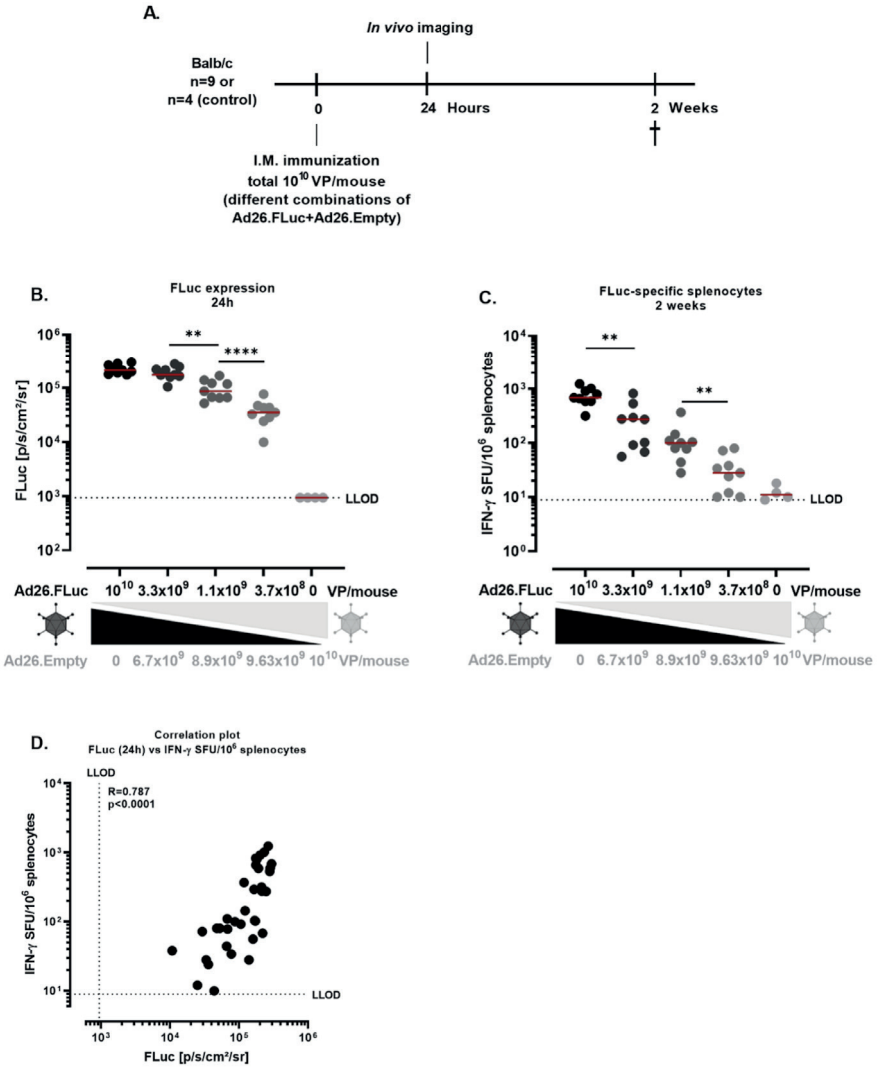


**Figure 4. FLuc expression and FLuc-specific cellular responses in Ad26 immunized mice. A.** Experimental design. BALB/c mice (n=9/ study group; n=4/ negative control group) were dosed IM with a total of $10^{10}$ VP/mouse, with decreasing concentrations of Ad26.FLuc and increasing concentrations of Ad26.Empty. Mice were injected subcutaneously with D-Luciferin 24h after dosing and FLuc signal was measured through *in vivo* imaging. **B.** Quantification of FLuc expression (p/s/cm$^2$/sr) 24h after dosing after background subtraction (background = mean of signals measured in the Ad26.Empty group). The dashed line defines the lower limit of quantification (LLOD) and corresponds to the average of the expression measured from the Ad26.Empty control group across timepoints + 3*STD. Data were analyzed using one-way ANOVA model (** = $p$<0.01; **** = $p$<0.0001) and a correction for multiple comparisons was applied (Bonferroni) **C.** FLuc-specific IFN-γ producing splenocytes (Spot forming units, SFU) were measured at 2 weeks after dosing. Splenocytes were stimulated with a peptide pool covering FLuc. The dotted line indicates the background level (95$^{th}$ percentile of the medium stimulation). Data were analyzed using a one-way ANOVA model (** = $p$<0.01) and a correction for multiple comparisons was applied (Bonferroni) **D.** Correlation analysis of FLuc signal and FLuc-specific IFN-γ producing splenocytes. Spearman correlation coefficient (R) and p-value (p) were calculated for the analysis.

Certain AdVs have been reported to induce low levels of FLuc-specific antibody responses [13]. This could potentially be due to the intracellular nature and processing of the FLuc protein. Therefore, to assess whether transgene expression also correlates with transgene-specific humoral responses, a similar experiment using a SARS-CoV-2 spike transgene-expressing Ad26 instead of Ad26.FLuc was performed. The Ad26.S.PP-PR vector used encodes a stabilized transmembrane spike protein with proline substitutions and a wild-type furin cleavage site, as previously described [18]. Mice were immunized IM with a total dose of $10^{10}$ VP/mouse with various ratios of Ad26.S.PP-PR and Ad26.Empty (**Fig 5A**). Spike protein was measured in the serum (24h after dosing) (**Fig 5B**). A dose-response trend in spike expression was observed across all groups immunized with Ad26.S.PP-PR ($p<0.0001$, Tobit model in all comparisons) (**Fig 5B**). Spike-specific IFN-γ producing splenocytes and spike-specific antibodies were measured 4 weeks after dosing (**Fig 5C** and **Fig 5D**). The number of spike-specific IFN-γ producing splenocytes were significantly higher in mice immunized with $10^8$ VP/mouse compared with the response seen at the $10^7$ VP/mouse ($p<0.0001$, Tobit model). There were no significant differences observed among the other groups. A dose-response trend in spike-specific IgG titers was observed in mice across all doses ($10^{10}$, $10^9$, $10^8$ and $10^7$ VP/mouse of Ad26.S.PP-PR). Correlations were observed between spike protein expression and spike-specific IFN-γ producing splenocytes (R=0.8122, $p<0.0001$, Spearman correlation) (**Fig 5E**) and between the spike-specific IgG titers and the spike protein expression (R=0.9051, $p<0.0001$, Spearman correlation) (**Fig 5F**).

IFN-γ expression in serum has been identified as a hallmark of innate immune activation 1 day after Ad26 immunization in non-human primates (NHPs) [15]. All study groups immunized with Ad26.S.PP-PR presented similar levels of IFN-γ in serum at 24h after dosing (**Suppl Fig S4**), indicating similar levels of innate immune activation across groups.

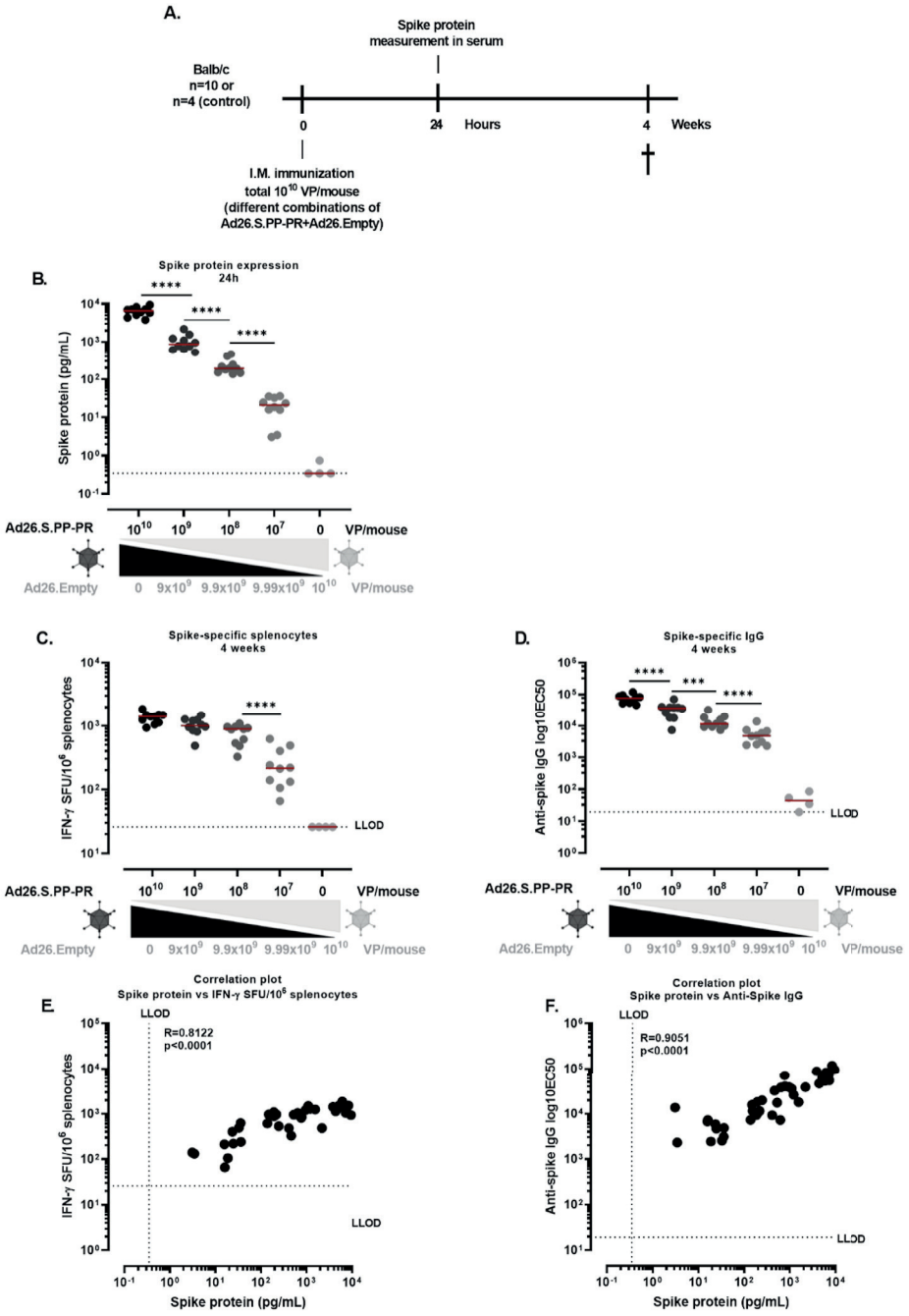


**Figure 5. Spike protein expression and spike-specific cellular and humoral responses in Ad26 immunized mice. A.** Experimental design. BALB/c mice (n=10/ study group; n=4/ negative control group) were dosed IM with a total of $10^{10}$ VP/mouse, with decreasing concentrations of Ad26.S.PP-PR and increasing concentrations of Ad26.Empty. **B.** Serum was collected at 24h after dosing and spike protein (picograms/milliliter, pg/mL) was

measured in the serum through electrochemoluminescence. Data were analyzed using the Tobit model (**** = $p<0.0001$) and a correction for multiple comparisons was applied (Bonferroni). **C.** Spike-specific IFN-γ producing splenocytes (SFU) were measured at 4 weeks after dosing using IFN-γ ELISpot. Splenocytes were stimulated with a pool of peptides of the Spike protein (Wuhan strain). The dotted line indicates the background level (95$^{th}$ percentile of the medium stimulation). Data were analyzed using the Tobit model (**** = $p<0.0001$) and a correction for multiple comparisons was applied (Bonferroni). **D.** Spike-specific IgG (half maximal effective concentration, EC50) was measured in the serum at 4 weeks after dosing by enzyme-linked immunosorbent assay (ELISA). The dotted line indicates the LLOD of the assay. Data were analyzed using the Tobit model (*** = $p<0.001$; **** = $p<0.0001$) and a correction for multiple comparisons was applied (Bonferroni). **E.** Correlation analysis of spike protein expression and spike-specific IFN-γ producing splenocytes, and **F.** Correlation analysis of spike protein expression and spike-specific IgG titers. Spearman correlation coefficient (R) and *p*-value (p) were calculated for the analysis as described in the method section.

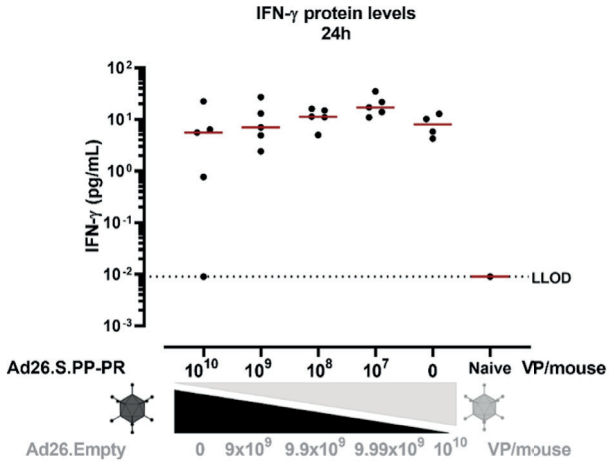


**Figure 6. IFN-γ protein levels in serum of Ad26 immunized mice.** BALB/c mice (n=10/ study group; n=4/ negative control group) were dosed IM with a total of $10^{10}$ VP/mouse, with decreasing concentrations of Ad26.S.PP-PR and increasing concentrations of Ad26.Empty. Serum was collected at 24h after dosing and IFN-γ levels were measured (picograms/milliliter, pg/mL) in randomly selected mice (n=5/group) in a 1 in 10 dilution. In a separate run, BALB/c naive pool serum (dilution 1 in 10) was used to measure IFN-γ levels. Each symbol represents the average of 3 technical replicates. The dotted line indicates the LLOD of the assay defined as 2 standard deviations above background. Data were analyzed using the Tobit model and a correction for multiple comparisons was applied (Bonferroni).

## 3. Discussion

Non-replicating adenovirus-based vectors have been extensively used for gene therapy and therapeutic vaccination, as well as prophylactic vaccines against infectious diseases that led to the licensed vaccines against COVID-19 disease and Ebola virus disease [1-5]. The development of transgene-specific adaptive immune responses is dependent on early events after adenovirus-vector vaccination, such as transgene expression [8-10,14], but there are few studies that address this question for other serotypes than Ad5. Here, we characterized the transgene expression and biodistribution after Ad26 vaccination

and demonstrated a clear correlation between peak magnitude of transgene expression and transgene-specific immune responses in Ad26-immunized mice, independent of the dose of viral particles administered.

We observed transgene expression for up to 77 days after Ad26 immunization and >363 days after Ad5 immunization. Consistent with our data, dosing of Swiss Webster mice with Ad5 has resulted in duration of transgene expression (luciferase) for over 150 days [23]. These datasets conflict with a previously published study in which complete clearance of luciferase expression was observed by day 20 after Ad5 dosing [24]. This may be due to the use of the C57BL/6 mouse model compared with the BALB/c mouse model used in our studies, since it has been shown that the pigmentation of the C57BL/6 mouse skin attenuates bioluminescent signals [25]. Notably, faster clearance of the vector has been reported for other virus-based vaccine platforms, such as Modified Vaccinia virus Ankara (MVA) compared with AdVs in mice, with undetectable levels of the MVA vector at 72h after dosing [26]. RNA-based vaccines have been reported to express the SARS-CoV-2 Spike transgene for over 9 days (mRNA) [27] or up to 63 days (saRNA) [28] in mice; and up to day 60 in humans [29]. This suggests that Ad26 is comparable to other vaccine platforms like saRNA in terms of vector clearance.

The lower magnitude and duration of transgene expression induced by Ad26 compared with Ad5 could be due to the cellular entry mechanisms or the innate immune responses triggered by the vector, which have been reported to play a role on the magnitude of transgene expression after AdV vaccination [21,22,26,30-33]. To this extent, Ad5 uses the coxsackie adenovirus receptor (CAR) to transduce cells [34], which is broadly expressed across tissues in mice (including endothelial and epithelial tissues) [35]; whereas Ad26 utilizes CD46 as the main receptor for transduction [36,37], which is mainly restricted to the testis in mice [38], and sialic acids [39] and integrins [40] as alternative receptors. The broader receptor availability at the site of immunization and draining organs could lead to higher transduction rates in Ad5 immunized mice, explaining the higher magnitude of peak transgene expression. Moreover, the resolution of *in vivo* bioluminescent imaging does not allow to distinguish whether the transgene signal is at the site of immunization or at the draining lymph nodes, where Ad5 immune complexes could be retained for an extensive period of time in combination with follicular dendritic cells or other immune cells, as has been described for other antigens such as ovalbumin and B-Phycoerythrin [41,42]. The retention of the antigen in the draining lymph nodes could explain the longer duration of transgene expression observed in Ad5 immunized mice.

Another potential factor that might explain the lower peak transgene expression induced by Ad26 compared with Ad5 is the antiviral response triggered after cellular transduction. Ad5 virions undergo endosomal escape after cellular entry, whereas Ad26

virions accumulate in late endosomes, triggering innate responses that can lead to the destruction of the virions [43] and potentially prevent some of the adenoviral DNA from reaching the nucleus and producing transgene copies. Moreover, Ad26 vectors trigger the release of higher levels of proinflammatory cytokines (e.g. IFNα2, IFN-γ, IL-1β) in multiple species (mice, NHPs and human PBMCs) compared with Ad5 [15,44], which may result in a faster clearance of the Ad26 vector. The precise innate responses that might influence transgene expression in mice after dosing with Ad26 remain to be further elucidated.

In our studies, Ad26 and Ad5 showed transgene expression at the site of immunization (quadriceps), whereas only Ad5 induced strong transgene expression in draining lymph nodes and liver, aligning with previous reports of transgene biodistribution in Ad5 immunized mice [24,45]. It is important to note that the lack of detection of transgene expression in the draining lymph nodes of the Ad26 vaccinated animals in our studies might be due to limitations in the detection sensitivity. Transgene expression in the liver after Ad5 immunization (intravenous and intramuscular) of mice and rats has been previously reported [10,24], but not after Ad26 immunization (intravenous or intramuscular) of mice [45]. Ad5 has been shown to transduce liver cells through factors IX [46] and X [45,47] mediated CAR interactions, whereas these interactions have not been shown for Ad26 so far, explaining the low or undetectable signal in the liver of the mice immunized with Ad26 compared with Ad5. Interestingly, Ad5 has been reported to distribute to the liver and spleen but not to draining lymph nodes in rabbits [48] indicating either a lower sensitivity of the method used in this report (qPCR) or differences in the tropism of the transduced trafficked cells between species. The tropism of Ad5 may differ across species due to differences in the biodistribution of its primary cellular receptor CAR. For instance, CAR expression has been detected in human erythrocytes but not mouse erythrocytes [49]. Future studies should investigate the expression and biodistribution of the AdV primary receptors across species, and their involvement in transgene biodistribution and development of transgene-specific adaptive immune responses.



Despite the lower transgene expression, differences in transgene biodistribution and lower short-term transgene-specific immune responses observed in Ad26 immunized mice compared with Ad5, Ad26 induces robust T-cell and antibody responses in preclinical models and humans. Although we did not perform a phenotypic characterization of the T-cell responses in our studies, it has been described that Ad26 induces more polyfunctional transgene-specific T-cell responses and enhanced memory T-cell differentiation than Ad5 in mice [50]. Additionally, high levels of preexisting anti-vector responses have been reported to impair immunogenicity against the transgene of interest in Ad5 vaccinees [51,52]. Preexisting Ad5 anti-vector immunity

can lower vaccine effectiveness by blocking transduction and transgene expression [53]. However, a recent study assessed the influence of subsequent Ad26-based vaccination on transgene-specific immune responses in NHPs [17]. No clear consistent effect of preexisting immunity was observed, aligning with the clinical data from homologous Ad26 or ChAdOx1 regimens showing consistent boosting after the second dose [16,17,54-59]. In addition, spike-expressing adenovirus-based vector vaccines Ad26.COV2.S and Ad5-nCoV have shown to elicit similar levels of neutralizing antibodies in humans [60].

Our studies showed that the dose-effect observed in transgene-specific adaptive immune responses after intramuscular one-dose immunization is tightly linked to the amount of transgene expressed, and not to the total number of adenoviral particles administered. These data suggest that co-stimulation of immune cells is directly dependent on the amount of transgene expression rather than on differences in proinflammatory cytokine levels. Specifically, transgene expression influences the potency of the cellular immune responses at least up to week 8 after dosing AdV vaccination, antigen duration beyond 77 days does not appear to improve the potency of the immune cellular response. This finding confirms the data reported by Finn et al., showing that termination of Ad5 transgene expression after 60 days does not influence CD8+ T-cell memory maintenance [9]. Moreover, we show that the potency of transgene-specific T-cell responses reaches a plateau at high doses of transgene-encoding adenoviral particles, suggesting there is a threshold in antigen expression after which cellular responses cannot be further enhanced, likely due to the saturation of antigen-loaded major histocompatibility complex class I (MHC-I). This plateau in transgene-specific T-cell responses has been previously shown after spike-expressing Ad26 vaccination in mice [18] and spike-expressing mRNA vaccination in humans [61], indicating this may be the case for different platforms across species. In another study [62], peak transgene (FLuc) expression reached comparable levels across different platforms (Ad5, MVA, DNA and recombinant vaccinia virus (rVAC)) but Ad5 elicited the highest magnitude of cellular immune responses, suggesting that there are other factors aside from peak transgene expression that influence cellular responses.

B-cell activation and antibody secretion is independent of antigen-loaded MHC-I molecules [63] and no plateau is observed in transgene-specific humoral responses in our studies or in previous reports after Ad26 or mRNA vaccination [18,61]. Our findings suggest that the potency of humoral responses can be further enhanced through the increase of peak transgene expression. A correlation between spike-specific IgG titers and virus-neutralizing antibodies has been observed in previous studies in hamsters and NHPs [64,65], therefore it is likely that peak transgene expression has a similar effect on virus-neutralizing antibodies. Moreover, humoral responses have been shown to

correlate with protection against the disease caused by the Ebola virus in NHPs [66] and COVID-19 in human vaccinees [67,68] after Ad26 administration, suggesting that an increase in the potency of humoral responses could lead to increased protection against disease. Previous reports have shown that repeated HIV protein-based vaccine administrations [69,70] and sustained HIV antigen release through microneedle array implants [71] resulted in enhanced humoral responses compared to one dose administration due to the increased antigen availability during germinal center induction. The maintenance of transgene expression during germinal center induction is likely a key factor in the development of humoral responses after adenoviral-based vaccination. Modifications of the adenoviral particles that lead to higher peak transgene expression and maintenance could be key in the development of vaccines that elicit effective humoral responses and convey protection against the disease of interest.

Overall, our studies provide further insights in transgene expression and distribution, their effect on adaptive immune responses after Ad26 vaccination in a preclinical model, and the potential to increase the potency of transgene-specific humoral responses after AdV vaccination (and potentially other vaccine platforms) by increasing the magnitude of transgene expression.



## 4. Material and methods

### Adenoviral vectors

E1/E3-deleted, replication-incompetent Ad26 or Ad5 vectors were engineered as described previously [72]. The firefly luciferase (FLuc) transgene is based on an intracellular FLuc [72], the Human Papilloma Virus (HPV) transgene is a fusion protein of E6 and E7 of HPV16 [73] and the spike protein is a stabilized SARS-CoV-2 spike protein (S.PP-PR, [18]). The transgene identity was validated through polymerase chain reaction (PCR) and sequencing of the products, and western blot analysis of infected A549 cell lysates or luciferase assay of infected A549 cells. The viral particle titers were measured by optical density at 260nm [74], and the infectivity was validated through $TCID_{50}$ assay [75,76]. The release criteria for animal experiments were met for bioburden and endotoxin levels.

### Animal experiments

Female BALB/c mice (specific pathogen-free), aged 5–12 weeks at the start of the study were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were immunized with varying doses of Ad26.FLuc, Ad5.FLuc, Ad26.HPV16 E6E7fus, Ad26.S.PP-PR, or Ad26.Empty in 50 µl total volume of vaccine per hind leg under isoflurane anesthesia (IM immunization; see dosing in each individual figure).

Intermediate blood samples were collected via submandibular bleeding (at 24h after dosing, as indicated in figure). At the end of each study (see individual figures), animals were exsanguinated by heart puncture under anesthesia and sacrificed by cervical dislocation. Blood was processed for serum isolation and spleens were collected for humoral and cellular assays respectively. Mice experiments were designed according to the European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation; and approved by the Central Authority for Scientific Procedures on Animals of the Netherlands (Centrale Commissie Dierproeven).

### *In vivo* imaging

Mice were immunized IM with different doses of Ad26.FLuc, Ad5.FLuc, Ad26.Empty or Ad26.HPV16 E6E7fus as indicated in the figure legends. At different timepoints, mice received 200µl of D-Luciferin Potassium Salt in PBS (15mg/mL) through subcutaneous administration in the scruff of the neck. After administration of luciferin, mice were kept awake for 5 minutes to allow distribution of the substrate before being imaged under anesthesia (isoflurane or ketamine/xylazine) using the IVIS Lumina II (Perkin Elmer). Regions of interest (ROI) were drawn for all animals covering the entire body for calculation of signal intensity. Light emission was measured in photons/s/cm$^2$/sr (photon flux). Acquisition and analysis were performed with Living Image Software, Version 4.5 (Calliper LifeSciences, Hopkinton, MA).

### *Ex vivo* imaging

Mice were immunized I.M. with a dose of $10^{10}$ VP/mouse of Ad26.FLuc, Ad5.FLuc, or saline buffer. At 24h, 3 days and 7 days after dosing, mice received 200µl of D-Luciferin Potassium Salt as described above. Mice were kept awake for 5 minutes to allow biodistribution of the substrate and sacrificed through cervical dislocation. Organs were collected in buffer containing luciferin, ATP, and $Mg^2$+ and imaged using the IVIS Lumina II (Perkin Elmer). ROIs were drawn for all organs covering the entire organ for calculation of signal intensity. Light emission was measured in photons/s/cm$^2$/sr (photon flux). Acquisition and analysis were performed with Living Image Software, Version 4.5 (Calliper LifeSciences, Hopkinton, MA).

### Luciferase detection in adenovirus vector batches

AdV batches (Ad26.FLuc, Ad5.FLuc, or Ad26.HPV16 E6E7fus) were diluted in buffer containing luciferin, ATP, and $Mg^2$+ (30µl in 2mL of buffer). Luciferase protein (Sigma) was reconstituted in phosphate-buffered saline (PBS) (final concentration 1µg/µl), and 30µl were added to 2mL of buffer containing luciferin, ATP, and $Mg^2$+ (positive control). The solutions were imaged using the IVIS Lumina II (Perkin Elmer). Light emission was measured in photons/s/cm$^2$/sr (photon flux). Acquisition and analysis were performed with Living Image Software, Version 4.5 (Calliper LifeSciences, Hopkinton, MA).

## Peptide pools

For the studies in which FLuc antigen was used, a peptide pool composed of 15mer peptides overlapping by 4 amino acids spanning the FLuc sequence [77] was used in the IFN-γ ELISpot.

For the studies in which spike antigen was used, a peptide pool composed of 156 15-mers peptides overlapping by 11 amino acids of the SARS-CoV-2 Wuhan-Hu-1 (B) spike protein [78] was used in the IFN-γ ELISpot.

## IFN-γ ELISpot

Splenocytes were processed and IFN-γ producing cells specific for FLuc or spike were measured using a mouse IFN-γ ELISpot-plus kit (MabTech) as described previously [18]. Briefly, splenocytes were stimulated with the FLuc peptide pool (1 µg/peptide/mL, 0.4% DMSO), the spike peptide pool (1 µg/peptide/mL, 0.4% DMSO), or 0.4% DMSO in medium (negative control). All samples were run in duplicates. Plates were counted on an AELVIS ELISpot reader, and the numbers of spot-forming units (SFU) per $10^6$ cells were calculated. Background was defined as 95$^{th}$ percentile of values from the 0.4% DMSO in medium.



## Detection of spike protein in serum by electrochemiluminescence

Complete EDTA-free protease inhibitor (Roche) was added to serum samples. Serum samples were centrifuged for 3 minutes, 2000x g. at 4°C to remove particulates before assay.

S-PLEX SARS-CoV-2 Spike detection assay (Mesoscale, detecting presence of the S protein RBD, direct communication from the manufacturer) was used to detect S protein in the serum samples, according to manufacture instruction, using PBS + 0.05% Tween-20 as washing buffer. All incubation steps were performed at 27°C.

The spike protein signal was measured using an MSD Sector S600 (model 1201) and the analysis was performed with the DISCOVERY WORKBENCH v4 software.

## Determination of spike-specific IgG in serum by ELISA

Total serum spike-binding IgG was measured by an ELISA. Briefly, ½ area 96-well OptiPlates (Perkin Elmer) were directly coated overnight at 4°C with SARS-CoV-2 S protein (COR200153, [64]) diluted in PBS at 2 µg/ml. Remaining S protein was removed and the plates were washed 3 times with PBS + 0.05% Tween-20 (PBS-T) and blocked with PBS 1% Casein for at least 1 hour at room temperature (RT), and then washed again.

Mouse serum was serially diluted (starting dilution 1:50) in sample buffer (PBS/1% Casein). Diluted samples were transferred to the coated Maxisorp 96-well ELISA plates (50µl/well in total), incubated for 60 minutes at RT and washed as described above. Bound IgG was detected with goat-anti-mouse IgG (H+L) conjugated to HRP (KLP/SeraCare) and detection substrate (electrochemiluminescence [ECL]) was added and incubated for 10 minutes. Luminescence was read on an BioTek Synergy Neo plate reader.

### Detection of IFN-γ in serum by ProQuantum ELISA

IFN-γ protein levels were measured in serum with a mouse IFN-γ ProQuantum ELISA detection assay (Thermofisher). The ProQuantum ELISA assay is based on antibody binding to the analyte that produces stabilized oligos that are amplified through qPCR. Serum was diluted 1 in 10 in assay dilution buffer and incubated with the antibody-conjugate mixture for 1h at RT. The qPCR protocol was performed according to manufacturer instruction. The qPCR was run in a ViiA 7 Real Time PCR Fast 96-well instrument. The data was analyzed with the ProQuantum software provided by the manufacturer.

### Statistical analysis

Data was log-transformed and groups were compared using a two-sample t-test or analysis of variance (ANOVA) in case of noncensored data, or a Tobit model in case of censored data. P-values $<0.05$ were considered statistically significant. A correction for multiple comparisons (Bonferroni adjustment) was applied where indicated. Correlation coefficients were calculated where indicated using the Spearman rank correlation.

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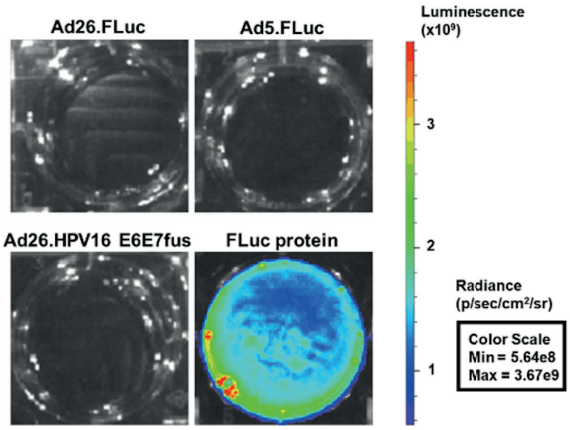
## Author contributions

S.M.M., S.K., N.S. and R.Z. conceived and designed the studies; S.M.M., S.K. and N.S. designed experiments; S.M.M. and N.S. performed experiments and analyzed data; S.M.M. and S.K. wrote the manuscript. All authors participated in the critical revision of

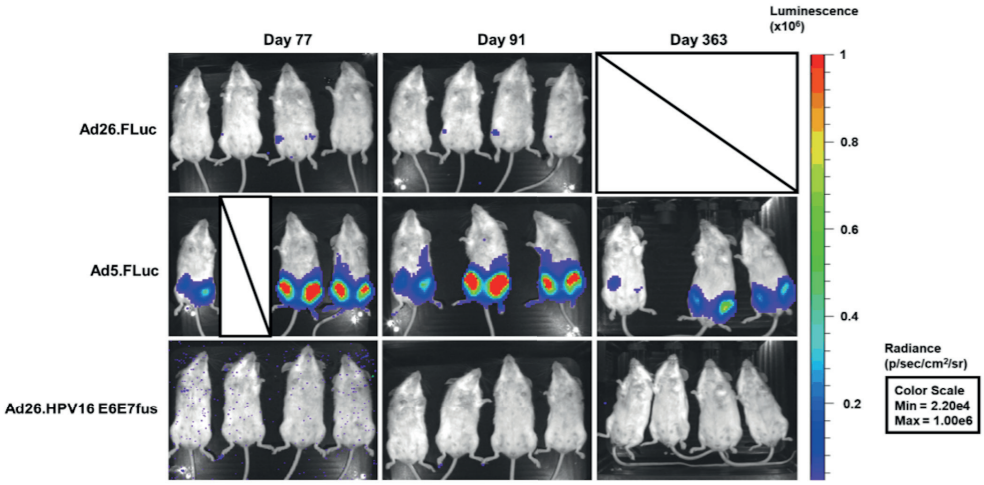
the article and approve its final version. All authors attest they meet the ICMJE criteria for authorship.

## 5. Supporting information

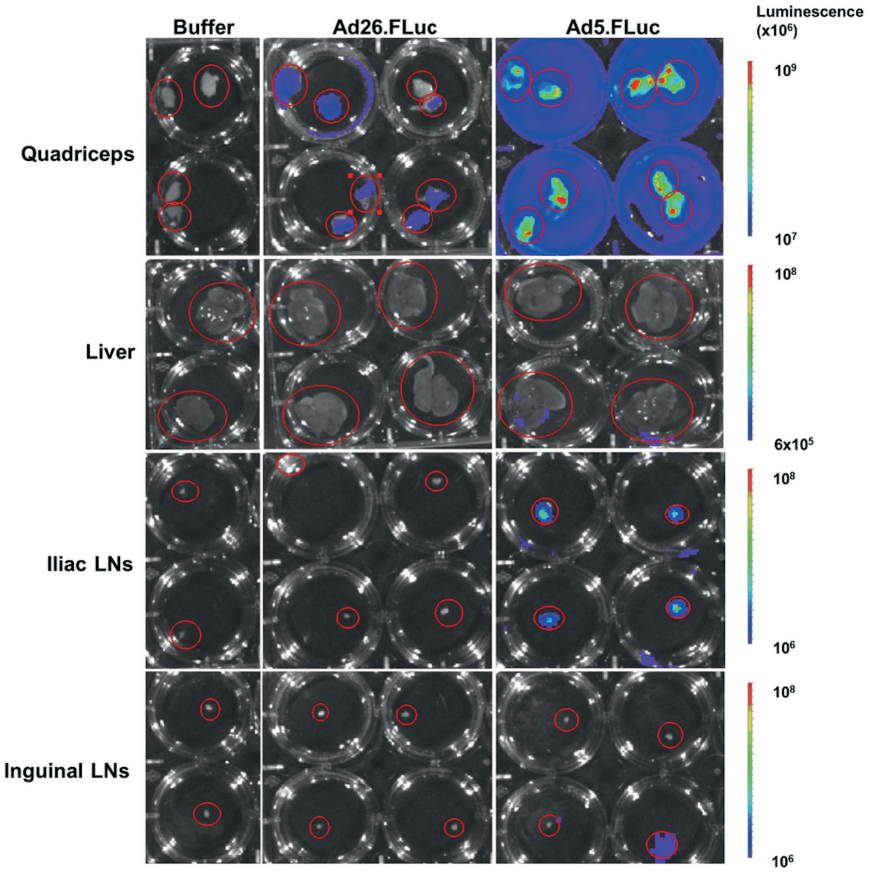



**Suppl figure S1. Residual FLuc protein in vector batches.** Vector batches (Ad26.FLuc, Ad5.FLuc, or Ad26.HPV16 E6E7fus) (30µL/batch) were diluted in 2mL of PBS and FLuc signal was measured through bioluminescence imaging. FLuc protein (30mg) was used as a positive control.






**Suppl figure S2. *In vivo* whole-body FLuc signal after AdV immunization in mice (late timepoints).** FLuc signal from day 77 onwards from study shown in **figure 1**. BALB/c mice (n=4 per group) were dosed I.M. with $10^{10}$ VP/mouse of Ad26.FLuc, Ad5.FLuc, or Ad26.HPV16 E6E7fus (19), and FLuc signal was measured through *in vivo* bioluminescence imaging at different timepoints (77, 91 and 363 days after immunization). Empty square with diagonal line: data not available. One animal in the group dosed with Ad5.FLuc died during the course of the study (at day 77, FLuc expression data of this mouse is included up to and including day 63).




**Suppl figure S3. Representative images of luciferase expression in organs of immunized mice.** FLuc signal from 24h from study shown in Fig 2. Mice were injected with D-Luciferin subcutaneously, sacrificed 24h after dosing and quadriceps, liver, iliac LNs and Inguinal LNs were collected. The organs were extracted and embedded in a buffer containing luciferin, ATP, and $Mg^{2}$+; and FLuc signal was measured.

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CHAPTER 4

# The biodistribution of the spike protein after Ad26.COV2.S vaccination is unlikely to play a role in vaccine-induced immune thrombotic thrombocytopenia

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## Abstract

Ad26.COV2.S vaccination can lead to vaccine-induced immune thrombotic thrombocytopenia (VITT), a rare but severe adverse effect, characterized by thrombocytopenia and thrombosis. The mechanism of VITT induction is unclear and likely multifactorial, potentially including the activation of platelets and endothelial cells mediated by the vaccine-encoded spike protein (S protein). Here, we investigated the biodistribution of the S protein after Ad26.COV2.S dosing in 3 animal models and in human serum samples. S protein was transiently present in draining lymph nodes after Ad26.COV2.S dosing. S protein was detected in serum in all species 1 day up to 21 days after Ad26.COV2.S vaccination, but it was not detected in platelets, the endothelium lining the blood vessels, or other organs. S protein S1 and S2 subunits were detected at different ratios and magnitude when comparing Ad26.COV2.S and COVID-19 mRNA vaccine. However, the S protein expressed by ChAdOx1 nCoV-19, another vaccine leading to VITT, induced a similar S1/S2 ratio as COVID-19 mRNA vaccine–derived transgenes, suggesting that the S1/S2 ratio is not VITT related. Overall, our data suggest that the S protein biodistribution and kinetic after Ad26.COV2.S dosing are likely not main contributors to the development of VITT, but other S-specific parameters require further investigation.

## 1. Introduction

The Ad26.COV2.S COVID-19 vaccine (Jcovden, Johnson & Johnson) has been broadly used in the prevention of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has been shown to elicit protection, lasting 9 months or longer, against severe disease [1].

Ad26.COV2.S is a recombinant and replication-deficient human adenovirus type 26 (Ad26) vector encoding the full-length spike protein (S protein) of SARS-CoV-2 and is based on the Wuhan strain [2,3]. The S protein encoded by this monovalent vaccine contains 2 stabilizing proline substitutions and mutations in the furin cleavage site to preserve the prefusion conformation [2]. The S protein expressed by all other COVID-19 vaccines originally licensed in Europe and North America is also based on the original SARS-CoV-2 Wuhan strain but contains a wild-type furin cleavage site [4].

Thrombosis with thrombocytopenia syndrome (TTS) has been reported following COVID-19 vaccination, and the term vaccine-induced immune thrombotic thrombocytopenia (VITT) has been used to describe cases that are likely vaccine related. VITT is a rare adverse event characterized by thrombocytopenia and thrombosis, often in atypical anatomical locations, and the presence of antibodies against platelet factor 4 (PF4) [5]. VITT has been reported in approximately 2.3 to 5.5 cases per 1 million vaccinees after Ad26.COV2.S dosing, depending on the definition of the syndrome [6] (eg, definitions from Centers for Disease Control and Prevention, USA [7,8]; Prevention of Pharmacovigilance Risk Assessment Committee of the European Medicines Agency [9]; and the Brighton Collaboration Case Definition [10]). VITT occurs 5 to 43 days [7,11,12] after the first dose of ChAdOx1 nCoV-19 [13] or Ad26.COV2.S [14]. It has also been reported sporadically after COVID-19 vaccination with mRNA-1273 [15], inactivated COVID-19 [16,17], or Gam-COVID-vac vaccinees [18].



The underlying mechanism of VITT has not yet been elucidated, but multiple hypotheses have been proposed. Some hypotheses focus on the role of the adenoviral particle in the development of VITT. It was originally reported that the binding of the adenoviral particle to PF4 could play a role in the development of VITT [19], but recent studies showed no binding between PF4 and Ad26.COV.2.S [20,21]. There is also conflicting literature associating human adenovirus infections with a prothrombotic disorder resembling VITT [22,23]. However, the high prevalence of natural human adenovirus infections without a high frequency of associated prothrombotic disorders, and the fact that TTS-like disease has also been described in patients with COVID-19 [24,25] suggests a multifactorial mechanism behind VITT. Other potential factors influencing the development of VITT include interactions of the S protein with platelets/endothelial

cells that might lead to the activation of coagulation pathways [26,27]. The SARS-CoV-2 S protein has been shown to cause vascular damage in hamsters [28] and has been detected within the thrombus and in the adjacent vessel wall in patients with VITT-induced cerebral venous thrombosis [29]. Moreover, the S protein may activate coagulation pathways through the binding of angiotensin-converting enzyme 2 (ACE2) directly on platelets and/or endothelial cells [30]. To gain further insight into the potential contribution of the S protein in VITT in the context of an Ad26-based vaccine, it is important to understand the distribution and composition of the S protein after Ad26.COV2.S dosing.

Here, we investigated the biodistribution of the S protein and characterized the circulating S protein after intramuscular (IM) dosing with Ad26.COV2.S in preclinical models and clinical samples.

## 2. Materials and methods

### Ethics statement

The rabbit study was conducted at JRD Belgium, according to the European guidelines (EU directive on animal testing 86/609/EEC) and Belgian guidelines, and with the principles of euthanasia as stated in the Report of the American Veterinary Medical Association Panel.

The mouse studies were conducted in JVP, according to the European guidelines (EU directive on animal testing 86/609/EEC) and Dutch national legislations; and approved by the Central Authority for Scientific Procedures on Animals (Centrale Commissie Dierproeven).

The non-human primate (NHP) study was conducted in Charles River Laboratories (Wilmington, MA, USA), according with the standard operating procedures by technical staff and approved by the Institutional Animal Care and Use Committee (IACUC) at Charles River Laboratories. The test facility is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and animal experiments were performed in accordance with the standards of the AAALAC International's reference resource [31,32].

### Participants

Levels of S protein were measured as part of exploratory assessments in serum samples from clinical studies COV1001, COV3003, COV3001, and COV3009. Samples from 5

participants, aged ≥18 to ≤55 years, who were dosed with $5\times10^{10}$ vp of Ad26.COV2.S and were enrolled in the study COV1001 (NCT04436276) at the Beth Israel Deaconess Medical Center (Boston, MA, USA) [33], were used for S protein analysis. Clinical study COV3003 (NCT04908722) was a randomized, double-blind, phase 3 study to evaluate 6 dose levels of Ad26.COV2.S administered as a 2-dose schedule in healthy adults ≥18 to ≤55 years of age. Levels of S protein were measured in participants who enrolled in the substudy and received 1 dose of $9\times10^{10}$ vp, $5\times10^{10}$ vp, or $1.25\times10^{10}$ vp of Ad26.COV2.S.

The serum samples obtained after mRNA COVID-19 vaccination were collected from vaccinees who received placebo within the clinical studies (COV3001 or NCT04505722 [1] and COV3009 or NCT04614948 [34]) but who self-reported that they received an mRNA-based COVID-19 vaccine outside the clinical studies. Although the participants followed the clinical study–defined blood sample collection schedule, specific post-mRNA vaccination timepoints were not preplanned. Therefore, serum samples from participants who received mRNA-based COVID-19 vaccines outside the clinical studies were collected at different post-mRNA vaccination timepoints.

All clinical study protocols were conducted following the Declaration of Helsinki and International Council for Harmonisation Good Clinical Practice Guidelines (ICH-GCP) and were approved by both local and national independent ethics committees, as well as institutional review boards (IRBs). All participants provided informed consent.

## Vaccines



Replication-incompetent, E1/E3-deleted recombinant Ad26 vectors were engineered using the AdVac® system as described elsewhere [35,36], with Ad26 encoding different versions of the SARS-CoV2 S protein from the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank accession number: MN908947). The constructs encoded a native full-length spike protein (Ad26.S) in which proline substitutions (K986P, V987P) were introduced (Ad26.S.PP-PR), a full-length spike in which the furin cleavage site was abolished by amino acid changes R682S and R685G and proline substitutions (K986P, V987P) were introduced (Ad26.COV2.S), or a native full-length spike protein with a tissue plasminogen activator (tPA) sequence upstream of the spike (Ad26.S.tPA.WT.S) (**Figure A1**) [2]. Ad26 vector Ad26.ZIKV.001 (encoding Zika virus envelope protein) was used as a control [35,37]. The Ad26-mediated expression of the various transgenes was confirmed by Western blot analysis of cell-culture lysates from infected A549 cells or by polymerase chain reaction.

BNT162b2 mRNA (Comirnaty, Pfizer/BioNTech) [38] encodes a wild-type prefusion-stabilized SARS-CoV2 S protein.

## Purified SARS-CoV-2 Spike protein and adjuvant

The S protein used for dosing (COR201225) contains amino acids 14-1208 of the Wuhan-Hu-1 SARS-CoV-2 spike (GenBank accession no. MN908947) and includes stabilizing mutations R682S, R685G, N532P, T572I, D614G, G880C, F888C, A944P, and V987P as described elsewhere [39]. The protein was produced in Exp293F cells and purified by a 2-step purification protocol by first applying cleared culture supernatant on a Galantus nivalis-lectin column (Vectorlabs, AL-1243) with 40 mM Tris, 500 mM NaCl pH 7.4 as a buffer. Elution was performed with the same buffer with additional 1M Mannopyranoside final pH of 7.4. Eluted protein was concentrated and subsequently loaded on a Superdex200 Increase column (GE) in 20 mM Tris, 150 mM NaCl pH 7.4 as buffer. Sucrose was added to a final concentration of 5% before snap freezing in liquid nitrogen. Protein was tested for bioburden and endotoxin levels before use. Aluminum hydroxide $Al(OH)_3$ was produced from Alhydrogel 2% at Janssen Vaccines. COR201225 protein and adjuvant were mixed by pipetting and incubation on a roller-bench for 1 hour at room temperature (RT) before dosing. The S protein used in the cell lysate *in vitro* test (COR200672) contains amino acids 14-1208 of the Wuhan-Hu-1 SARS-CoV-2 spike (GenBank accession no. MN908947) and similar stabilizing substitutions to COR201225 (R682S, R685G, N532P, T572I, D614N, G880C, F888C, A942P, K986P, and V987P). The protein was produced and purified according to the same protocol as COR201225 described above.

## Animals and housing

A total of 40, 14-week old, healthy male New Zealand white (NZW) rabbits (body weight 2.4-3.4 kg at study start) were included. Animals were supplied by Charles River Laboratories (France). Animals were kept in a biosafety level 2 (BSL-2) facility under specific pathogen-free conditions after screening negative for Mycobacterium tuberculosis, simian immunodeficiency virus, simian retrovirus, and simian T-lymphotropic virus. Screening included herpes B virus and measles serology. Animals were single-housed in stainless steel cages placed in study-dedicated rooms.

Female BALB/c and C57BL/6 mice (specific pathogen-free), aged 5 to 12 weeks at the start of the study, were purchased from Charles River Laboratories (Sulzfeld, Germany). Female Jh C57BL/6NTac-Igh-Jem1Tac and C57BL/6 control mice (specific pathogen-free), aged 10 weeks at the start of the study, were purchased from Taconic Biosciences. Animals were kept in a BSL-2 facility under specific pathogen-free conditions.

The NHP study was conducted at Charles River Laboratories (Wilmington, MA, United States). Cynomolgus macaques (*Macaca fascicularis*) of Cambodian origin were aged 5.32 to 9.22 years and weighted 5.8 to 8 kg (males) and 3.3 to 4.4 kg (females) at the

initiation of dosing. The evaluations were performed in accordance with the standard operating procedures by technical staff.

For all animal studies, animals were kept under controlled, recorded environmental conditions of humidity, temperature, and 12-hour light cycle. Animals were provided with sensory and cognitive environmental enrichment including occupational material. Animals were fed a standard diet ad libitum and tap water was provided ad libitum through an automated system. Animal well-being and health surveillance was monitored at least daily by husbandry staff. Preset humane endpoints were used to define study-unrelated sacrifice criteria by a veterinarian. All measures were taken to minimize pain, distress, and suffering, and all procedures were performed by trained personnel.

## Animal study designs and procedures

In the rabbit study, NZW rabbits were divided into 5 study groups with 8 animals per group. The animals received a single IM dose of $5\times10^{10}$ vp of Ad26.COV2.S, Ad26.S (encodes wild-type SARS-CoV2 S protein [2]) or Ad26.S.PP-PR (encodes SARS-CoV2 S protein with 2 prolines in hinge region [2]) (**Figure 1A**). An IM injection with an $Al(OH)_3$ adjuvanted soluble S protein (COR201225) (50 mg) was included as a positive control and IM injection of an empty Ad26 vector (Ad26.Empty) as a negative control (**Figure 1A**). All vaccines were administered IM in a 0.5-mL volume. Blood sampling was performed from the central ear artery. The total blood volume and sampling frequency was performed according to good ethical practices. Minimal to slight erythema at the administration site was noted in the groups (including vehicle groups) receiving an IM injection, and was considered to represent the normal, expected reaction related to the IM injection procedure [40]. At the end of the study, animals were anesthetized by an intravenous injection of pentobarbital and sacrificed by exsanguination via the inguinal blood vessels. Terminal blood sampling was performed via the inguinal blood vessels.



Mice were immunized IM with different doses in 50 µL of vaccine preparation as indicated in the text (Sections 3.3 and 3.5). Intermediate blood samples were collected via submandibular bleeding at different timepoints (**Figure 7** and **Figure A4**). At the end of each study, mice were anesthetized with isoflurane, exsanguinated through heart puncture, and sacrificed by cervical dislocation. Blood was processed for serum isolation and spleens were collected for humoral and cellular assays, respectively. Control mice received a buffer solution (15 mM citric acid, 75 mM NaCl, 2-hydroxylpropyl-β-cyclodextrin 5% (w/w), 0.03% PS-80 pH 6.2).

In the NHP study, 8 cynomolgus macaques (4 females and 4 males) were immunized IM (left thigh) on days 0 and 56 with $5\times10^{10}$ vp/animal of Ad26.COV2.S in 0.5 mL of vaccine

preparation. The total blood volume and sampling frequency was performed according to good ethical practices.

## Processing of whole blood for serum and plasma generation

Rabbit serum samples were prepared from clotted blood drawn into serum tubes after centrifugation at 1900 g for 5 minutes at RT. Serum was stored at -80°C until time of analysis. Rabbit plasma was prepared drawing whole blood into anticoagulant-containing tubes (ethylenediaminetetraacetic acid (EDTA)) and centrifuging at 2000 g for 15 minutes at 4°C. The pellet (referred to as blood cellular fraction) was collected and stored at -80 °C. The supernatant (platelet-depleted plasma) was collected into sterile 15-mL Falcon tubes, mixed by inversion, and stored at -80 °C.

Mouse serum samples were prepared from clotted blood drawn into eppendorf tubes after centrifugation at 2660 g for 4 minutes followed by 20,800 g for 1 minute. Serum was stored at -20°C until time of analysis.

## Detection of S protein in tissues by immunohistochemistry staining

Rabbit samples from the administration site (skin, muscle—left biceps femoris), draining lymph nodes (iliac and popliteal), and vein (lateral saphenous/cava caudalis) were fixed in 10% formalin and embedded in paraffin and sectioned at 5-μm thickness. Sections of administration site, draining lymph nodes, spleen, and veins were stained immunohistochemically (Ventana Discovery Ultra autostainer, Roche Diagnostics) using a hapten multimer horseradish peroxidase (HRP)–based technology and diaminobenzidine (DAB) tetrahydrochloride detection method, by a monoclonal S protein antibody; SARS-CoV-2 Spike S1 Subunit Antibody (clone 1035206, R&D, catalog no. MAB105403) at 5 mg/mL (S1 antibody). Isotype control (Mouse IgG1 Abcam ab18443) and the Ad26.Empty group served as negative controls. The sections were scored semiquantitatively for S protein (S1)-immunoreactive cells.

## Detection of spike mRNA in tissues by RNAscope

Formalin-fixed paraffin-embedded blocks from the administration site and lymph nodes were processed at 5-mm thickness for in situ detection of SARS-CoV-2 S mRNA (Advanced Cell Diagnostics, catalog no. 1116539-C1). For this, we used the RNAscope VS Universal AP assay for Ventana Discovery Ultra (Advanced Cell Diagnostics) according to the manufacturer's protocol. The housekeeping gene peptidylpropyl isomerase B (PPIB) was used as positive control probe and the *Bacillus subtilis* strain SMY methylglyoxal synthase (mgsA) gene, partial cds dihydrodipicolinate reductase (DapB) gene was used as negative control probe. The signal was amplified using an alkaline phosphatase (AP)–based hybridization and was detected using Fast Red as chromogenic substrate.

Punctate red dots representing transcripts were evaluated using a standard brightfield microscope.

## Detection of the S protein in blood by electrochemiluminescence

Complete EDTA-free protease inhibitor (Roche) was added to plasma samples. Plasma samples were centrifuged for 3 minutes, 2000 g at 4°C to remove particulates before assay. For blood cellular fraction, samples were lysed with cold lysing buffer (RIPA buffer; complete EDTA-free protease inhibitor, Roche, 04693116001; benzonase) on ice for 1 hour, inverting the tubes every 15 minutes. Cellular fraction samples were centrifuged down for 5 minutes, 600 g at 4°C to clarify the lysate of cell debris and larger membrane fractions.

S-PLEX SARS-CoV-2 Spike detection assay (Mesoscale, detecting the presence of the S protein RBD, direct communication from the manufacturer) was used to detect S protein in the plasma, serum, or blood cellular fraction samples, according to manufacturer's instruction, using phosphate-buffered saline (PBS) + 0.05% Tween-20 as washing buffer, all incubation steps were performed at 27°C. Analysis was done with the MDS Discovery Workbench version 4.0.

In the experiment presented in **Figure 4A**, mouse serum was diluted 1/100 for the samples in the Ad26.COV2.S and buffer control groups, and 1/10,000 for the samples in the BNT162b2 group. In **Figure A5** (A), all serum samples were diluted 1/100. All other samples were measured undiluted. In **Figure 7B**, all serum samples were diluted 1/5.



## Detection of S1-S2 protein in blood by electrochemiluminescence

The S-PLEX SARS-CoV-2 Spike detection assay was used to detect S protein containing the S1 and S2 domains (S1-S2) in serum samples by exchanging the anti-RBD capture antibody with an antibody against the conserved S2 stem helix region of the S protein (CC40.08). Briefly, plates were coated overnight with CC40.08 (1.7 µg/mL) in coating reagent and diluent supplied by the assay kit. After coating, the assay was followed as described by the manufacturer (Mesoscale). The washing buffer was PBS + 0.05% Tween-20, and an automated plate washer was used for the washing steps (washing protocol as described in the kit manual). All incubation steps were performed at RT except for the TURBO-TAG detection solution (27°C). Analysis was done with the MSD Discovery Workbench version 4.0.

Mouse serum was diluted 1/2 for the samples in the Ad26.COV2.S and buffer control groups, and 1/10 for the samples in the BNT162b2 group (**Figure 4B**). In the experiment presented in **Figure A5** (B), serum samples were diluted 1/2.

## Detection of spike-specific immunoglobulin (IgG) in serum by enzyme-linked immunosorbent assay (ELISA)

Spike-binding IgG was measured in total mouse serum through ELISA. Briefly, ½ area 96-well OptiPlates (Perkin Elmer) were coated with SARS-CoV-2 S protein at 2 µg/mL (COR200153, [41] in 1X PBS) overnight at 4°C. Remaining S protein was removed and the plates were washed 3 times with PBS + 0.05% Tween-20 (PBS-T) and blocked with PBS 1% casein for at least 1 hour at RT, followed by another wash. Serum from mice was serially diluted (starting dilution 1:50) in sample buffer (1X PBS/1% Casein). Diluted samples were transferred to the coated Maxisorp 96-well ELISA plates (50 mL/well in total), incubated for 60 minutes at RT and washed as described above. Bound IgG was detected with goat-anti-mouse IgG (H+L) conjugated to HRP (KLP/SeraCare) and detection substrate (electrochemiluminescence [ECL]) was added and incubated for 10 minutes. Luminescence was read on a BioTek Synergy Neo plate reader and a 4-parameter logistic curve fitting was performed. The final reportable values ($log_{10}$EC50) are derived from the fitted curve.

Serum from cynomolgus macaques was used to assess IgG binding to SARS-CoV-2 S protein by ELISA using a recombinant soluble S protein-based on the Wuhan-Hu-1 SARS-CoV-2 strain (MN908947) and stabilized by 2 point mutations in the S1/S2 junction that knock out the furin cleavage site, and by 2 newly introduced prolines in the hinge region in S2. In addition, the transmembrane and cytoplasmic regions were replaced by a foldon trimerization domain followed by a His-tag, allowing the trimeric protein to be produced and purified as soluble protein. Briefly, 96-well microplates were coated with the S protein for a minimum of 16 hours at 4°C. Plates were washed in PBS/0.05% Tween-20 and blocked with 5% skim milk in PBS/0.05% Tween-20 for 1 hour at RT. Samples were serially diluted starting at 1:50, added to the plates, and incubated for 1 hour at RT. After washing, the plates were incubated with peroxidase conjugated goat anti-human IgG for 1 hour at RT, washed again, and developed with tetramethylbenzidine substrate for 30 minutes at RT. The optical density was read at 450/620 nm and a 4-parameter logistic curve fitting was performed. The antibody titers (expressed in ELISA units [EU]/mL) were determined in relation to the standard based on all dilutions tested.

Serum samples from human vaccinees were assessed for SARS-CoV-2 S-specific binding antibody concentrations as previously described [3]. In brief, purified SARS-CoV-2 pre-S antigen was adsorbed to the wells of a microplate and diluted serum samples (test samples, standard, and quality controls) were added. Unbound sample was washed away, and enzyme-conjugated anti-human IgG added. After washing excess conjugate away, 3,3′,5,5′-tetramethylbenzidine colorimetric substrate was added. After the established time period, the reaction was stopped. A reference standard on each test

plate was used to quantify the amount of antibodies against SARS-CoV-2 pre-S in the sample according to the unit assigned by the standard (EU/mL).

### Serum coincubation study

The interference of anti-spike antibodies in the detection of S protein was assessed in an *in vitro* serum coincubation study. Rabbit sera obtained from a different study [42] predosing or 28 days postdosing with either Ad26.COV2.S or Ad26.ZIKV001 were mixed with serum obtained 1 day postdosing with Ad26.COV2.S. Serum samples were mixed in a 1:1 ratio (20 mL + 20 mL). Complete EDTA-free protease inhibitor (Roche) was added to all samples (including single controls) and samples were shaken at 700 rpm for 3 hours at RT. S-PLEX SARS-CoV-2 Spike detection assay (Mesoscale) was used to determine the concentration of S protein in the samples.

#### *Statistical analysis*

Data were log-transformed and groups were compared using a 2-sample t-test (paired or unpaired) or analysis of variance (ANOVA) in case of noncensored data. For censored data, a Tobit model analysis with correction for multiple comparisons (Bonferroni adjustment) was applied where indicated. P-values <0.05 were considered statistically significant. Correlation coefficients were calculated where indicated using the Spearman rank correlation.

## 3. Results



### 3.1. S protein and S mRNA were detectable at the site of administration and in draining lymph nodes at day 1 but not day 11 after IM Ad26.COV2.S vaccination in rabbits

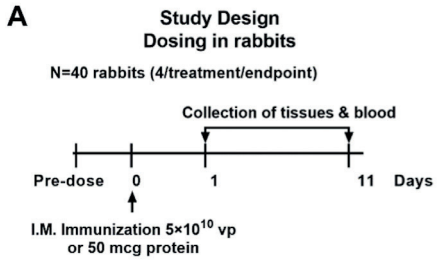
To study the biodistribution of the S protein after Ad26-based COVID-19 vaccination and understand the effect of stabilizing mutations of the S protein, rabbits were dosed IM with Ad26 vectors encoding different versions of the SARS-CoV2 S protein (Ad26.COV2.S, Ad26.S, or Ad26.S.PP-PR) (**Figure A1**), Ad26.Empty (negative control), or 50 mg of a subunit S protein vaccine (COR201225) together with 250 mg $Al(OH)_3$ (positive control) (**Figure 1A**). Rabbits were sacrificed 1 or 11 days after dosing (4 rabbits/treatment/endpoint) and samples were collected (**Figure 1A**).

On day 1 post IM injection, S protein was present at the administration site (**Figure 1B**) and in the draining lymph nodes (iliac and/or popliteal) (**Figure 1C**) of groups dosed with an S-encoding Ad26 vector as evaluated by immunohistochemistry (IHC). At the injection site, membranous/cytoplasmic S protein staining was mainly observed in round to elongated cells (considered macrophages and/or fibroblasts) in connective

tissue, while the S protein could not be detected in myocytes at the administration site. In draining lymph nodes, S protein was detected in presumed macrophages and dendritic cells, according to their morphology. The S protein was not detected in arteries/veins (ie, blood vessels at the injection site, or lateral saphenous vein and vena cava caudalis) (**Figure 1D**). At day 11 after the IM injection with Ad26-based vaccines, the S protein was no longer detected in any of the tissues examined (**Figure 1**).

In animals dosed with the recombinant soluble S protein COR201225 (+$Al(OH)_3$), S protein was only detected at the administration site, mainly without cellular association, on day 1 after an IM injection and it was no longer detected in any of the tissues examined on day 11. No S protein signal was detected in the Ad26.Empty group (Figure 1) or the isotype control (**Figure A2**).

As an alternative method to identify vector-transduced cells, S mRNA was investigated through in situ hybridization (ISH) in Ad26.COV2.S-immunized animals. S mRNA was detected at the site of administration (**Figure 2A**) and draining lymph nodes (**Figure 2B**) 1 day after dosing with Ad26.COV2.S consistent with the S protein expression as detected by IHC. Comparable morphology of positive cells was observed across the 2 techniques. No S mRNA was detected in any of the tissues at day 11 after immunization, similar to the S protein detection through IHC, suggesting the clearance of transduced cells (**Figure 2**).




| Group | Treatment | Spike antigen |
|---|---|---|
| 1 | Ad26.COV.2.S | Wild-type SARS-CoV-2 spike + stabilizing mutations + mutated furin cleavage site |
| 2 | Ad26.S | Wild-type SARS-CoV-2 spike |
| 3 | Ad26.S.PP-PR | Wild-type SARS-CoV-2 spike + stabilizing mutations |
| 4 | S protein + Al(OH)$_3$ | COR201225 |
| 5 | Ad26.Empty | None |

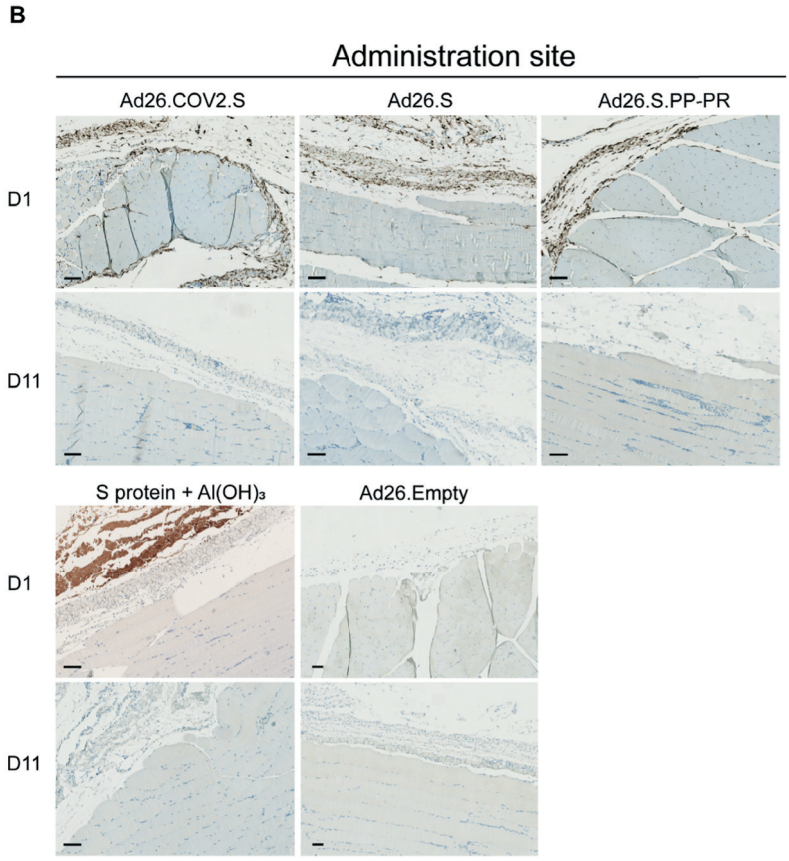



**Figure 1. (A-B)**

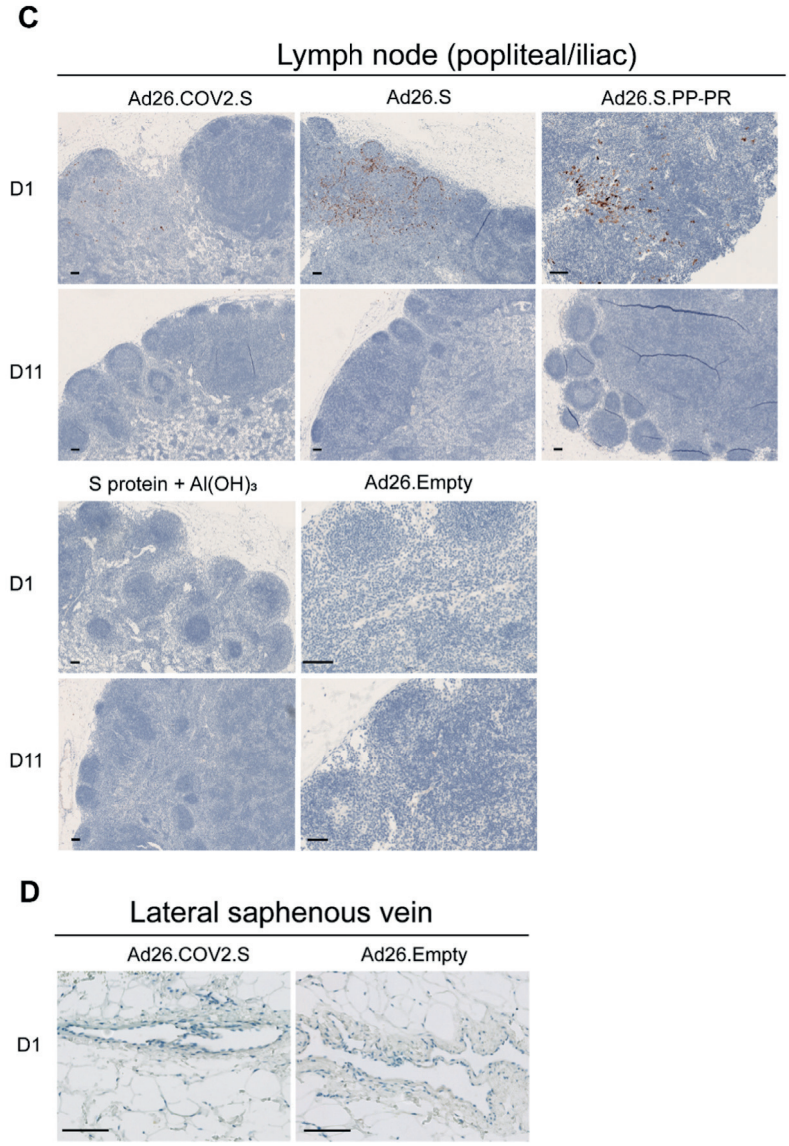



**Figure 1. The spike (S) protein was detected at site of injection and draining lymph nodes, but not in the veins after intramuscular (IM) Ad26.COV2.S dosing in rabbits. (A)** Rabbits (N=40) were dosed with $5\times10^{10}$ vp of Ad26.COV2.S, Ad26.S, Ad26.S.PP-PR, Ad26.Empty or 50 mg of S protein + $Al(OH)_3$. Anti-SARS-CoV2 S1 staining by immunohistochemistry of **(B)** muscle (administration site) and **(C)** draining lymph nodes (popliteal and iliac) at day 1 and day 11 postdosing, and **(D)** lateral saphenous vein at day 1 postdosing. The black bars represent the magnification of the images (100 mm).

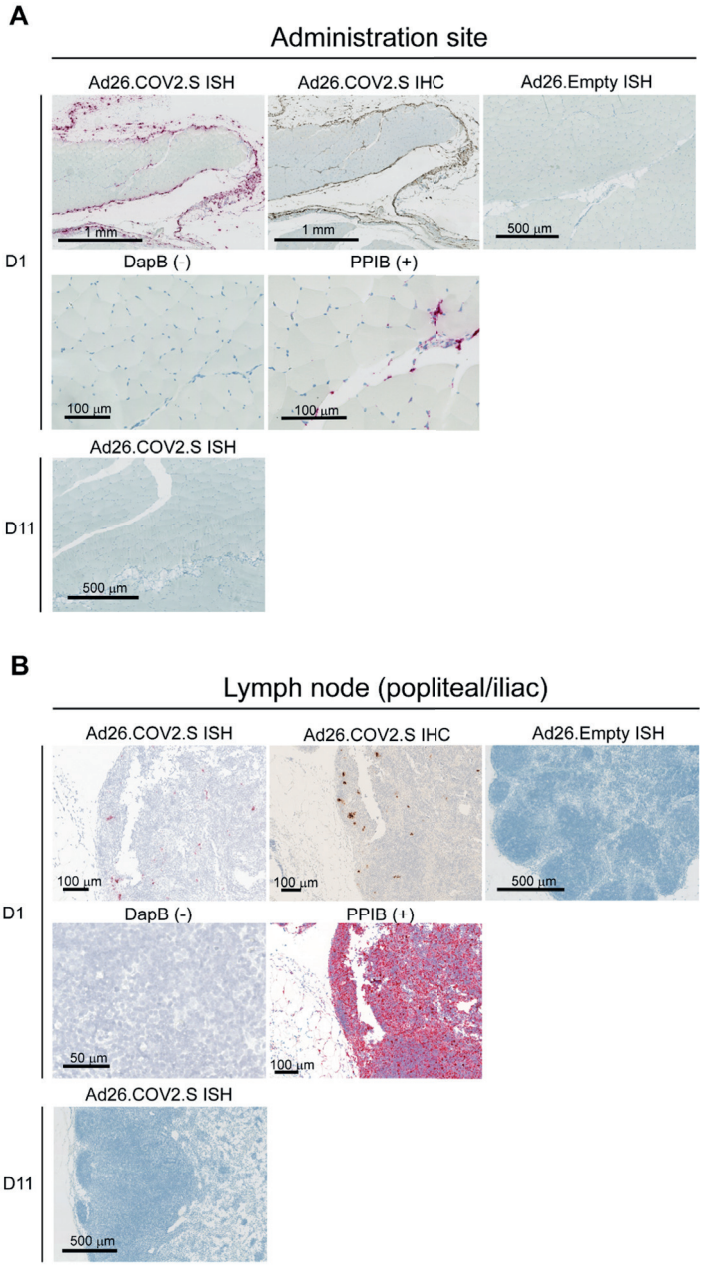



**Figure 2. Spike mRNA in situ hybridization (ISH) detected at site of injection and draining lymph nodes after IM Ad26.COV2.S dosing in rabbits.** Anti-SARS-CoV2 S1 staining by immunohistochemistry (IHC) and ISH of spike mRNA at **(A)** administration site and in **(B)** lymph nodes from rabbits (n=4 per group) at day 1 after dosing with Ad26.COV2.S or Ad26.Empty. Bacterial gene dihydrodipicolinate reductase (dapB) (negative control) and housekeeping gene cyclosporine-binding protein peptidylpropyl isomerase B (PPIB) (positive control) shown in the same tissues. The black bar represents the magnification of the image.

### 3.2. S protein was detectable in plasma/serum after IM administration of Ad26.COV2.S and other S protein-encoding Ad26-based vectors in rabbits, but not in platelet-rich cell fraction

We compared S protein levels in blood (both in plasma and in cell fraction) of rabbits dosed IM with different S-encoding Ad26-based vaccines, a subunit S protein vaccine or Ad26.Empty (**Figure 1A**). Levels of S protein were detected in plasma sampled 1 day after administration of the Ad26 vectors encoding the S protein or of the subunit S protein vaccine (**Figure 3A**). There were significantly lower S protein levels in Ad26.COV2.S-dosed animals (group geomean 31.3 pg/mL ± 1.26) compared with animals receiving Ad26.S (group geomean 86.9 pg/mL ± 1.30; $p$=0.0006, Tobit model with Bonferroni adjustment) or Ad26.S.PP-PR (group geomean 161.8 pg/mL ± 2.04; $p$<0.0001 Tobit model with Bonferroni adjustment). On day 11 after administration, the S protein levels in plasma of all groups were back to background levels (**Figure 3B**).

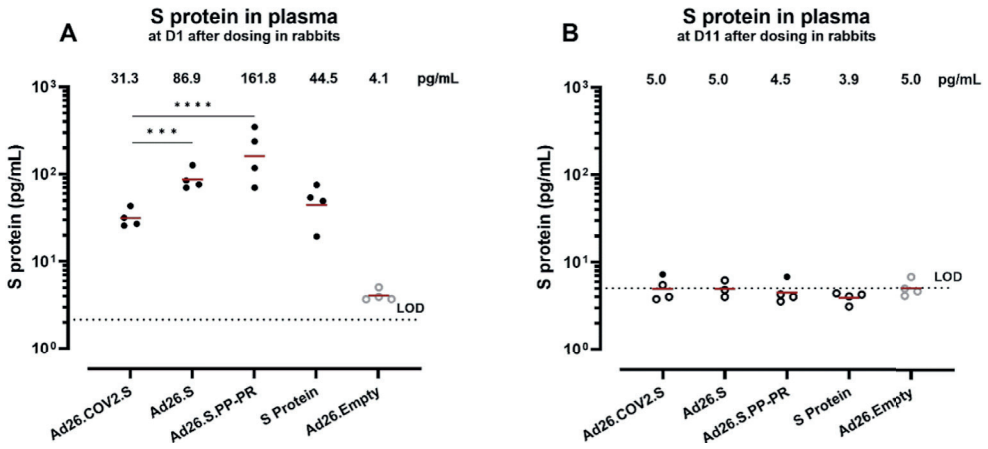



**Figure 3. Concentration of S protein in plasma after administration of S protein or Ad26-based vectors encoding S protein in rabbits.** Plasma from rabbits (n=4 per group/timepoint) dosed with Ad26.COV2.S, Ad26.S, Ad26.S.PP-PR, recombinant S protein COR201225 + aluminum hydroxide ($Al(OH)_3$), or Ad26.Empty was analyzed at **(A)** day 1 and **(B)** day 11 after dosing. Symbols in (A) represent the mean response per animal of 2 independent assays. Comparison of the S protein concentration induced by Ad26.COV2.S and Ad26.S or Ad26.S.PP-PR was done using a Tobit model with a Bonferroni adjustment for multiple comparisons, *** $p$=0.0006, **** $p$<0.0001. The dotted line represents the lower limit of detection (LOD) of the assay based on the standard curve. The background is defined by responses measured after dosing with Ad26.Empty, which does not include or encode SARS-CoV-2 S. Open symbols represent values below the 95$^{th}$ percentile of the Ad26.Empty group. The geometric mean is represented with a red line.

In contrast, S protein levels in the blood-derived cell fraction collected 1 day postdosing were in the range of the background measured in the control animals dosed with Ad26.Empty (**Figure A3** (**A**)). We confirmed that cell lysis buffer does not interfere with

the S protein detection assay by spiking purified S protein into the blood-derived cell fraction of naïve rabbits prior to cell lysis (**Figure A3** (**B**)).

The S protein was also detectable in serum and the levels were comparable to the levels detected in plasma (**Figure A3** (**C**)), suggesting that the blood sample preparation method had no major influence on S protein detection.

### 3.3. Circulating S protein presents similar expression kinetics but different magnitude of expression and subunit composition after IM administration of S-encoding mRNA compared with Ad26.COV2.S

To follow up on the potential role of the circulating S protein in the development of VITT and make a cross-platform comparison, we assessed the level of S protein after IM administration of S-encoding mRNA vaccines or Ad26.COV2.S in mouse and human serum samples. First, S protein expression kinetics were determined in the serum of mice dosed with $1\times10^9$ vp of Ad26.COV2.S (**Figure A4**). S protein levels increased by day 1 (26.7 pg/mL), remained detectable until day 6, and dropped to background levels by day 10 after dosing.

To compare the S protein levels after Ad26.COV2.S with an mRNA-based vaccine platform containing a wild-type furin cleavage site, we dosed mice with $1\times10^{10}$ vp of Ad26.COV2.S/mouse (1/5 of approved human dose), 6 mg of BNT162b2 (1/5 of approved human dose) or saline buffer. Serum was collected 24 hours after dosing to determine S protein levels as measured by an S1 antibody. The S protein levels were more than 100-fold higher in mice dosed with BNT162b2 (group geomean 32,477 pg/mL ± 1.318) compared with mice dosed with Ad26.COV2.S (group geomean 264.2 pg/mL ± 1.382) ($p<0.0001$, Mann Whitney test) 1 day after dosing (**Figure 4A**). To determine whether the S protein detected in circulation consists of the S1 subunit only or an S protein containing the S1 and S2 domains (S1-S2 protein), serum samples from day 1 after dosing were evaluated for S protein containing S1-S2 protein (**Figure 4B**). The S1-S2 protein levels were similar between Ad26.COV2.S and BNT162b2, suggesting a lower degree of S1 shedding for this vaccine.

To determine whether the differences observed in the S1 shedding between Ad26.COV2.S and BNT162b2 were due to differences in the S furin cleavage site, we dosed mice with $1\times10^{10}$ vp of Ad26.COV2.S, Ad26 encoding an S protein similar to that of BNT162b2 (Ad26.S.PP-PR), Ad26 encoding an S protein similar to that of ChAdOx1 (Ad26.tPA.WT.S), or saline buffer. Ad26.S.PP-PR and Ad26.tPA.WT.S both encode an S protein containing a wild-type furin cleavage site (**Figure A1**). Serum was collected at 24 hours postdosing and the S protein (as measured by an S1 antibody) and S1-S2 protein levels were measured (**Figure A5**). In the Ad26.tPA.WT.S and Ad26.S.PP-PR groups, the

S protein levels were higher compared with Ad26.COV2.S while the S1-S2 protein levels were lower, suggesting that the degree of S1 shedding is dependent on the encoded S furin cleavage site.

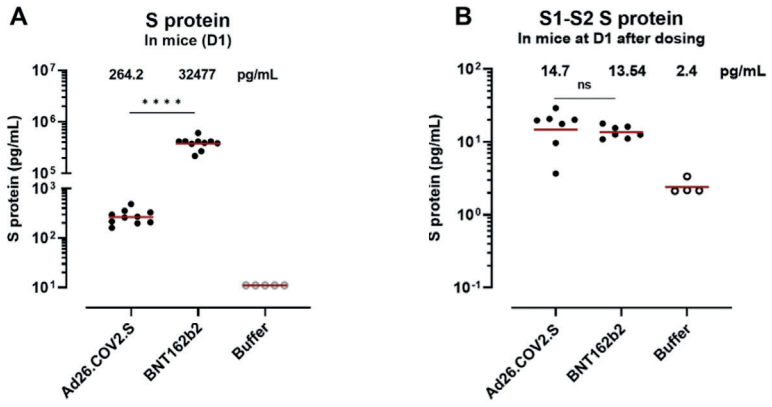



**Figure 4. S protein concentration in mouse serum after Ad26.COV2.S or S-encoding mRNA vaccination.** **(A)** S protein was measured in mouse serum at 24 h afte dosing with Ad26.COV2.S or BNT162b2. Open symbols represent values below the 95$^{th}$ percentile of the buffer (negative control) samples. **(B)** S1-S2 protein was measured at 24 hours after dosing in serum. The geometric mean is represented with a red line.

Next, S protein expression kinetics were determined in sera from COVID-19 human vaccinees that were seronegative for SARS-CoV-2 before vaccination. Vaccinees received an IM dose of $5\times10^{10}$ vp of Ad26.COV2.S (approved human dose) (**Figure 5A**) or mRNA (30 mg BNT162b2 or 100 mg mRNA-1273, approved human dose) (**Figure 5B**). The S protein expression presented similar kinetics between the 2 groups, with a peak in expression between 3 and 7 days post-administration. At 7 days after Ad26.COV2.S vaccination (group geomean 2.584 pg/mL ± 1.918) the levels were 7.3-fold lower than 7 days after mRNA vaccination (group geomean 21.45 pg/mL ± 7.122). Overall, the levels of the S protein were approximately 10-fold lower in Ad26.COV2.S vaccinees compared with mRNA vaccinees across timepoints. Due to the sensitivity of the assay, S1-S2 protein levels could not be determined in human serum. However, higher levels of S1 shedding are expected in mRNA vaccinees compared with Ad26.COV2.S due to the presence of a wild-type furin cleavage site in the S protein, consistent with the results observed in mice.

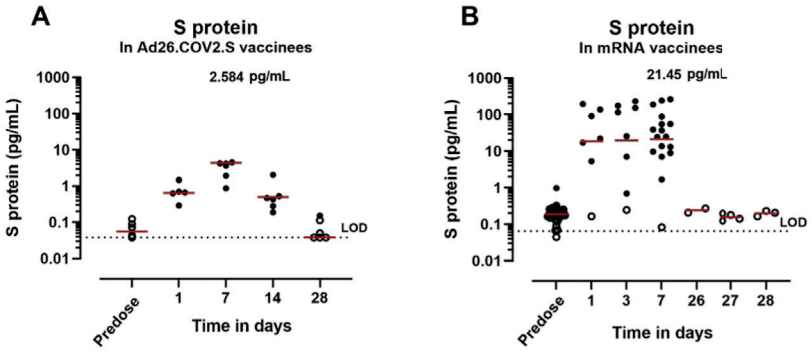



**Figure 5. S protein concentration in human serum after Ad26.COV2.S or S-encoding mRNA vaccination.** S protein expression kinetics in serum from **(A)** Ad26.COV2.S vaccinees or **(B)** from mRNA vaccinees at different timepoints. Open symbols represent values below the 95$^{th}$ percentile of the predose samples. The geometric mean is represented with a red line and, at day 7 after dosing, is represented in pg/mL.

## 3.4. Anti-spike antibodies interfere with S protein detection in serum after IM administration of Ad26.COV2.S in NHPs and humans

To assess whether the drop of S protein to background levels after Ad26.COV2.S immunization is due to the interference of anti-spike antibodies with the detection assay or due to a decrease in transgene expression alone, we analysed samples from a study in cynomolgus macaques (N=8). The macaques received 2 full human doses of $5\times10^{10}$ vp/animal of Ad26.COV2.S at days 0 and 56, and serum samples were evaluated for S protein and anti-spike antibodies over time (**Figure 6A**). The S protein concentration peaked at day 1 after the first dose (group mean 60.45 pg/mL ± 40.78) and was still detectable at day 7 (group mean 24.87 pg/mL ± 9.11). A decrease to background levels in 7/8 animals was observed at day 28 after the first dose. The decrease in detectable S protein levels coincides with the first detection of anti-spike IgG antibody titers on day 28 (group mean 2208.31 ELISA units/mL ± 1878.61). The anti-spike antibody titers remain constant at subsequent timepoints and further increase at day 64 (day 8 after the second dose; group mean 13,202.56 ELISA units/mL ± 5324.64). In contrast, the S protein was not detectable in serum at any timepoint after the second Ad26.COV2.S dose where, according to primary exposure in naïve animals, S expression would be expected.

To assess S protein levels in anti-spike antibody seropositive human trial participants, sera from Ad26.COV2.S vaccinees from a dose range study (COV3003) were analyzed. This study was conducted later during the pandemic, therefore 9 out of 15 participants were anti-spike seropositive at baseline. The S protein concentration was determined in serum of vaccinees that received an IM dose of $9\times10^{10}$ vp, $5\times10^{10}$ vp, or $1.25\times10^{10}$ vp of Ad26.COV.2.S. Anti-spike antibody levels were measured in the same vaccinees before

dosing. In baseline seronegative individuals, S protein levels peaked at 3 days after dosing (2.78 pg/mL ± 4.41), and remained detectable by day 7 after dosing (**Figure 6B**). The S protein levels decreased to near background levels by 28 days after dosing, similar to the S protein expression kinetics observed in cynomolgus macaques. In contrast, only 1 out of 9 seropositive individuals showed detectable levels of S protein at day 3 (**Figure 6C**). The magnitude of anti-spike binding titers in the serum of this individual was relatively low ($log_{10}$ 2.15 ELISA units/mL) (**Figure A6**).

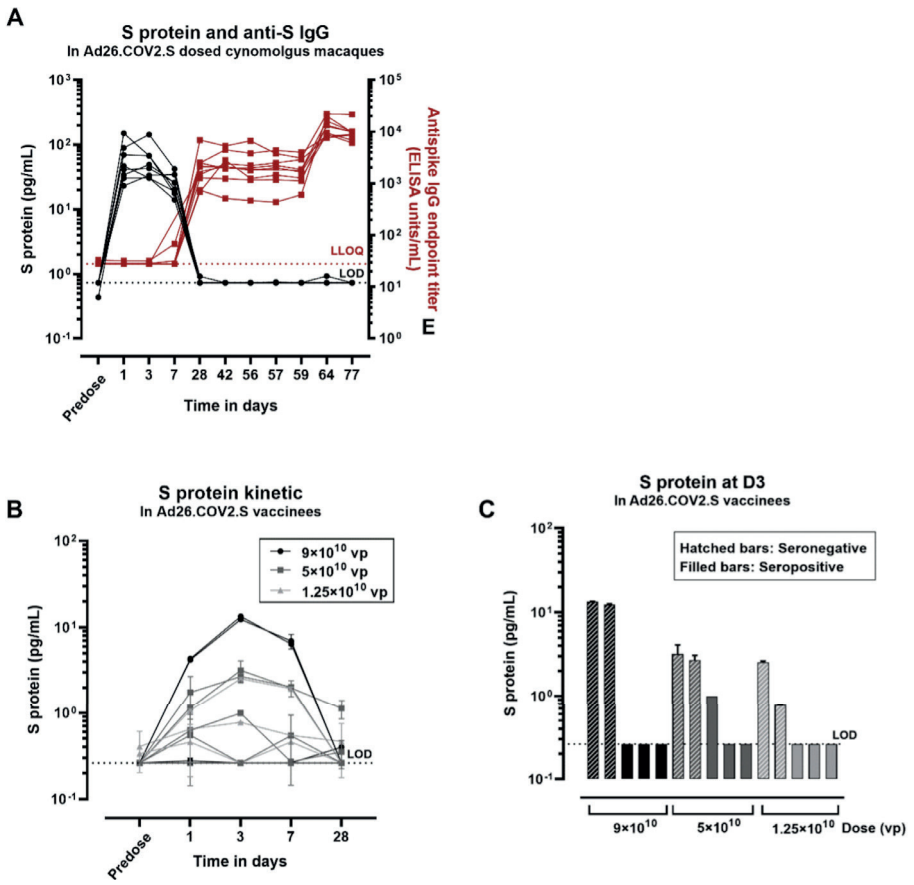



**Figure 6. Concentration of S protein in serum after dosing with Ad26.COV2.S in macaques and humans.** **(A)** Macaque serum samples were analyzed for S protein detection and for anti-spike immunoglobulin G (IgG) antibody titers. Black symbols correspond to S concentration expressed in pg/mL and red symbols correspond to anti-spike IgG titers expressed in endpoint titer ELISA (1 symbol/animal). The black dotted line represents the lower limit of detection (LOD) of the S assay based on the standard curve. The red dotted line corresponds to the lower limit of quantification of the anti-spike assay (LLOQ). **(B)** The concentration of S protein was measured in Ad26.COV2.S vaccinees at different timepoints before and after dosing. **(C)** The concentration of S protein was measured in serum from seropositive vaccinees (containing anti-spike neutralizing antibodies) or seronegative vaccinees 3 days after dosing with Ad26.COV.2.S. The black dotted line represents the lower limit of detection (LOD) of the assay based on the standard curve. The error bars represent the standard deviation of 2 technical replicates in **(B)** and **(C)**.

Since S protein expression was undetectable upon induction of anti-spike antibodies in NHP and human serum samples, we determined whether anti-spike antibodies interfere with the detection of S protein through the formation of anti-spike IgG – S protein immune complexes *in vitro*. Serum containing S protein was coincubated with serum containing anti-spike antibody. Rabbit serum (n=4) taken on day 1 postdosing with $5\times10^{10}$ vp of Ad26.COV2.S was coincubated with predose serum (anti-spike antibody negative) or serum from day 28 after dosing with $5\times10^{9}$ vp/kg of Ad26.COV2.S (anti-spike antibody positive) or Ad26.ZIKV.001 (anti-spike antibody negative) from a different study [42] (**Figure A7**). The S protein readouts after coincubation with day 28 serum (group mean 0.09 pg/mL ± 0.07) were significantly lower than the S protein readouts after coincubation with predose serum (group mean 7.29 pg/mL ± 2.04) ($p$=0.001, unpaired t-test) (**Figure A7**). As a control, S protein containing serum from day 1 after dosing with Ad26.COV2.S was coincubated with serum from day 28 after dosing with Ad26.ZIKV.001. S protein readouts were comparable between samples coincubated with predose serum or Ad26.ZIKV.001 immune serum, suggesting that the anti-spike antibodies, and not other components in the serum after Ad26 vaccination, interfere with the S protein detection assay.

### 3.5. Low levels of circulating S protein were detectable for a prolonged period after Ad26.COV2.S administration in antibody-deficient mice

In vaccinees who do not mount sufficient anti-spike antibody responses after vaccination, the free circulating S protein might be present for a prolonged period of time and could be an additional factor in the development of VITT. To further investigate the duration of the S protein expression in an anti-spike antibody free model Jh (C57BL/6NTac-Igh-Jem1Tac) knockout or C57BL/6 (background control), mice were dosed with $5\times10^{9}$ vp/mouse of Ad26.COV2.S (n=6/group) (**Figure 7A**). Jh mice carry a deletion of the endogenous murine J segments of the Ig heavy chain locus and therefore contain no mature B-cells and produce no antibodies [43].

S protein was detected at days 1, 3, and 6 after dosing with Ad26.COV2.S at comparable levels across groups and timepoints (**Figure 7B**). Ten days after dosing with Ad26.COV2.S, S protein expression was detectable in Jh mice (group mean 10.83 pg/mL ± 4.36) ($p$<0.0001, paired t-test) and not in C57BL/6 mice (group mean 0.50 pg/mL ± 0.28) (ns, paired t-test) (**Figure 7B**). At day 15 postdosing, the S protein levels were higher in Jh (1.05 pg/mL ± 0.36) compared with C57BL/6 mice (0.32 pg/mL ± 0.1) ($p$=0.0001, unpaired t-test). The S protein was detectable at low levels until day 35 after dosing in the Jh (group mean 1.05 pg/mL ± 0.36), but not in C57BL/6 mice.

Anti-spike IgG antibodies were measured in serum at day 10 and day 35 after dosing. Anti-spike antibody levels were higher at day 35 after dosing compared with day 10 in

C57BL/6 mice ($p$=0.0135, unpaired t-test) and anti-spike antibodies were not detectable in Jh mice at any of the timepoints (**Figure 7C**). These data suggest the interference of anti-spike antibodies with the S protein detection or the clearance of the S protein and/or S-expressing cells by anti-spike immune complexes.

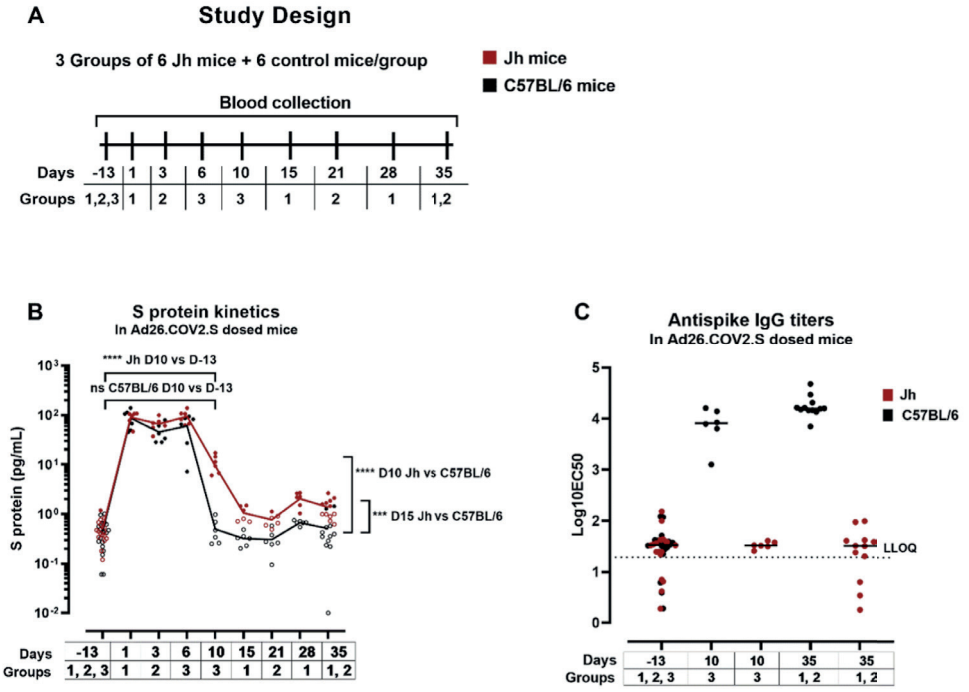



**Figure 7. S protein expression and S-specific IgG titers in antibody-deficient and wild-type mice. (A)** Antibody-deficient mice (Jh) or control mice (C57BL/6) were dosed with $5\times10^9$ vp/mouse of Ad26.COV2.S. **(B)** Expression of S protein was measured in serum at different timepoints. The open symbols represent the values below the 95$^{th}$ percentile of the predose values. The red (Jh mice) and black (control mice) lines represent the trend of the mean S protein expression across timepoints. **(C)** S-specific IgG titers were measured in serum from predose sampling, 10 days and 35 days after dosing in 2 ELISA runs. The dotted line represents the lower limit of quantification (LLOQ) of the assay. Each symbol (circles, squares, and triangles) corresponds to a different group of mice. Statistical comparisons were done using paired t-tests or unpaired t-tests when comparing different mice.

## 4. Discussion

VITT is a rare adverse event characterized by thrombosis in atypical anatomical locations and severe thrombocytopenia. The mechanism behind VITT has not yet been elucidated, but it may result from a combination of factors, including the activation of platelets/endothelial cells and the inflammatory signatures induced by the SARS-CoV-2 S protein after vaccination. Here we assessed the distribution of the S protein and characterized

the circulating S protein after Ad26.COV2.S vaccination in animal models. Additionally, we evaluated circulating S protein levels in clinical samples.

We detected S protein and S mRNA at the site of administration (muscle) and in draining lymph nodes 1 day after dosing of rabbits with Ad26.COV2.S, but not in the blood vessel wall or lining endothelium (arteries/veins). The SARS-CoV-2 S protein has been shown to cause vascular damage in hamsters [28] and has been detected within the thrombus and in the adjacent vessel wall in patients with VITT-induced cerebral venous thrombosis [29]. In our studies, we did not detect S protein in blood vessel walls or observe evidence of endothelial damage in rabbits. However, effects of the circulating S protein on endothelial cells may still play a role on VITT as published [27,44,45], and may depend on exposure kinetics and subunit composition of the circulating S protein.

Eleven days after dosing with Ad26.COV2.S, we did not detect S protein by immunohistochemistry or S mRNA by in situ hybridization in any of the tissues examined. The circulating S protein was not detectable by day 10/11 in the serum of animal models or by day 29 in the serum of human vaccinees. Consistent with our results, Stebbings et al found that S protein was rapidly undetectable (within days 7-14) in the circulation of mice dosed intravenously or via IM with ChAdOx1 nCoV [46]. This decrease in the S protein detection is likely mainly due to the clearance of the transduced cells and the adenoviral vaccine. However, our results demonstrate the interference of anti-spike antibodies with the detection of S protein. In an antibody-deficient mouse model, we measured low but detectable levels of circulating S protein up to day 35 after dosing, suggesting partial antibody-mediated clearance of the S protein. This would explain the early timing of induction of VITT and the lower incidence of VITT after a second dose of the vaccine [12], if S protein plays a role in the process. In alignment with this, prolonged bioavailability of the S protein may play a role in the development of VITT. In some human cases of VITT, the circulating S protein has been detected for a prolonged period of time, up to 35 days after vaccination [47]. The lack of anti-spike antibody complex formation with the S protein and the persistence of free circulating S protein for a prolonged period of time due to host-specific factors may drive prolonged proinflammatory signatures via S binding and activation of specific cell types, such as endothelial cells, as previously hypothesized [28].

In our studies, significantly lower levels of S protein, as detected by an S1 antibody, were observed after Ad26.COV2.S administration compared with the levels observed after administration of an mRNA vaccine in mice and human vaccinees, while the S1-S2 protein presented similar levels across these 2 vaccine platforms in mice. The mRNA vaccines for COVID-19 (BNT162b2 and mRNA-1273) express an S protein that contains a wild-type furin cleavage site, which has been shown to result in the shedding of the

S1 portion of the S protein in the plasma of mRNA-1273 vaccinees, consistent with our results [4]. VITT has been reported after ChAdOx1 vaccination in humans [13]; however, ChAdOx1 encodes a wild-type S that presented similar S1 shedding properties compared with mRNA vaccines in our studies. Therefore, this factor is unlikely the main trigger of VITT.

## 5. Conclusions

Overall, we showed no detection of the S protein in the endothelium or bound to platelets after Ad26.COV2.S vaccination in animal models. We have demonstrated similar kinetics of transient S protein expression after Ad26.COV2.S vaccination in preclinical models and humans, with a comparable kinetic but lower magnitude as observed after mRNA COVID-19 vaccination. The S subunit composition was different in serum after Ad26.COV2.S compared with mRNA BNT162b2 dosing in preclinical models, however, and this is likely linked to mutation of the furin cleavage site in Ad26.COV2.S, which is not present in the other VITT-related vaccine, ChadOx1. The observations of the S protein biodistribution, kinetic, and composition after Ad26.COV2.S vaccination do not provide conclusive evidence for the absence or presence of a direct association of S protein with the development of VITT. The S protein, in the context of Ad26 vaccination, cannot be excluded as a potential contributing factor in the pathogenesis of VITT in combination with other factors such as previous infections, genetic predisposition, or preexisting health conditions that likely influence the development of the VITT.

***Author Contributions***

Sonia Márquez Martínez: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing—original draft, writing—reviewing and editing. Selina Khan: Conceptualization, data curation, formal analysis, project administration, supervision, validation, writing—reviewing and editing. Joan van Lubbe: Data curation, formal analysis, methodology, validation, writing—reviewing and editing. Laura Solforosi: Data curation, formal analysis, validation, writing—reviewing and editing. Lea Costes: Data curation, formal analysis, validation, writing—reviewing and editing. Ying Choi: Investigation. Satish Boedhoe: Investigation. Mieke Verslegers: Investigation, methodology, visualization, writing—reviewing and editing. Marjolein van Heerden: Investigation, methodology, visualization, writing—reviewing and editing. Wendy Roosen: Investigation, writing—reviewing and editing. Sandra de Jonghe: Supervision, writing—reviewing and editing. Hendy Kristyanto: Formal analysis, investigation, writing—reviewing and editing. Veronica Rezelj: Formal analysis, investigation, writing—reviewing and editing. Jenny Hendriks: Formal analysis, investigation, writing—reviewing and editing. Jan Serroyen: Formal analysis.

Jeroen Tolboom: Formal analysis. Frank Wegmann: Conceptualization, formal analysis, project administration, supervision, writing—reviewing and editing. Roland C. Zahn: Conceptualization, supervision, writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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***Informed Consent Statement***
Informed consent was obtained from all subjects involved in the study.

***Data Availability Statement***
The data presented in this study are available on request from the corresponding author.

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***Conflicts of Interest***
The authors declare the following competing interests: all authors are or were employees of Janssen Vaccines & Prevention B.V. or of Janssen Research & Development, a division of Janssen Pharmaceutica NV while engaged in the research project S.K., L.S., A.V., S.B., J.S., J.T., F.W., and R.Z. held or still hold stock in Johnson & Johnson.

# Appendix A

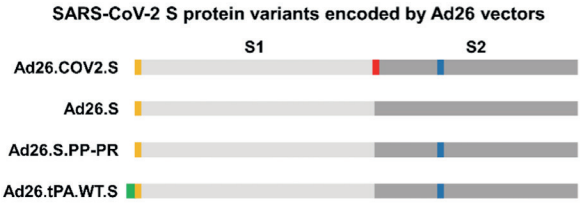


**Figure A1. Ad26 vectors encoding variants of the SARS-CoV-2 S protein.** From top to bottom: (1) full-length S in which furin cleavage site mutations and proline substitutions have been introduced, (2) native full-length S, (3) full-length S with only the proline substitutions, and (4) full-length S in which tissue plasminogen activator signal peptide is added upstream of the wild-type signal peptide. Green bar represents tissue plasminogen activator signal peptide, yellow bars represent wild-type signal peptide, red vertical lines represent furin cleavage site mutations, and blue vertical lines represent proline substitutions.

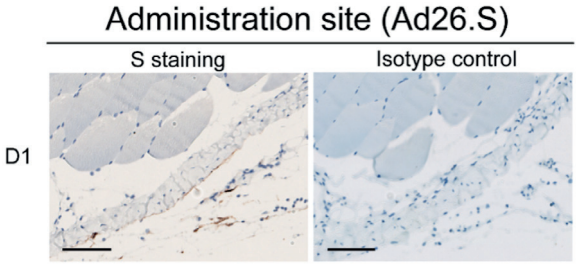


**Figure A2. Isotype control in muscle after IM Ad26.S dosing in rabbits.** Anti-SARS-CoV2 S1 or isotype control staining by immunohistochemistry of muscle (administration site) at day 1 post Ad26.S dosing. The black bars represent the magnification of the images (100 mm).

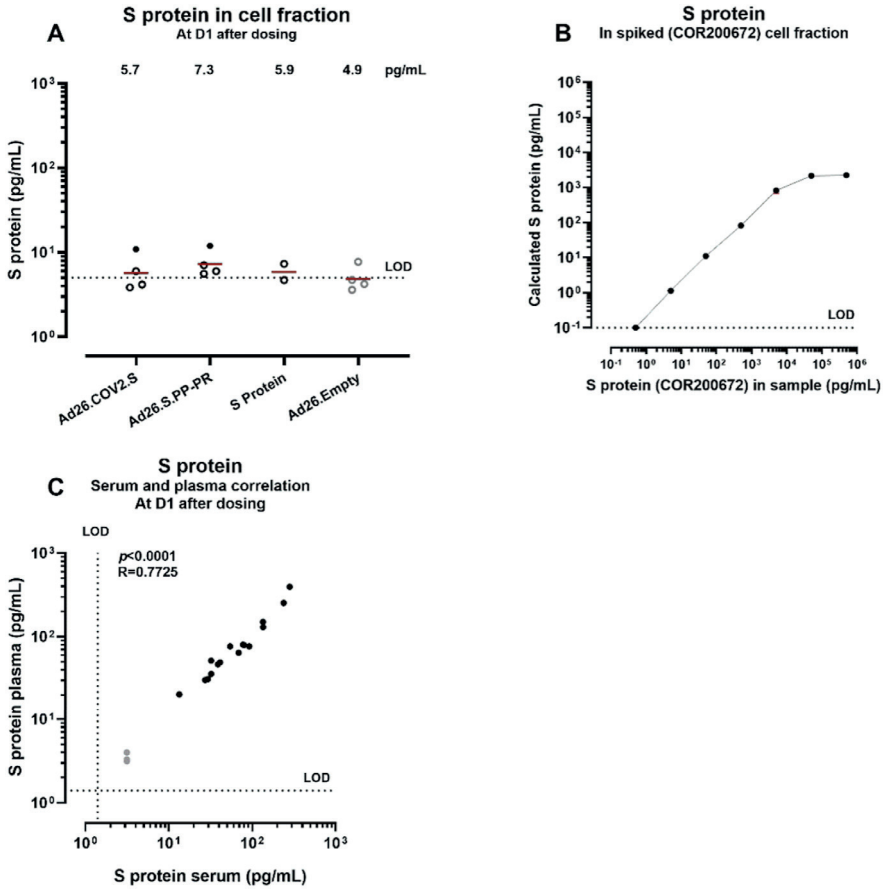


**Figure A3. S protein concentration in cellular fraction after dosing with S protein or Ad26-based vectors encoding S protein in rabbits and correlation of S protein concentration in plasma and serum. (A)** Blood-derived cell fraction was analyzed at day 1. The background is defined by responses measured after dosing with Ad26.Empty, which does not include or encode SARS-CoV-2 S. Open symbols represent values below the 95$^{th}$ percentile of the Ad26.Empty group. The geometric mean response per group is indicated with a red line and the numbers above the graph. **(B)** Blood-derived cell fraction was isolated before dosing and was spiked with purified S protein COR200672 at increasing concentrations. **(C)** Correlation of S protein concentration in plasma and serum. Black symbols correspond to the samples from rabbits dosed with Ad26.COV2.S, Ad26.S, Ad26.S.PP-PR, or recombinant S protein COR201225 + $Al(OH)_3$. Gray symbols correspond to the samples from rabbits (n=4 per group) dosed with Ad26.Empty analyzed at day 1. Spearman correlation coefficient (R) and p-value were calculated for the correlation analysis. The dotted lines represent the lower limit of detection (LOD) of the assays based on the standard curves.

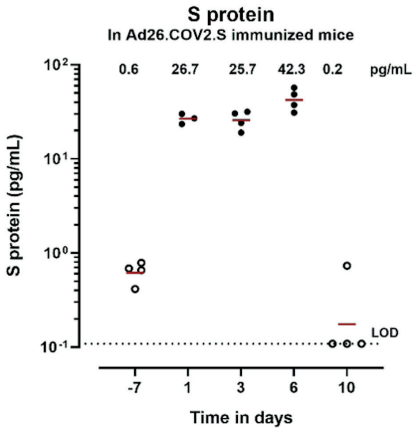


**Figure A4. S protein concentration in serum of mice dosed with Ad26.COV2.S. (A)** BALB/c mice (4 mice/group, 1 group/timepoint) were dosed with $1\times10^{9}$ vp/mouse and **(B)** S protein was measured in serum at different timepoints (1 symbol/mouse). The dotted line represents the lower LOD of the assay based on the standard curve. Open symbols represent values below the 95$^{th}$ percentile of the predose samples. The geometric mean is represented with a red line.

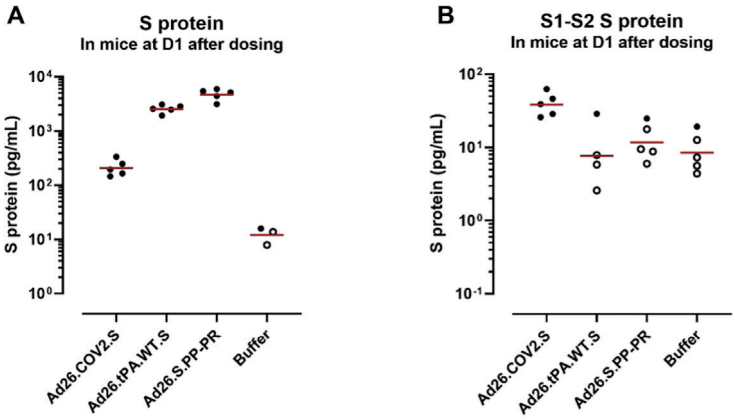


**Figure A5. S protein and S1/S2 protein levels in mice immunized with Ad26 constructs encoding different S protein variants.** Mice were immunized with $10^{10}$ vp of Ad26.COV2.S, Ad26tPA.WT.S, Ad26.S.PP-PR or saline buffer. **(A)** S protein and **(B)** S1-S2 protein were measured at 24 h after dosing in serum. Open symbols represent values below the 95$^{th}$ percentile of the buffer control samples. The geometric mean is represented with a red line.

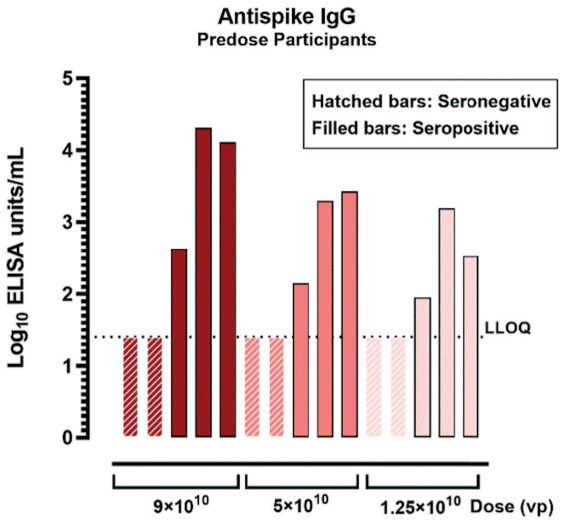


**Figure A6. Anti-spike IgG titers in serum before dosing with Ad26.COV2.S in humans.** S-specific IgG titers were measured in serum from predose sampling. The dotted line represents the LLOQ of the assay. Each bar corresponds to a different individual. Hatched bars correspond to seronegative individuals (below LLOQ) and filled bars correspond to seropositive individuals (above LLOQ).

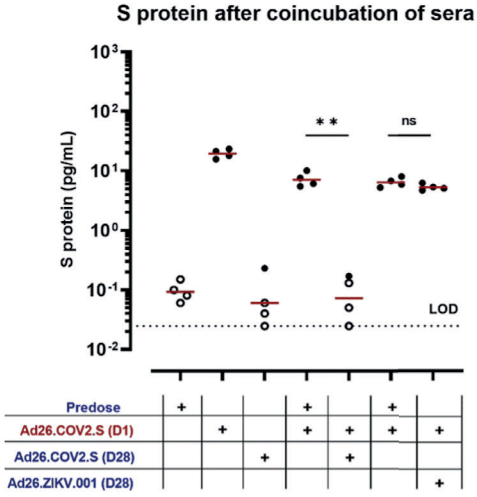


**Figure A7. S protein in rabbit serum samples after coincubation with anti-spike antibody containing serum.** Spike-containing serum from day 1 after dosing with Ad26.COV2.S (in red) was coincubated with anti-spike antibody containing serum from 28 days after dosing with Ad26.COV2.S (in blue). Spike-containing serum from day 1 after dosing (in red) was coincubated with predose serum (from Ad26.COV2.S immunized group—column 4, or Ad26.ZIKV.001 immunized group—column 6) or anti-spike antibody-deficient serum of rabbits dosed with Ad26.ZIKV.001 (in blue) (negative controls). The dotted line represents the lower limit of detection (LOD) of the assay based on the standard curve. Comparison of the S protein concentration was done using unpaired t-tests with Welch's correction. The geometric mean is represented with a red line.

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CHAPTER 5

# Intravenous administration of Ad26.COV2.S does not induce thrombocytopenia or thrombotic events or affect SARS-CoV-2 spike protein bioavailability in blood compared with intramuscular vaccination in rabbits

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## Abstract

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a very rare but serious adverse reaction that can occur after Ad26.COV2.S vaccination in humans, leading to thrombosis at unusual anatomic sites. One hypothesis is that accidental intravenous (IV) administration of Ad26.COV2.S or drainage of the vaccine from the muscle into the circulatory system may result in interaction of the vaccine with blood factors associated with platelet activation, leading to VITT. Here, we demonstrate that, similar to intramuscular (IM) administration of Ad26.COV2.S in rabbits, IV dosing was well tolerated, with no significant differences between dosing routes for the assessed hematologic, coagulation time, innate immune, or clinical chemistry parameters and no histopathologic indication of thrombotic events. For both routes, all other non-adverse findings observed were consistent with a normal vaccine response and comparable to those observed for unrelated or other Ad26-based control vaccines. However, Ad26.COV2.S induced significantly higher levels of C-reactive protein on day 1 after IM vaccination compared with an Ad26-based control vaccine encoding a different transgene, suggesting an inflammatory effect of the vaccine-encoded spike protein. Although based on a limited number of animals, these data indicate that an accidental IV injection of Ad26.COV2.S may not represent an increased risk for VITT.

# 1. Introduction

Ad26.COV2.S is a monovalent vaccine composed of a recombinant, replication-incompetent human adenovirus type 26 (Ad26) vector, encoding a full-length, membrane-bound severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) spike (S) protein (Wuhan strain), stabilized in its prefusion conformation [1].

Ad26.COV2.S has been used in millions of individuals for the prevention of COVID-19 and is highly effective against severe and critical COVID-19, COVID-19–related hospitalization, and death, with protection lasting ≥6 months [2].

Thrombosis with thrombocytopenia syndrome (TTS) has been reported following vaccination with ChAdOx1 nCoV-19, Ad26.COV2.S, [3-6] and to a lesser extent, with COVID-19 mRNA-1273 [7], inactivated COVID-19 vaccines [8,9], and Gam-COVID-Vac [10]; some similar cases were observed following COVID-19 disease [11,12]. TTS has occurred as a very rare event in approximately 2.3 to 5.5 cases per 1 million doses of Ad26.COV2.S administered, depending on the definition of TTS (Centers for Disease Control [USA] and Prevention of Pharmacovigilance Risk Assessment Committee [EMA]) [5,13,14]. The very low incidence rate complicates the identification of a causal pathway leading to this adverse clinical outcome. The term vaccine-induced immune thrombotic thrombocytopenia (VITT) has been used to describe cases that are likely vaccine adverse effect related.

The hallmarks of VITT are thrombosis at unusual anatomic sites, such as brain venous sinuses or splanchnic vein; severe thrombocytopenia with the presence of platelet-activating antibodies targeting platelet factor 4 (PF4); and high D-dimer levels [15]. Onset of symptoms is usually within 5 to 43 days following vaccination, which can be fatal; however, with appropriate treatment the symptoms can be managed [3-6,16].



The mechanistic relevance of PF4 antibodies was shown recently by demonstrating PF4-VITT antibody complex–induced thrombus formation *in vitro*, which was mediated through neutrophilic activation and NETosis in a FcγRIIa-dependent manner. Adoptive transfer of purified VITT IgG into a human PF4 and FcγRIIa transgenic mouse model confirmed these data [16,17]. Furthermore, anti-PF4 antibodies from patients with VITT bind to a highly restricted epitope site on PF4 that corresponds to the heparin-binding site, are reported to have the same single lambda light chain IGLV3-21 [18], and therefore have a distinct specificity compared with antibodies found in heparin-induced thrombocytopenia [17,19].

Multiple hypotheses have been proposed for potential mechanisms underlying VITT post-vaccination with adenovirus vector-based COVID-19 vaccines. One hypothesis is that systemic exposure of adenovirus vector-based vaccine particles and/or the S protein encoded by the vector-based vaccines (due to either accidental intravenous (IV) injection or leakage from the muscle injection site into the blood) may lead to interaction of the adenovector with platelets [3,20-22]. Nicolai and colleagues reported that IV, but not intramuscular (IM), injection of a high dose of ChAdOx1 nCoV-19 in mice resulted in platelet-adenovirus aggregate formation and platelet activation [20,22]. A possible contributing factor to this effect may be IV dosing–related systemic biodistribution of the vaccine-encoded S protein, which has been shown to activate platelets and cause thrombus formation and inflammatory responses in mice [20,23].

Here we investigated whether IV dosing (as a model for accidental systemic exposure) of Ad26.COV2.S could induce signs of thromboembolic disease or changes in platelet counts, other clinical pathology parameters, histopathology findings (of thrombotic events and their sequelae), or systemic S protein exposure when compared with IM dosing in rabbits.

## 2. Materials and methods

### 2.1 Ethics Statement

The rabbit study was conducted at Janssen Research & Development Belgium, in facilities approved by the Institute of Health Office of Animal Welfare and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Animal research protocols were approved by the Institutional Ethical Committee, and the studies were conducted in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and Belgian guidelines, and with the principles of euthanasia as stated in the guidelines from the American Veterinary Medical Association Panel [24]. Import and export permits for vectors and rabbit biospecimens were obtained in compliance with European Union federal regulations.

The mouse study was performed at Janssen Vaccines & Prevention, The Netherlands, and was conducted according to the Dutch Animal Experimentation Act and the Guidelines on the Protection of Animals for Scientific Purposes by the Council of the European Committee after approval by the Centrale Commissie Dierproeven and the Dier Experimenten Commissie.

## 2.2 Vaccines

Replication-incompetent, E1/E3-deleted recombinant Ad26 vectors were engineered using the AdVac® system, as described elsewhere [25,26], with Ad26 encoding stabilized SARS-CoV-2 S protein (Ad26.COV2.S) [1], or Envelope (Env) of Zikavirus (Ad26.ZIKV.001) [26,27]. The vectors were clinical grade material produced under the same process.

Ad26-mediated expression of the various transgenes was confirmed by Western blot analysis of cell-culture lysates from infected A549 cells or by polymerase chain reaction (PCR).

The commercial clinical grade MMR (M-M-RVAXPRO, Merck Sharp & Dohme B.V) was used as controls in the rabbit study.

## 2.3 Animals and housing

A total of 45, eleven-week-old, healthy female New Zealand white rabbits (body weight [BW] 2.1-2.8 kg at study start) were included. Female rabbits were used since at the time of start of the rabbit study, TTS cases observed appeared primarily in female patients, however this imbalance is presently less clear. The animals were supplied by Charles River Laboratories (France). Rabbits were kept in a biosafety level 2 facility under specific pathogen-free conditions after screening negative for *Mycobacterium tuberculosis*, simian immunodeficiency virus, simian retrovirus, and simian T-lymphotropic virus. Screening included herpes B virus and measles serology. Rabbits were single-housed in stainless steel cages with a slotted plastic floor and placed in study-dedicated rooms.

For our study in mice, six- to 8-week-old, naïve specific-pathogen-free female BALB/c mice (Charles River Laboratories) were housed with 10 animals per cage.



For all animal studies, animals were kept under controlled, recorded environmental conditions of humidity, temperature, and 12-hour light cycle. Animals were provided with sensory and cognitive environmental enrichment. Animals were fed a standard diet ad libitum, and tap water was provided ad libitum through an automated system. Animal well-being and health surveillance was monitored at least twice daily by husbandry staff. Preset humane end points were used by a veterinarian to define sacrifice criteria not related to the study. All measures were taken to minimize pain, distress, and suffering, and all procedures were performed by trained personnel.

## 2.4 Study design and animal procedures

Rabbits were divided into 9 study groups with 5 animals per group (**Figure A1**(A)). The animals received a single dose of Ad26.COV2.S IV ($0.2\times10^9$ viral particles [vp]/kg, $1\times10^9$ vp/kg, or $5\times10^9$ vp/kg), Ad26.COV2.S IM ($5\times10^9$ vp/kg), Ad26.ZIKV.001 IV

($5\times10^9$ vp/kg), Ad26.ZIKV.001 IM ($5\times10^9$ vp/kg), or a full human dose (FHD) of measles-mumps-rubella (MMR) vaccine ($10^2$ measles 50% tissue culture infectious dose [$TCID_{50}$]/kg, $12\times10^2$ mumps $TCID_{50}$/kg, $10^2$ rubella $TCID_{50}$/kg; Sanofi Pasteur MSD).

Control rabbits received the buffer (15 mM citric acid, 75 mM NaCl, 2-hydroxylpropyl-β-cyclodextrin 5% (w/w), and 0.03% polysorbate-80 pH 6.2 [referred to as vehicle]) given either IM or IV. On average, the animals dosed with $5\times10^9$ vp/kg of Ad26 vector received a total dose of $1.25\times10^{10}$ vp (based on an average BW of 2.5 kg).

All vaccines (of clinical grade) were administered in a 0.1 mL/kg BW volume either IM (in the biceps femoris) or IV (via ear vein). Blood sampling was performed from the central ear artery. The total blood volume and sampling frequency was performed according to good ethical practices. At the end of study, rabbits were anesthetized by an IV injection of pentobarbital and euthanized by exsanguination via the inguinal blood vessels. Terminal blood sampling was performed via the inguinal blood vessels.

Mice were used to confirm observations from the rabbit study in a second species. In a separate study, mice Animals were bled prior to dosing by submandibular bleeding to obtain serum. Mice were then given a single IM immunization (50 µL/hind leg) with Ad26.COV2.S ($1\times10^{10}$ vp, N=10) or Ad26.ZIKV.001 ($1\times10^{10}$ vp, N=10). Twenty-four hours later mice were exsanguinated by cardiac puncture (serum was collected) followed by cervical dislocation under isoflurane anesthesia.

## 2.5 Droplet digital PCR to measure adenovirus DNA copies in blood

DNA from 100 µL blood of immunized rabbits was extracted using the DNeasy Blood & Tissue Kit (Qiagen), and the isolation procedure was optimized from the original manufacturer's instruction. DNA was eluted by performing 2 subsequent elution steps using 25 µL elution buffer per step. DNA quantity and quality were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Adenovirus DNA copies were measured with droplet digital PCR (ddPCR) in 3 technical replicates. In total, 5 µL of DNA per reaction was measured in a 22 µL reaction, including 10 µL 2× ddPCR Supermix for probes (Bio-Rad), 900 nM forward primer (GATAGCGGTTTGACTCACG), 900 nM reverse primer (AATGGGGCGGAGTTGTTAC), and 250 nM probe (VIC-TCCCGTTGATTTTGGTGCC-MGB), added up to 22 µL total volume with distilled $H_2O$. Samples were incubated for 10 minutes at room temperature before droplet generation using the Automated Droplet Generator (Bio-Rad) following the manufacturer's instruction. PCR was performed with 40 PCR cycles (30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 68°C), preceded by incubation for 10 minutes at 94°C and followed by incubation for 10 minutes at 98°C. Droplets were read in the QX

200 Droplet Reader (Bio-Rad) and analyzed using QuantaSoft™ Analysis Pro software (version 1.0; Bio-Rad).

### 2.6 Hematology, clinical chemistry, coagulation, and C-reactive protein (CRP) analysis

Rabbit blood samples for hematology and coagulation were collected at pretreatment and at 24 hours, 48 hours, 72 hours, 7 days, 14 days, 21 days, and 28 days post vaccine administration. These were analyzed for hematology or coagulation parameters on an Advia2120 Hematology Analyzer (Siemens) and an ACL TOP500 Coagulation Analyzer (Instrumentation Laboratory), respectively. Clinical chemistry and CRP analysis was performed on serum (1.1 mL Z-gel Microtube, Sarstedt) collected at pretreatment and at 24 hours (CRP only), 48 hours (clinical chemistry only), 7 days, 14 days, 21 days, and 28 days post-vaccination. Clinical chemistry was analyzed on a Cobas6000 analyzer (Roche), while CRP was determined in serum using a rabbit CRP enzyme-linked immunosorbent assay (ELISA; Life Diagnostics Inc.) on an Infinite M1000 PRO instrument (Tecan) according to the manufacturer's instructions.

### 2.7 Serum Amyloid A Analysis

Serum amyloid A was measured in mouse serum samples using the Mouse Serum Amyloid A Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

### 2.8 Histopathology

Necropsy and gross/macroscopic examinations were conducted. Histopathologic evaluation was performed in the following formalin-fixed paraffin-embedded tissues from IV dosed rabbits: macroscopically abnormal tissues, administration site (ear vein), adrenal glands, aorta, brain, heart, intestines (colon and duodenum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mesentery with blood vessels, spleen, and stomach. Histopathology was not conducted on the IM groups in the present study, as it was already assessed in regulatory toxicity studies.



### 2.9 Detection of S protein in blood by electrochemiluminescence

Complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche) was added to rabbit serum samples. Serum samples were centrifuged for 3 minutes (2000 × g at 4°C) to remove particulates before assay.

S-PLEX SARS-CoV-2 S detection assay (Mesoscale) was used to detect S protein in the serum samples; this assay detects the presence of the S protein receptor-binding domain (RBD; direct communication from the manufacturer). According to manufacturer instruction, phosphate-buffered saline + 0.05% Tween-20 was used as a washing buffer.

### 2.10 Statistical Analysis

Responses were log-transformed, and groups were compared using analysis of variance (ANOVA) in cases of noncensored data or a Tobit model in cases of censored data. P-values <0.05 were considered statistically significant. A correction for multiple comparisons was applied where indicated.

## 3. Results

### 3.1 Scaling to body weight is required to mimic accidental systemic exposure in humans

To assess potential effects of systemic Ad26.COV2.S on platelet counts, coagulation, histopathology, and acute phase immune response parameters, rabbits received IV vaccine dose levels that were scaled to BW. Considering that the IV dosing route leads to systemic distribution of the vaccine, scaling to BW is necessary to mimic a comparable tissue exposure to that in humans. The clinical Ad26.COV2.S dose is $5\times10^{10}$ vp, which translates to $1\times10^{9}$ vp/kg for a 50 kg adult. This dose/kg was used as the middle dose for IV dosing in rabbits. To assess a possible dose relationship of any finding, we also tested a high dose ($5\times10^{9}$ vp/kg) and a low dose ($0.2\times10^{9}$ vp/kg). To compare the effects of IV dosing with the intended IM route of administration, the high dose of $5\times10^{9}$ vp/kg was also dosed IM. The comparator adenovirus vector-based vaccine, Ad26.ZIKV.001, was administered at the high dose of $5\times10^{9}$ vp/kg (IV and IM). Ad26.ZIKV.001 encodes the Zika virus M and E Env proteins [26,27]. As a reference control, the MMR vaccine was used at a dose level based on a 1-year-old child scaled to BW. The rabbit study design is shown in **Figure A1(A)**.

### 3.2 IV or IM administration of Ad26.COV2.S was not associated with changes in hematologic, coagulation time, or clinical chemistry parameters

IV and IM vaccine administrations were well tolerated by all rabbits across groups. There were no unscheduled mortalities and no vaccine-related systemic clinical signs or effects on body temperature. Minimal to slight erythema at the administration site was noted in the groups (including vehicle groups) receiving an IM injection and was considered to represent the normal, expected reaction related to the IM injection procedure [27,28].

No clear vaccine-related changes in platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT; **Figures 1A-1C**), or any other clinical pathology parameters (except for fibrinogen and CRP, discussed in the paragraph below) were observed compared with the vehicle groups (**Table A1**). In addition, no major differences in these clotting parameters (platelet count, PT, and APTT) or any other

clinical pathology parameters were observed between the IV and IM routes for any of the vaccines.

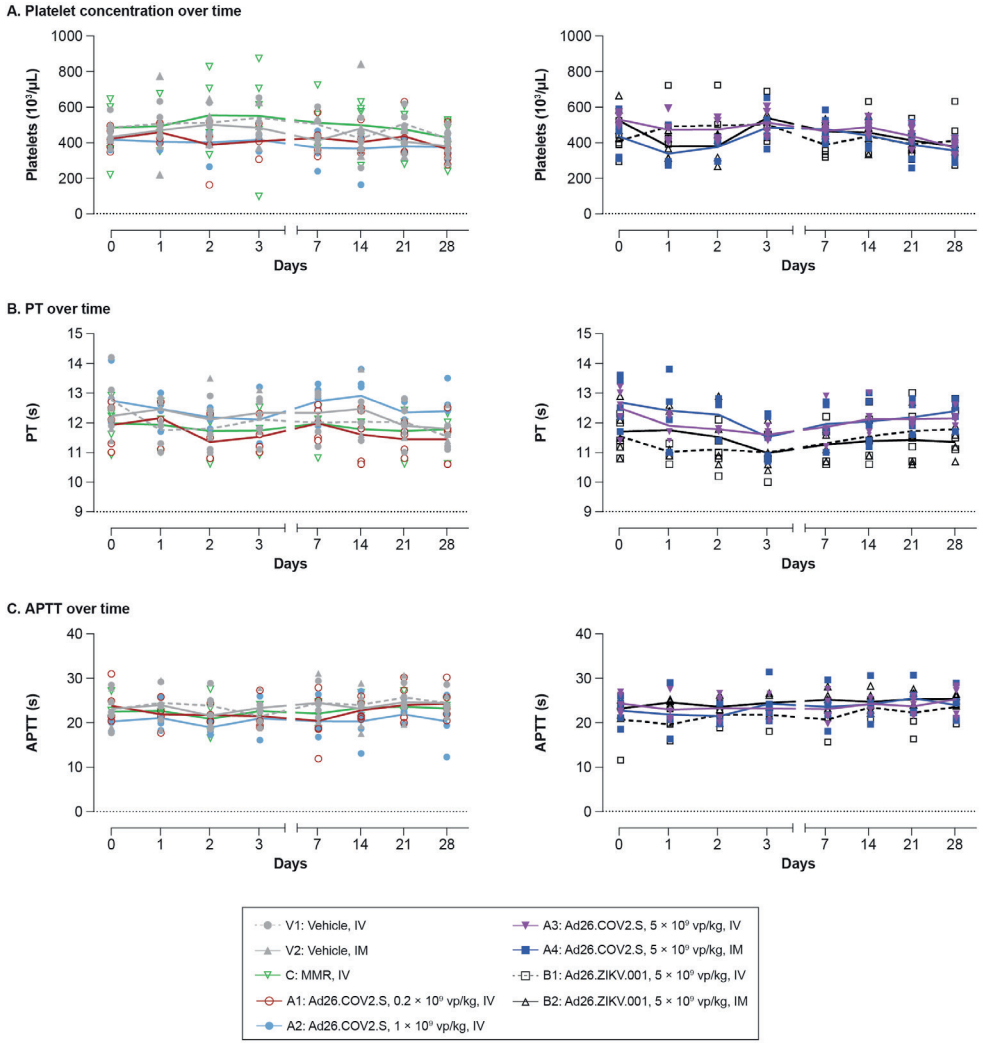
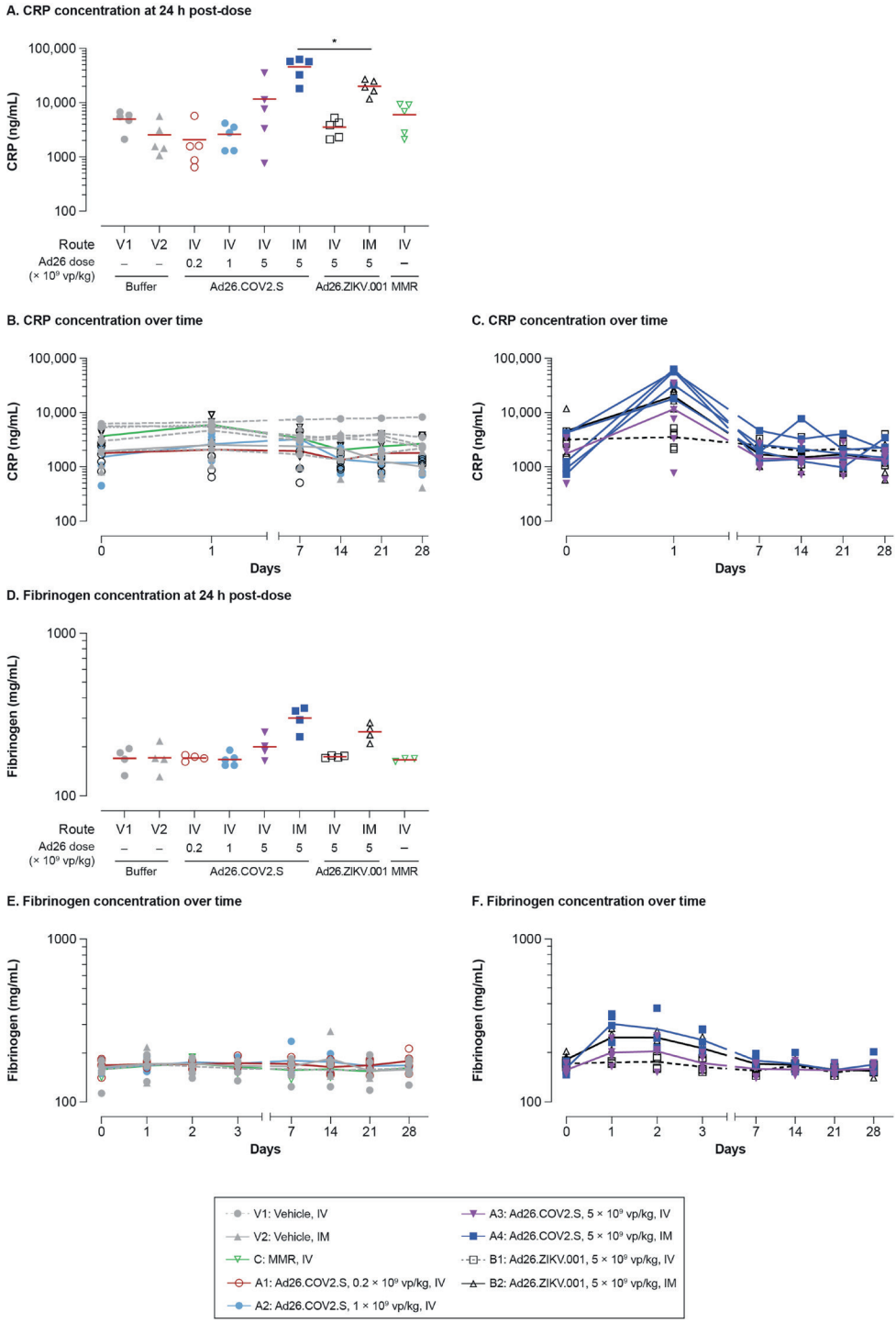



**Figure 1. Time course of platelets, PT, and APTT levels after single IV or IM dosing with Ad26-based vaccines in rabbits.** Levels of **(A)** platelet counts, **(B)** PT, and **(C)** APTT were measured in plasma taken at the indicated time points pre and post dosing with vehicle IV (V1); vehicle IM (V2); Ad26.COV2.S IV at $0.2\times10^9$ vp/kg (A1), $1\times10^9$ vp/kg (A2), or $5\times10^9$ vp/kg (A3); Ad26.COV2.S IM at $5\times10^9$ vp/kg (A4); Ad26.ZIKV.001 IV at $5\times10^9$ vp/kg (B1); Ad26.ZIKV.001 IM at $5\times10^9$ vp/kg (B2); or MMR IV (C). The time course of each parameter is shown, with lines representing the group mean and symbols corresponding to individual animals (n=5/group) for each time point evaluated. Left graphs in each panel show the data from groups V1, V2, C, A1, and A2. Right graphs in each panel show the data from groups A3, A4, B1, and B2. APTT, activated partial thromboplastin time; IM, intramuscular; IV, intravenous; MMR, measles-mumps-rubella; PT, prothrombin time; vp, viral particles.

### 3.3 Administration of Ad26.COV2.S led to a transient increase in acute phase proteins

Transient CRP increases (4.4- to 20.3-fold) were observed in IV and IM dosed rabbits 24 hours post dosing with $5\times10^9$ vp/kg Ad26.COV2.S (**Figures 2A-2C**). The CRP response was more pronounced after IM dosing, but the difference between routes was not statistically significant. There was no statistically significant difference in the CRP levels induced by IV Ad26.COV2.S ($5\times10^9$ vp/kg) compared with the IV reference vaccine MMR. One-week post dosing, CRP levels in all groups had returned to baseline. Overall, the CRP increases by Ad26 vaccination were mirrored by transient increases in fibrinogen (≤1.9-fold) observed 24 to 72 hours post dosing (**Figures 2D-2E**). Interestingly, IM administration of Ad26.COV2.S induced significantly higher CRP levels compared with IM administration of Ad26.ZIKV.001 ($P<0.03$, ANOVA), suggesting that the vaccine transgene influences this parameter. This observation was confirmed in a separate mouse study (the study design is shown in **Figure A1**(B)), where serum amyloid A protein levels (a mouse major acute phase protein) were significantly higher 24 hours post dosing with $1\times10^{10}$ vp/mouse Ad26.COV2.S compared with $1\times10^{10}$ vp/mouse Ad26.ZIKV.001 ($P<0.0035$, Tobit model; **Figure A2**).

**Figure 2. (opposite) CRP and fibrinogen levels after IV and IM administration with Ad26.COV2.S and other vaccines in rabbits.** Individual animal CRP **(A-C)** and fibrinogen **(D-F)** levels were measured in serum taken at the indicated time points pre and post dosing with the same vaccines as indicated in the Figure 1 legend. **A** and **D** show CRP and fibrinogen levels at 24 hours; horizontal lines represent respective group means. ANOVA testing was done by comparing IM Ad26.COV2.S (A4) with IM Ad26.ZIKV.001 (B2). **B** and **E** show the time course data from groups V1, V2, C, A1, and A2, with lines representing the group mean and symbols corresponding to individual animals (n=5/group) for each time point evaluated. **C** and **F** show the time course data from groups A3, A4, B1, and B2. *$P<0.05$. ANOVA, analysis of variance; CRP, C-reactive protein; IM, intramuscular; IV, intravenous; MMR, measles-mumps-rubella; vp, viral particles.


A. CRP concentration at 24 h post-dose
CRP (ng/mL)
100,000
10,000
1000
100
*
Route V1 V2 IV IV IV IM IV IM IV
Ad26 dose – – 0.2 1 5 5 5 5 –
(× 10⁹ vp/kg)
Buffer
Ad26.COV2.S
Ad26.ZIKV.001
MMR
B. CRP concentration over time
C. CRP concentration over time
Days
0 1 7 14 21 28
D. Fibrinogen concentration at 24 h post-dose
Fibrinogen (mg/mL)
1000
100
E. Fibrinogen concentration over time
F. Fibrinogen concentration over time
0 1 2 3 7 14 21 28
V1: Vehicle, IV
V2: Vehicle, IM
C: MMR, IV
A1: Ad26.COV2.S, 0.2 × 10⁹ vp/kg, IV
A2: Ad26.COV2.S, 1 × 10⁹ vp/kg, IV
A3: Ad26.COV2.S, 5 × 10⁹ vp/kg, IV
A4: Ad26.COV2.S, 5 × 10⁹ vp/kg, IM
B1: Ad26.ZIKV.001, 5 × 10⁹ vp/kg, IV
B2: Ad26.ZIKV.001, 5 × 10⁹ vp/kg, IM

### 3.4 IV Administration of Ad26.COV2.S and Vehicle Control Induced Comparable Histopathology Findings in Rabbits

To study potential pathologic effects associated with IV dosing of Ad26.COV2.S, necropsy and gross/microscopic examinations were conducted on all IV dosed rabbits.

IV dosing with Ad26.COV2.S, Ad26.ZIKV.001, or the MMR vaccine was not associated with any gross or histopathologic evidence of thrombosis, thromboembolic disease, or their sequelae, as assessed following necropsy on day 28 post immunization in comparison with vehicle controls. A comparable minimally or mildly increased cellularity of germinal centers in the spleen was observed in animals dosed IV with Ad26.COV2.S and Ad26.ZIKV.001, which is part of the normal immune response to vaccine administration (**Figure A3**) [28,29]. There were no systemic pathologic findings associated with IV Ad26.COV2.S or Ad26.ZIKV.001 administration. Locally, at the IV administration site (ear vein), minimal or mild, procedure-related perivenous hemorrhage, inflammatory infiltrates/inflammation, or fibrosis were observed for all IV dosed vaccines, at comparable incidences to the vehicle controls.

### 3.5 Ad26 DNA copies detected in the blood 30 minutes after IV and IM administration of Ad26.COV2.S or Ad26.ZIKV.001 in rabbits

To confirm IV dosing and to investigate if IM dosing resulted in distribution of vaccine components into the circulation, we quantified Ad26-derived DNA in whole blood early after administration. Ad26 vector DNA copies in blood drawn 30 minutes post IV Ad26.COV2.S administration were detected at higher levels with increasing doses ($0.2\times10^9$, $1\times10^9$, and $5\times10^9$ vp/kg for groups A1, A2, and A3, respectively). Moreover, a similar number of Ad26 vector DNA copies was detected in the blood after administration with Ad26.COV2.S (IM) or Ad26.ZIKV.001 (IM and IV) at a dose of $5\times10^9$ vp/kg (**Figure A4**).

### 3.6 IV and IM Administration of Ad26.COV2.S Vector Induced Detectable Levels of S Protein in Rabbit Serum

Considering the hypothesis for a potential role of the SARS-CoV-2 S protein (fragments) in VITT [20,21], we assessed the level of S protein in the serum of rabbits immunized with Ad26.COV2.S using a commercial S-PLEX SARS-CoV-2 S protein detection assay, based on an electrochemiluminescence readout that detects the presence of the S protein RBD.

Sera from rabbits dosed with $5\times10^9$ vp/kg of Ad26.COV2.S showed a significantly increased S protein concentration at 24 and 48 hours post IV dosing and IM injection compared with baseline. Post IV dosing, the geometric mean was 18.4 ± 1.53 pg/mL at 24 hours and 31.1 ± 1.82 pg/mL at 48 hours (both $P<0.05$; ANOVA). Post IM injection, the

geometric mean was 18.6 ± 1.32 pg/mL at 24 hours ($P$<0.004; ANOVA) and 25 ± 1.12 pg/mL at 48 hours ($P$<0.0001; ANOVA). No statistically significant difference was observed between the IV and IM routes of vaccine administration (ANOVA; **Figure 3**).

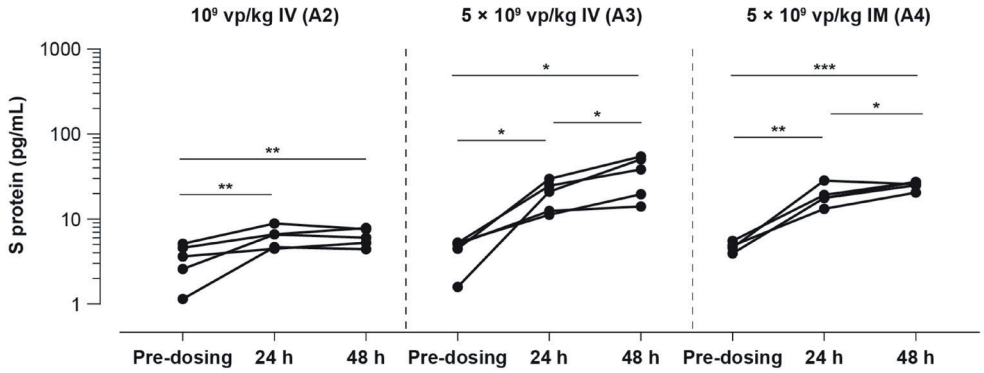



**Figure 3. S protein concentration in serum post dosing with Ad26.COV2.S vector encoding S protein.** Serum from New Zealand white rabbits rabbits (n=5/group) dosed with Ad26.COV2.S $1\times10^9$ vp/kg IV (**A2**), $5\times10^9$ vp/kg IV (**A3**), or $5\times10^9$ vp/kg IM (**A4**) were analyzed using a commercial S-PLEX SARS-CoV-2 S detection assay. Serum was sampled pre-dosing and Day 1 (24 h) and Day 2 (48 h) post dosing. Comparison of the S protein concentration measured at Day 1 and Day 2 was performed using an ANOVA model, with a significance level of 0.05. Since the SARS-CoV-2 S protein detection assay is developed, but not qualified to test rabbit serum samples, no lower limit of detection is available for this assay. ANOVA, analysis of variance; IM, intramuscular; IV, intravenous; S, spike; SARS-CoV-2, severe acute syndrome coronavirus-2; vp, viral particles.

## 4. Discussion

One hypothesis that was put forward to explain VITT observed with COVID-19 vaccines is unintended systemic exposure to vaccine particles resulting subsequently in their interaction with platelets, thereby inducing thromboembolic events [3,20-22].



Our data demonstrate that systemic exposure following IV administration of Ad26.COV2.S had no relevant impact on hematologic and coagulation parameters, including platelet count, PT, or APTT clotting times compared to vehicle control in rabbits. IV dosing of Ad26.COV2.S and Ad26.ZIKV.001 resulted in an increased cellularity of germinal centers in the spleen, which is however part of a normal immune response to the injection of a vaccine [29-31]. Our data contrast with those published by Nicolai and colleagues [20] showing that IV injection with ChAdOx1 nCoV-19 led to a decrease in platelet count when compared with IM dosing in mice. This effect was most pronounced at dose levels above $2.5\times10^8$ vp/mouse [20,32]. Similarly, previous studies in rabbits [33] and non-human primates [34] with high doses of Ad5 vectors encoding LacZ β-galactosidase showed a decrease in platelet count upon IV dosing with Ad5. A

possible explanation for the difference between these studies and our findings is the systemic exposure dose, which requires adequate scaling between test species for the IV dosing route. In the above studies, systemic dose levels of ≥$1.25\times10^{10}$ vp/kg (mice, 20 g), $5\times10^{11}$ vp/kg (rabbits, 2-3 kg), or $1\times10^{12}$ vp/kg (non-human primates, 2.6-3.5 kg) were used when scaled to BW. In contrast, we tested a dose level range of $0.2\times10^{9}$ vp/kg to $5\times10^{9}$ vp/kg, which was selected based on the assumption that the FHD of $5\times10^{10}$ vp Ad26.COV2.S is given to a 50 kg adult (corresponding to $1\times10^{9}$ vp/kg) and a dose of $2.5\times10^{10}$ vp for a 5 kg child (corresponding to $5\times10^{9}$ vp/kg). We consider doses scaled to BW as more relevant for assessing IV toxicity in rabbits given the systemic exposure associated with this route compared with the local exposure associated with the IM route. Scaling to BW corresponds to a worst-case scenario where a FHD of Ad26.COV2.S would accidentally be dosed IV. Of note, no indication for (pro)thrombotic events was observed in regulatory toxicology studies with Ad26.COV2.S, in which IM administration of a FHD ($5\times10^{10}$ vp) was assessed (data not shown).

A second explanation for the discrepancy between our findings and those published with other adenovirus vectors might be related to the highly disparate biologic mechanisms of cell entry, receptor binding, and cell or receptor tropism used by the different vectors, as well as differences in the electronegative surface charge and vector-backbone characteristics [22,35]. These differences could potentially influence the interaction of different adenovirus-based vectors with platelets *in vivo*. Interestingly, a recent study showed that IV bolus injection with $10\times10^{11}$ vp (~$5\times10^{13}$ vp/kg) of replication-competent Ad26 vectors encoding a fusion protein of green fluorescent protein and luciferase protein in human-CD46 transgenic mice failed to provoke notable changes in platelets when compared with controls [36], suggesting that even high-dose systemic exposure to Ad26-based vectors relative to BW may be tolerable, which is in line with the results of our studies.

A third explanation for the discrepancy between our findings and those published with other adenovirus vectors in the context of thrombocytopenia/VITT could be related to the levels of impurities in the vaccines. Michalik and colleagues recently showed that the Ad26.COV2.S vaccine contains much lower amounts of impurities, e.g. host cell protein, compared with ChAdOx1 nCoV-19 [37]. Additionally, no EDTA is present in the Ad26.COV2.S vaccine preparation [37], while ChAdOx1-S contains EDTA [38], which is known to activate platelets and may lead to PF4 release [39].

In our rabbit study, we observed a transient increase in acute phase proteins (CRP and fibrinogen) 24 hours post dosing, which is considered a normal response after vaccine administration, and this was more pronounced after IM immunization of Ad26.COV2.S compared with IV administration for both Ad26.COV2.S and Ad26.ZIKV.001 at a dose

level of $5\times10^9$ vp/kg. A potential explanation for this observation is that the IV route leads to a faster dilution of the vaccine formulation in blood compared with local injection into muscle tissue, leading to exposure of target cells to higher concentrations of the vaccine after IM administration. In addition, the procedure of IM administration causes a local inflammatory reaction at the injection site, which contributes to the change in acute phase proteins. These data are not suggestive for the use of the IV route for Ad26-based vaccines, as that would require a thorough clinical safety, immunogenicity and efficacy assessment, which has not been conducted for IV administration of Ad26.

IM dosing of Ad26.COV2.S induced significantly higher levels of CRP (rabbits) and of serum amyloid A (mice) compared with IM dosing of Ad26.ZIKV.001, despite the fact that the adenovirus particle structure and composition is similar for both vaccines. The only difference is the genetically encoded vaccine transgene, suggesting a more inflammatory effect of the S protein encoded by Ad26.COV2.S compared with the Zika Env protein encoded by Ad26.ZIKV.001. This is supported by recent publications showing that S protein can initiate an inflammatory phenotype in endothelial cells, induce leukocyte adhesion, and promote proinflammatory cytokine secretion after IV S protein administration in mice [30,40]. Furthermore, as reviewed by Trougakos and colleagues [31,41], S protein may influence prothrombotic and inflammation-related signaling and is thus hypothesized to contribute to many adverse effects of COVID vaccination.

The development of VITT-like antibodies and adenovirus-associated thrombocytopenia and thrombosis has also been reported in 2 individuals experiencing natural adenovirus infection who were not previously vaccinated with adenovector-based vaccine, but who either had a prior SARS-COV2 infection or had received 2 doses of Spikvax (mRNA-based COVID-19 vaccine) [42]. The high prevalence of adenoviral infections, particularly in the developing world [43], and the limited incidence of only 2 VITT-like cases reported in the context of natural adenovirus infection underscores the possibility that multifactorial determinants such as individual genetics or prior health status may play a role in the development of VITT.



The presence of soluble S protein after vaccination with the BNT162b2 (BioNTech) vaccine was recently shown in plasma in humans [40,44] and in mice [41,45] and has been associated with the occurrence of myocarditis in young male patients dosed with the mRNA-based COVID-19 vaccine [44,46]. Notably, prolonged detection of S protein was seen by immunohistochemistry in humans up to day 60 post second dosing with BNT162b2 in the lymph nodes [40,44]. Moreover, after BNT162b2 dosing of mice, nanogram ranges (~100-400 ng/mL) of S protein were detected in the serum within 1 day after immunization and returned to background level by day 7 [41,45].

In the present study, S protein was detected in blood on day 1 and day 2 post IV and IM dosing with Ad26.COV2.S at approximately the same level for both routes, and in the same range as seen for mBNT162b2, a mRNA COVID-19 vaccines in another animal model [45]. It remains to be determined whether a similar level of S protein expression is seen following Ad26.COV2.S vaccination in humans. Our data suggest that soluble S protein generated in the context of adenovectors is likely not sufficient on its own for the induction of VITT, since no adverse effect was observed in our study and since mRNA vaccines also induce detectable soluble S protein in the circulation without causing a similar frequency of VITT in human vaccinees [7,44,47]. Nevertheless, the exact location of S protein expression; the duration of expression; and the conformation, membrane presentation, or glycosylation of the S protein could be different between the vaccine platforms [46,48]. Therefore, S protein may not be ruled out as a potential contributing factor in a multifactorial scenario of VITT pathogenesis that may also include other risk factors, such as previous infections, genetic predispositions, or preexisting health conditions. Future studies need to characterize the S protein detected in the circulation in greater depth, including clarification of the S protein biodistribution in tissues other than blood and its potential influence on inflammatory processes, and comparison between different COVID-19 vaccines to assess a potential role in VITT.

## 5. Conclusions

In conclusion, we have shown that Ad26.COV2.S, independently of the administration route, did not have a relevant impact on platelet counts and other blood parameters, such as coagulation times and clinical chemistry parameters, in rabbits. Moreover, IV and IM dosing did not induce any major changes in safety parameters compared with vehicle controls or the childhood MMR vaccine when administered IV. Although based on a limited number of animals, these data indicate that an accidental IV injection of Ad26.COV2.S by itself is unlikely to represent a direct risk that could be associated with VITT pathogenesis. The very low incidence of VITT in humans suggests that this clinical outcome may be associated with Ad26 vector-related factors in combination with other factors, potentially including inflammatory activity of the S transgene and a predisposition of the host.

***Author Contributions***

Conceptualization, Selina Khan, Roland C Zahn and Frank Wegmann; Validation, Selina Khan, Sonia Márquez Martínez, Tim Erkens, Adriaan De Wilde, Lea MM Costes and Ronnie Chamanza; Formal analysis, Selina Khan, Sonia Márquez Martínez, Tim Erkens, Adriaan De Wilde, Lea MM Costes, Petra Vinken, Sandra De Jonghe, Wendy Roosen, Chiara Talia, Ronnie Chamanza, Jan Serroyen, Jeroen Tolboom and Frank Wegmann; Investigation,

Sonia Márquez Martínez, Adriaan De Wilde and Lea MM Costes; Data curation, Selina Khan, Sonia Márquez Martínez, Tim Erkens, Adriaan De Wilde, Lea MM Costes and Ronnie Chamanza; Writing – original draft, Selina Khan; Writing – review & editing, Selina Khan, Tim Erkens, Jan Serroyen, Roland C Zahn and Frank Wegmann; Visualization, Selina Khan, Sonia Márquez Martínez, Tim Erkens, Adriaan De Wilde, Lea MM Costes and Ronnie Chamanza; Supervision, Selina Khan, Petra Vinken, Sandra De Jonghe, Roland C Zahn and Frank Wegmann; Project administration, Selina Khan, Wendy Roosen, Chiara Talia and Frank Wegmann.

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***Institutional Review Board Statement***
The rabbit study was conducted at Janssen Research & Development Belgium, in facilities approved by the Institute of Health Office of Animal Welfare and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Animal research protocols were approved by the Institutional Ethical Committee, and the studies were conducted in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and Belgian guidelines, and with the principles of euthanasia as stated in the guidelines from the American Veterinary Medical Association Panel protocol number 736, approval date 22 June 2021 [24]. Import and export permits for vectors and rabbit biospecimens were obtained in compliance with European Union federal regulations.



The mouse study was performed at Janssen Vaccines & Prevention, The Netherlands, and was conducted according to the Dutch Animal Experimentation Act and the Guidelines on the Protection of Animals for Scientific Purposes by the Council of the European Committee after approval by the Centrale Commissie Dierproeven and the Dier Experimenten Commissie (protocol number AVD 21300202115215-22004 approval date.17 May 2022)

***Data Availability Statement***
The data presented in this study are available on request from the corresponding author.

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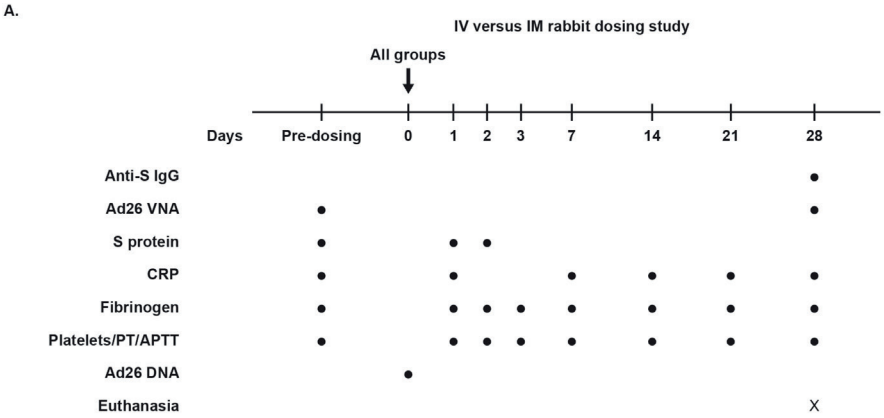
***Conflicts of Interest***

The authors declare the following competing interests: all authors are or were employees of Janssen Vaccines & Prevention B.V or Janssen Research & Development, a division of Janssen Pharmaceutica NV, while engaged in the research project. S.K., T.E., A.d.W., P.V., J.S., J.T., R.C.Z., and F.W. held or still hold stock in Johnson & Johnson. The funders of Johnson and Johnson had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

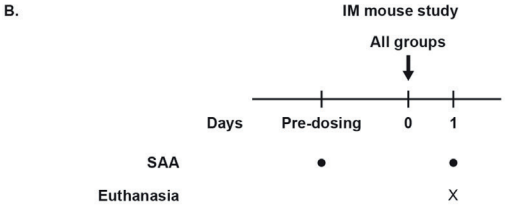
# Appendix A

A.




| Group | Dosage group | Dose (vp/kg) | Route | S protein | CRP/fibrinogen/ platelets/PT/ APTT | Ad26 DNA |
|---|---|---|---|---|---|---|
| V1 | Vehicle | 0 | IV | ● | ● | ● |
| V2 | Vehicle | 0 | IM | ● | ● | ● |
| A1 | Ad26.COV2.S | $2 \times 10^8$ | IV | | ● | ● |
| A2 | Ad26.COV2.S | $1 \times 10^9$ | IV | ● | ● | ● |
| A3 | Ad26.COV2.S | $5 \times 10^9$ | IV | ● | ● | ● |
| A4 | Ad26.COV2.S | $5 \times 10^9$ | IM | ● | ● | ● |
| B1 | Ad26.ZIKV.001 | $5 \times 10^9$ | IV | | ● | ● |
| B2 | Ad26.ZIKV.001 | $5 \times 10^9$ | IM | | ● | ● |
| **Group** | **Dosage group** | **Dose ($TCID_{50}$/kg)** | **Route** | **S protein** | **CRP** | **Ad26 DNA** |
| C | MMR | $1 \times 10^2$ measles<br>$12.5 \times 10^2$ mumps<br>$1 \times 10^2$ rubella | IV | | ● | |

B.




| Group | Dosage group | Dose (vp) | Dose (vp/kg) | Route | SAA |
|---|---|---|---|---|---|
| 1 | Ad26.COV2.S | $1 \times 10^{10}$ | $5 \times 10^{11}$ | IM | ● |
| 2 | Ad26.ZIKV.001 | $1 \times 10^{10}$ | $5 \times 10^{11}$ | IM | ● |

**Figure A1. Study design. (A)** Rabbits received Ad26.COV2.S, Ad26.ZIKV.001, or MMR vaccines, or vehicle at the indicated dose levels either IV or IM. N=8 per group. Serum and plasma were collected at the indicated time points and processed for the assays listed. Rabbits were euthanized on Day 28 post dosing. **(B)** Mice were IM dosed with $10^{10}$ vp of either Ad26.COV2.S or Ad26.ZIKV.001, and serum were collected 24 hours later, and mice were then euthanized. APTT, activated partial thromboplastin time; CRP, C-reactive protein; IM, intramuscular; IV, intravenous; MMR, measles-mumps-rubella; PT, prothrombin time; S, spike; SAA, serum amyloid A; $TCID_{50}$, 50% tissue culture infectious dose; VNA, virus neutralization assay; vp, viral particles.

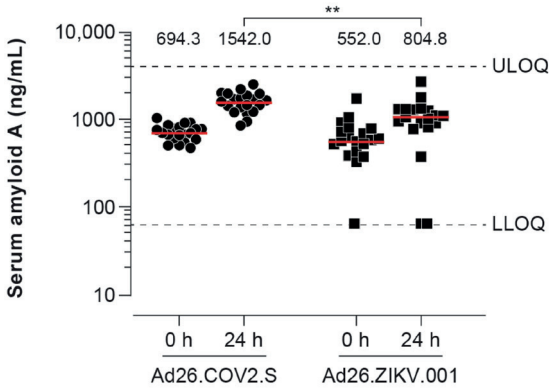



**Figure A2. Serum amyloid A levels in mice after IM dosing with Ad26.COV2.S or Ad26.ZIKV.001.** Serum amyloid A was measured in serum of mice pre-dosing and 24 hours post dosing with Ad26.COV2.S (N=20) or Ad26.ZIKV.001 (N=19). Each dot corresponds to an individual mouse. The numbers above refer to the geometric mean values per group. The difference at 24 hours post dosing between Ad26.COV2.S and Ad26.ZIKV.001 was assessed (Tobit model on $\log_{10}$ transformed response). $^{**}P<0.01$. LLOQ, lower limit of quantification; IM, intramuscular; ULOQ, upper limit of quantification.

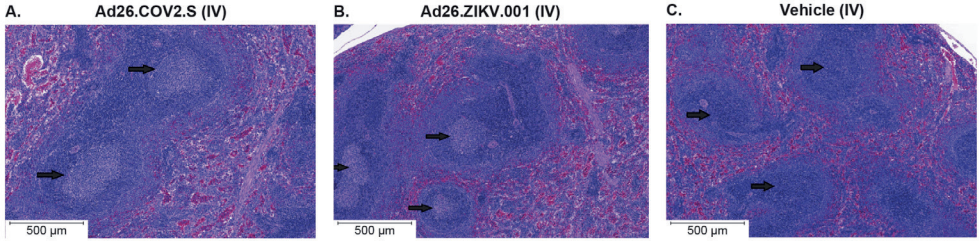



**Figure A3. Increased cellularity and size of germinal center cellularity in spleen upon IV dosing with Ad26.COV2.S and Ad26.ZIKV.001, but not with vehicle.** Representative H&E staining of spleens from New Zealand white rabbits (N=5/group) dosed IV with **(A)** Ad26.COV2.S $5\times10^9$ vp/kg, **(B)** Ad26.ZIKV.001 $5\times10^9$ vp/kg, or **(C)** vehicle. Arrows indicate the germinal centers. H&E, hematoxylin and eosin; IV, intravenous; vp, viral particles.

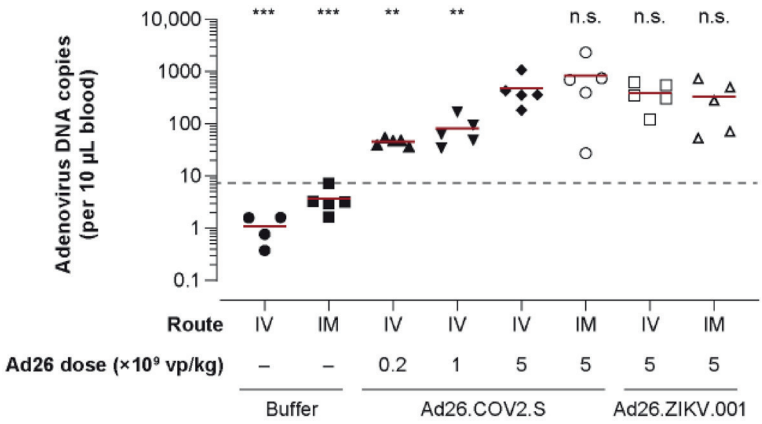



**Figure A4. Adenovirus DNA copies in rabbit blood upon immunization.** Each dot represents DNA copies/10 µL blood from an individual rabbit, of which the presence of vector DNA is measured in 3 technical replicates (n=3 per rabbit). Both adenovirus vectors used in the study (Ad26.COV2.S and Ad26.ZIKV.001) contained a transgene cassette with a CMV promoter. The primer-probe set used in the ddPCR assay was designed to amplify a sequence from this CMV promoter. For each group (n=5 rabbits per group, except for the IV buffer control group, where 4 samples were available), the difference with the group dosed IV with $5\times10^9$ vp/kg of Ad26.COV2.S has been assessed (ANOVA on $\log_{10}$ [average per rabbit] with separate variance per group). Each line represents the arithmetic mean of the DNA copies per rabbit in each group. Threshold (dotted line) is defined as the highest value of the negative control. $^{**}P<0.01$. $^{***}P<0.001$. Ad26, adenovirus type 26; ANOVA, analysis of variance; CMV, cytomegalovirus; ddPCR, droplet digital polymerase chain reaction; n.s., not significant; IM, intramuscular; IV, intravenous; vp, viral particles.

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CHAPTER 6

# Discussion

Sonia Márquez Martínez

# 1. Summary

Replication-incompetent adenoviral vectors present properties that make them a good platform for vaccines, such as a large packaging capacity and broad tropism, and can induce potent and durable immune responses against the transgene of interest. Although generally well tolerated, there are recent safety concerns regarding vaccine-induced thrombotic thrombocytopenia (VITT), a very rare but serious condition that occurs after adenovirus-based COVID-19 vaccination. Moreover, there is a gap in knowledge regarding early events after adenovirus-based vaccination, such as transgene expression or innate immune responses, which likely play an important role in shaping adaptive immune responses and in the development VITT.

In this thesis, the early events after adenovirus-based vaccination were investigated (cellular entry, transgene expression, and innate immune responses), with a focus on adenovirus-type differences, and their role in the development of transgene-specific adaptive immune responses was summarized (**Chapter 2**). This project provided insights into the specific effect of transgene expression on adaptive immune responses after intramuscular (IM) vaccination with an adenovirus 26-based vector (Ad26) in mice (**Chapter 3**). Additionally, the contribution of the transgene (spike protein) (**Chapter 4**) and accidental systemic exposure of the adenoviral vector (**Chapter 5**) in the development of VITT after Ad26.COV2.S dosing was evaluated.

## 1.1. Cell entry and innate sensing shape adaptive immune responses to adenovirus-based vaccines

Adenovirus-based vaccines are engineered by modifying the adenoviral genome to produce replication-incompetent virial particles capable of carrying a transgene of interest, with the aim of eliciting immune responses against the transgene product. Adenoviral vectors retain some characteristics of the adenovirus in terms of cellular entry, intracellular trafficking, and triggering of antiviral pathways. Key differences have been identified between adenovirus types, including primary cellular receptors, internalization process, and endosomal escape [1]. The interaction of proteins on the virion surface with cellular receptors (see **Chapter 2, Table 1**) is dependent on the adenovirus species, and these interactions shape their cellular tropism, distribution, and innate immune recognition, which are key in the development of transgene-specific adaptive immune responses [2,3]. Upon receptor interaction, adenoviruses are captured into endosomes, after which some adenovirus types (Ad5) escape the endosome through structural changes caused by receptor-virion interactions [3-7] and other adenovirus types (Ad26 and Ad35) accumulate in late endosomes and trigger innate immune sensors, such as toll-like receptors (TLRs) [8]. The innate response triggered by adenovirus vectors needs to be tightly balanced in preclinical models,

with stronger type I IFN and NF–kB responses having a detrimental effect on transgene expression and the development of adaptive immune responses against the transgene [9-12], whereas other responses (such as activation of MyD88) are required to induce an adaptive immune response [13].

## 1.2. Peak transgene expression after intramuscular immunization of mice with adenovirus 26-based vaccines correlates with transgene-specific adaptive immune responses

Adenovirus-based vaccines encode one or more disease-specific transgenes to induce protective immunity against the target disease. The development of transgene-specific adaptive immune responses depends on early events after adenovirus-based vaccination, such as transgene expression [10,11,14,15]. However, few studies have addressed this question for serotypes other than Ad5 (human type C) [1]. The magnitude and duration of transgene expression in the host after Ad5 vaccination are critical factors influencing the development of adaptive immune responses. To address whether key serotype differences between Ad5 and Ad26 lead to different magnitude, duration, and/or organ biodistribution of transgene expression, these signatures were evaluated through *in vivo* imaging after a single IM administration in mice (**Chapter 3**).

The magnitude of transgene expression was higher in Ad5 immunized mice and essential differences were observed in the transgene biodistribution, with only Ad5 inducing strong transgene expression in the draining lymph nodes and liver. However, Ad26 and Ad5 induced a similar magnitude of cellular immune responses 1 year after dosing. This finding indicates that adenovirus serotype-specific factors other than transgene expression, such as innate immune responses, may play a role in the magnitude of memory immune responses. Although phenotypic characterization of the T-cell responses was not performed in our studies, it has been described that Ad26 induces more polyfunctional transgene-specific T-cell responses and enhanced memory T-cell differentiation than Ad5 in mice [16]. The differences in the magnitude and phenotype of cellular immune responses after adenovirus-based vaccination may rely on the serotype-specific mechanisms of cellular transduction and/or the interplay between innate immune responses and transgene expression, along with the magnitude of transgene expression. Further investigation is required to evaluate the impact of serotype-specific differences.

A correlation between peak transgene expression early after Ad26 vaccination and transgene-specific cellular and humoral immune responses was observed for a model antigen and SARS-CoV-2 spike protein, independent of innate immune activation (**Chapter 3**) [1]. We showed that the potency of transgene-specific T-cell responses reaches a plateau at high doses of transgene-encoding adenoviral particles, suggesting

that there is a threshold in antigen expression after which cellular responses cannot be further enhanced, likely due to the saturation of antigen-loaded major histocompatibility complex class I (MHC-I). However, we did not assess the durability of T-cell responses in this study, which may be influenced by transgene expression at high doses of transgene-encoding adenoviral particles. A plateau in early transgene-specific T-cell responses has been previously shown after spike-expressing Ad26 vaccination in mice [17] and spike-expressing mRNA vaccination in humans [18], indicating that this may be the case for different platforms across species. B-cell activation and antibody secretion are independent of antigen-loaded MHC-I molecules [19], and no plateau was observed in transgene-specific humoral responses in our studies or in previous reports after Ad26 or mRNA vaccination [17,18]. Our results suggest that the potency of humoral responses can be further enhanced by increasing peak transgene expression. The magnitude of humoral responses has been shown to correlate with protection against Ebola virus disease in NHPs [20] and COVID-19 in human vaccinees [21,22] after administration of Ad26 or other vaccine platforms, including mRNA, protein-based, and inactivated viral vaccines. Thus, our findings can be used to further improve current vaccine platforms to generate novel protective vaccines.

## 1.3. The mechanism of induction of vaccine-induced immune thrombotic thrombocytopenia

The global COVID-19 pandemic and the high morbidity and mortality associated with these infections led to the rapid development and approval of multiple vaccines [23]. Post-marketing safety surveillance in large populations led to the investigation of several safety signals that had not been detected in clinical trials due to their low incidence. Rare adverse effects were identified across vaccine platforms, including VITT after adenovirus-based vaccination (Ad26.COV2.S – Jcovden, and ChAdOx1 – Vaxzevria) [24].

Ad26.COV2.S, a recombinant and replication-deficient human Ad26 vector encoding the full-length spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been broadly used for the prevention of COVID-19. VITT has been reported in approximately 2.3 to 5.5 cases per 1 million vaccinees in the period of 5 to 43 days after the first dose of ChAdOx1 nCoV-19 [25] or Ad26.COV2.S [26]. VITT is characterized by thrombocytopenia and thrombosis, often in atypical anatomical locations, and the presence of antibodies against platelet factor 4 (PF4) [27]. The underlying mechanism of VITT has not yet been elucidated, but the early onset of VITT suggests that its development relies on early events after vaccination. Due to the safety implications of this rare but severe adverse event and the potential involvement of early events after vaccination in its development, some possible factors involved in the development of VITT were investigated in this thesis (**Chapters 4** and **5**).

Some potential factors influencing the development of VITT include interactions between the vaccine-expressed spike protein and platelets/endothelial cells, which might lead to the activation of coagulation pathways [28,29]. The SARS-CoV-2 spike protein has been shown to cause vascular damage in hamsters [30] and has been detected within the thrombus and in the adjacent vessel wall in patients with VITT-induced cerebral venous thrombosis [31]. Moreover, the spike protein may activate coagulation pathways by binding to the angiotensin-converting enzyme 2 (ACE2) receptor on platelets and/or endothelial cells, which could trigger thrombus formation [32]. In **Chapter 4**, we investigated the biodistribution of the spike protein and characterized the circulating spike protein after IM dosing with Ad26.COV2.S in preclinical models and clinical samples. Spike protein was not detected in the endothelium or bound to platelets after Ad26.COV2.S vaccination in preclinical models. We demonstrated similar kinetics of transient spike protein expression after Ad26.COV2.S vaccination in preclinical models and humans, with a comparable kinetic, but lower magnitude, as observed after mRNA COVID-19 vaccination. Spike subunit composition was different in serum after Ad26.COV2.S vaccination compared with mRNA BNT162b2 dosing in preclinical models. Lower levels of S1 shedding after Ad26.COV2.S vaccination are likely linked to the mutation of the furin cleavage site of the S1 and S2 spike subunit, which is, however, not mutated in the the ChAdOx1 vector, that is also associated with VITT. Based on the biodistribution, kinetic, and serum composition of spike after Ad26.COV2.S vaccination, a direct association of spike with the development of VITT was not identified. However, it cannot be excluded that spike contributes to the pathogenesis of VITT in the context of Ad26 vaccination and in combination with other influencing factors, such as previous infections, genetic predisposition, and preexisting health conditions.

Other hypotheses behind the mechanism of development of VITT focus on the role of the adenoviral particle. It was proposed that accidental intravenous (IV) administration of Ad26.COV2.S or the drainage of the vaccine from the muscle into the circulatory system may result in the interaction of the vaccine with blood factors associated with platelet activation. In **Chapter 5**, we demonstrated that, similar to IM administration of Ad26.COV2.S in rabbits, IV dosing was well tolerated, with no significant differences between dosing routes for the assessed hematologic, coagulation time, innate immune, or clinical chemistry parameters, and no histopathological indication of thrombotic events. For both routes, although based on a low number of animals, all other non-adverse findings observed were consistent with a normal vaccine response and comparable to those observed for unrelated or other Ad26-based control vaccines. However, Ad26.COV2.S induced significantly higher levels of C-Reactive protein on day 1 after IM vaccination compared with an Ad26-based control vaccine encoding a different transgene. This suggests an inflammatory effect of the vaccine-encoded spike protein,

which should be further studied in the context of VITT as a potential contributing factor in a multifactorial scenario.

# 2. Discussion and future perspectives

## 2.1. The mechanism of action of adenovirus-based vaccines

Adenovirus-based vaccines elicit strong immune responses against the transgene that they carry. However, the mechanism behind this induction is not well understood and depends heavily on early events following vaccination. Understanding the mechanism of action could lead to the development of new modified adenoviral vectors that induce more potent and protective immune responses.

### *2.1.1. Innate immune responses to the adenovirus vector*

The development of transgene-specific immune responses after adenovirus-based vaccination requires activation of innate immune signaling pathways [13]. The interplay between innate immune responses, such as the activation of pattern recognition receptors, cytokine production, and immune cell recruitment, shapes the adaptive immune responses after adenovirus-based vaccination. Innate immune recognition is a necessary adjuvating response, but an excess of proinflammatory signals can dampen adaptive immune responses either directly by clearing transduced cells or indirectly through cytokine production [10,11,33].

The balance between innate immune suppression and stimulation is essential for the development of potent adaptive immune responses after adenovirus-based vaccination [1]. The quality and magnitude of innate immune responses depend on the adenovirus type and the anatomical site of the response [34]. Unraveling the innate immune response at the site of immunization and draining lymph nodes after adenovirus-based vaccination and the adenovirus-type differences could bring knowledge to develop new modified adenoviral vectors that elicit more potent and protective immune responses.

### *2.1.2. Transduced and infiltrating cells*

One component of innate immune signaling upon vaccination is the activation of immune cells (such as NK-cells, neutrophils, monocytes/macrophages, and dendritic cells) that are directly transduced by the vector or recruited to the site of immunization [33-35]. At the same time, these cells can have a direct effect on the development of adaptive immune responses after vaccination by draining into the lymph nodes and modulating B and T-cell priming in the context of inflammatory cues [36].

However, little is known about the phenotypes of infiltrating cells and transduced resident cells at the site of immunization and their contribution to innate immune signals after adenovirus-based vaccination. The proinflammatory environment triggered by adenoviral vectors has been described for chimpanzee adenovirus vector ChAd55 in preclinical models and is characterized by type I IFN release (IFN-α), cytokines, and chemokines (CXCL9, CXCL10, CCL2). The release of proinflammatory signals coincided with the recruitment of immune cells to the site of immunization (monocytes and macrophages) and subsequently to the draining lymph nodes (natural killer cells, dendritic cells, monocytes). At the site of immunization (muscle), both hematopoietic and non-hematopoietic cells expressed the transgene, indicating the transduction of infiltrating and muscle-resident cells [35]. This is supported by our studies (**Chapter 4**) with a different adenoviral vector (Ad26.COV2.S), where we described the phenotype of a limited number of transgene-expressing cells based on their morphology. Spike-expressing macrophages and fibroblasts were identified by immunohistochemistry at the site of immunization (muscle) in rabbits, and no spike expression was detected in the endothelium or myocytes in the muscle after Ad26.COV2.S vaccination.

One of the goals of my thesis was to perform a full characterization of infiltrating and transduced cells after Ad26 vaccination. Several approaches were used to analyze mouse tissues, including immunofluorescence and flow cytometry (data not shown in the thesis), but the low sensitivity resulted in a low yield of transgene-expressing cells.

To circumvent this, a new and more sensitive model was set up, in which the Ad26-mediated expression of a Cre recombinase protein leads to the expression of a reporter gene in the transduced cells. This model is based on the existing Ai9 (B6. Cg-Gt*(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J) mouse strain [37]. Ai9 is a Cre reporter strain designed to have a loxP-flanked STOP cassette that prevents the transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato), all inserted into the Gt(ROSA)26Sor locus. Ai9 mice express tdTomato fluorescent protein following Cre-mediated recombination. We cloned and produced an Ad26.CRE.RSV.F vector and a self-amplifying RNA vector based on the Synthetically Modified Alpha Replicon RNA Technology (SMARRT) [38], SMARRT.CRE.RSV.F, to compare cellular transduction across vaccine platforms by flow cytometry. SMARRT is based on a modified sequence of the Venezuelan Equine Encephalitis Virus (VEEV) genome and is designed to drive more robust protein expression while maintaining the full triggering of innate immune pathways that drive the stimulation of the immune system. The VEEV genome was modified through the deletion of structural genes and insertion of restriction sites downstream of the subgenomic promoter, which enables the insertion of the transgene.

Our unpublished data demonstrated that transduced tdTomato+ cells were detectable in all groups to varying degrees at the site of immunization (muscle), except for the buffer control (**Figure 1**). After immunization with Ad26 and SMARRT vectors, a higher proportion of tdTomato+ cells in the muscle were of non-hematopoietic origin (CD45-). Additionally, the frequency of macrophages among the immune cells in the muscle that were CD45+ tdTomato+ was consistently the highest, regardless of the type of vector administered or timepoint, consistent with our findings presented in **Chapter 4**. Neutrophils, defined as CD11b+ and Ly6c+, were also positive for tdTomato protein after Ad26 and SMARRT vaccination (data not shown).

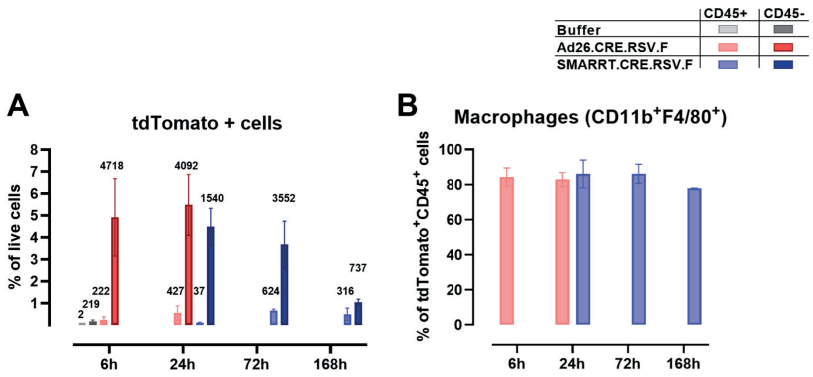


**Figure 1. Phenotyping of Ad26 and SMARRT target cells at the site of immunization.** Ai9 mice were immunized IM with $10^{10}$ viral particles of Ad26.CRE.RSV.F, 1 micogram SMAART.CRE.RSV.F, or saline buffer (AP-1). Mice were sacrificed at 6 (Ad26.Cre.RSV.F and saline buffer), 24 (Ad26.Cre.RSV.F, SMAART.Cre.RSV.F), 72 or 168 h (SMAART.Cre.RSV.F) after immunization and muscles (site of immunization) were collected. Organs were dissociated into single cell suspensions in DMEM containing 1% FCS, 100 µg/ml DNase I (Roche) and 0.1 U/ml Liberase ™ (Roche). Muscles were dissociated by 2 incubations of 30 minutes at 37°C, each followed by mechanical dissociation on a gentleMACS (Miltenyi Biotec). Cell suspension was stained for flow cytometry with Live/Dead Aqua (Invitrogen), BUV737 anti-Mouse CD45 (BD Bioscience), BV785 anti-Mouse F4/80 (Biolegend), and BV711 anti-Mouse CD11b (Biolegend). **A)** Percentages of CD45+ or CD45- and tdTomato+ cells in total live population. The average numbers of CD45+ or CD45- and tdTomato+ cells per group are depicted on top of each corresponding bar. **B)** Percentages of macrophages (CD11b+ and F4/80+) in total live tdTomato+ and CD45+ population. Data generated by Sonia Márquez Martínez, Miranda Baert, and Aneesh Vijayan (unpublished).



Overall, our proof-of-concept study revealed the potential of the Ai9 mouse model to characterize target cells upon vaccination in mice and provided some preliminary insights into the mode of action of Ad26 and SMARRT vectors. Interestingly, most transgene-expressing cells were non-hematopoietic in both groups, but whether these are the same cell types for Ad26 and SMARRT remains to be determined. Additionally, other studies have shown that structural cells (epithelium, endothelium and fibroblasts) have the potential to regulate or prime the immune system [39,40] and further research on non-hematopoietic transduced cells in the Ai9 model could provide insights into the

role of these cells in the development of adaptive immune responses. Macrophages were identified as the main transgene-expressing hematopoietic cell population after vaccination with Ad26 and SMARRT, which may be due to the recruitment of these cells to the site of inflammation and their subsequent Ad26 transduction or SMARRT internalization. Future studies should include a broader antibody panel and collection of draining lymph nodes and other organs to gain insights into the immunophenotype and migration of cells upon adenovirus-based vaccination.

### *2.1.3. Transgene expression*

One of the key findings of this project was the potential to enhance the potency of humoral responses by increasing peak transgene expression early after adenovirus-based vaccination (**Chapter 3**). Humoral responses have been shown to correlate with protection against the disease caused by the Ebola virus in NHPs [20] and COVID-19 in human vaccinees [21,22] after Ad26 administration, suggesting that an increase in the potency of humoral responses could lead to increased protection against disease.

Multiple parameters influence transgene expression after adenovirus-based vaccination, including innate immune responses and cellular tropism. For example, early excessive stimulation of type I IFN pathways following ChAdV-68 immunization has been shown to decrease transgene expression and subsequently reduce antigen-specific antibody responses [10,41]. Moreover, another study showed that abrogation of type I IFN and STING could increase transgene expression after adenovirus-based vaccination, and that IFN pathway induction was different across adenovirus types, with Ad5 and ChAd3 presenting the weakest transcriptional activation [11]. The differences in innate immune recognition may be explained by differences in cellular entry and trafficking, with CAR-specific Ad5 undergoing early endosomal escape while adenoviral vectors that utilize other primary receptors (e.g., Ad26 or Ad35) trigger TLRs in late endosomes and subsequent innate responses [8]. Modifications in the fiber knob of adenoviral vectors that do not utilize CAR to achieve retargeting toward this cellular receptor could lead to the targeting of a broader spectrum of different cell types and the triggering of early endosomal escape, which has been associated with lower innate activation. Broader tropism and decreased proinflammatory responses could lead to an increase in transgene expression.

Notably, an increase in transgene expression and subsequent humoral responses could lead to a decrease in the total adenoviral vector dose required to achieve protection through vaccination. This could lead to a reduction in reactogenicity and adverse effects directly related to adenoviral vector dose.

## 2.2. Vaccine-induced immune thrombotic thrombocytopenia

VITT is a rare multifactorial adverse effect after COVID-19 adenovirus-based vaccination. Understanding the underlying mechanism is critical for the development of safe adenovirus-based vaccines. The hallmarks of VITT are thrombosis at unusual anatomic sites, such as brain venous sinuses or splanchnic vein, and severe thrombocytopenia with the presence of platelet-activating antibodies targeting PF4 [42]. The early onset of VITT suggests that the underlying mechanism relies on early events after vaccination. VITT is a complex, multifactorial syndrome, and several factors have been identified as potential players in its induction, including the spike protein and/or other adenoviral vector vaccine components.

One hypothesis behind the development of VITT focuses on the role of the spike protein in the induction of PF4 antibodies. Endothelial cells may be activated through the binding of the spike protein to the ACE2 receptor, thereby recruiting and activating platelets [43]. At the same time, platelets might be directly activated by the circulating spike protein via the ACE2 receptor. Platelets then release PF4 molecules that can bind to anti-PF4 memory B-cells. Activation of pre-primed B-cells along with a proinflammatory co-signal would trigger the release of anti-PF4 antibodies into the circulation [44,45] (**Chapter 1, Figure 5A**).

The data presented in this thesis showed no detection of spike protein in the platelet-rich blood fraction or endothelial cells after Ad26.COV2.S vaccination in rabbits, suggesting no direct transduction or presence of spike in these cells (**Chapter 4**). Additionally, the spike protein levels detected in preclinical models and humans were lower after Ad26.COV2.S vaccination compared with mRNA. The subunit composition of the spike protein was different between Ad26.COV2.S and an mRNA vaccine. Additionally, we showed that an Ad26 vaccine encoding a spike protein with a wild-type furin cleavage site (similar to the spike encoded by ChAdOx1) led to the expression of a circulating spike with a similar subunit composition compared with mRNA. This suggests that the differences observed between Ad26.COV2.S and the other vaccines is linked to the mutation of the furin cleavage site in Ad26.COV2.S, which prevents the shedding of S1. Overall, the data presented here suggest that the magnitude, distribution, or composition of the spike protein are unlikely the main triggers of VITT. Follow-up studies should investigate the potential spike binding or passive internalization in endothelial cells and platelets after Ad26.COV2.S dosage in different species.



Another important aspect that may play a role in VITT is the contribution of the spike protein to proinflammatory responses after vaccination [28,29]. A recent report describes the promotion of inflammation by SARS-CoV-2 spike protein in collagen-induced arthritis mice injected with a plasmid encoding spike [46]. We demonstrated

higher levels of C-reactive protein and amyloid A in serum (**Chapter 5**) after Ad26.COV2.S vaccination when compared to an Ad26 vaccine expressing a different antigen, despite the fact that the adenovirus particle structure and composition is similar for both vaccines. These non-clinical data suggest the contribution of the spike protein to proinflammatory signatures. The combination of the proinflammatory signatures induced by the adenoviral vector along with the contribution of the spike protein might lead to an inflammatory milieu involved in the development of VITT. Aid et al. proposed that the triggering of platelet activation, coagulation, and innate immune pathways may be necessary for the development of VITT but is not the sole contributor [47]. They showed similar induction of these pathways after Ad26.COV2.S compared with 2 mRNA vaccines in humans, but they also observed a reduced proinflammatory response after the second dose of Ad26.COV2.S compared with the first dose, which is consistent with a lower incidence of VITT after the second dose of Ad26.COV2.S. The implications of proinflammatory signatures in VITT remain to be elucidated and future studies should investigate differences across vaccine platforms and the signatures triggered at the site of immunization.

Lastly, it has been proposed that the binding of the adenoviral particle to platelets might trigger platelet activation and a proinflammatory milieu linked to the development of VITT. Platelet activation could lead to the release of PF4 and binding to B-cell receptors (**Chapter 1, Figure 5C**). Subsequently, activation of PF4 memory B-cells along with a proinflammatory co-signal, could lead to the release of high titers of anti-PF4 antibodies into the circulation [44,45]. Accidental systemic exposure of adenoviral vectors (due to either accidental intravenous injection or leakage from the muscle injection site into the blood) may lead to interaction of the vectors with platelets [25,48-50]. CAR and integrins, such as αVβ3, are expressed on the platelet surface which would allow the attachment of ChAdOx1 and Ad26 [51-53]. However, in this project we showed that accidental IV exposure is unlikely a direct risk that could be associated with VITT pathogenesis (**Chapter 5**), as it did not have a relevant impact on platelet counts, other blood parameters or safety parameters in rabbits. It is important to note that the work was performed with a low number of animals and that there be might differences across species, so further investigation should be performed.

Overall, the work presented in this thesis has shed some light on the development of VITT, excluding or reducing the likelihood of certain underlying mechanisms. The events that lead to the activation of B-cells and the induction of PF4-antibodies in VITT remain to be elucidated. Other parameters that might contribute to the development of this syndrome, including neutrophilic or complement activation, were not addressed in this thesis and should be investigated to understand this multifactorial pathology. Future research should focus on understanding the exact molecular interactions that drive

this syndrome to support future clinical development of prophylactic and therapeutic adenovirus-based vaccines.

## 2.3. The future of adenovirus-based vaccines

Vaccines play a crucial role in human health and their impact has become even more evident during the COVID-19 pandemic, with an estimated 14.4 million deaths prevented through vaccination between 8th December 2020 and 8th December 2021 [54]. Traditional licensed vaccines consist of inactivated/attenuated pathogens or subunits of the pathogen but, more recently, vaccine platforms employing nucleic acids to produce the antigen of interest have proven to be valuable alternatives to traditional vaccines [55]. These include the induction of durable immune responses and ease of large-scale manufacturing.

Over recent years, adenovirus-based and mRNA nucleic acid-based vaccine platforms have received increased attention due to their use in the prevention of Ebola virus disease and/or COVID-19. Both vaccine platforms induce potent immune responses against the transgene of interest in preclinical models and humans [11,14,56-66] [67-70]. Licensed mRNA vaccine regimens induce higher neutralizing antibody titers against the SARS-CoV-2 virus than licensed adenovirus-based vaccine regimens, which correlate with immune protection against the disease [71,72] and would explain the higher efficacy of mRNA vaccine regimens against COVID-19. In terms of durability, single-dose Ad26.COV2.S elicits durable humoral and cellular responses with minimal decreases for at least 8 months after vaccination. Moreover, the binding and neutralizing antibodies show a considerable increase after a second dose of Ad26.COV2.S [68,73]. mRNA vaccines can also stimulate durable immune memory, however, a steady decrease in antibody responses and subsequent effectiveness of vaccine-mediated protection was observed after the 2nd dose [74-79].

Adenovirus-based and mRNA-based platforms are both valuable tools in vaccine development, and each presents different advantages. Adenovirus-based vaccines are more cost effective, can be administered in one-dose regimens and present higher stability than mRNA vaccines, so they do not require freezing storage conditions, making them easy to distribute in resource-limited settings [80]. Additionally, adenovirus-based vaccines can be used in the alternative mucosal vaccination route to elicit robust humoral responses (IgG, IgA and neutralizing antibodies) in the respiratory tract compared with IM vaccination, which was associated with enhanced protection and reduced transmission of infection [81-85]. Another important aspect of adenovirus-based vaccines is their potential in the development of vaccine candidates for cancer immunotherapies due to the induction of potent cellular immune responses. Further

insights into the mode of action of adenovirus-based vaccines would bring essential knowledge for the development of more potent prophylactic and therapeutic vaccines.

Adenovirus-based vaccines are generally well tolerated; however, in the case of the COVID-19 vaccines, they can cause rare but severe systemic adverse effects. The emergence of VITT as a rare adverse event associated with adenovirus-based COVID-19 vaccines has raised a general safety concern regarding the adenovirus-based vaccine platform. Although VITT is an extremely rare event, it is a life-threatening syndrome that questions the risk/benefit ratio of adenovirus-based prophylactic vaccines for the use against infectious diseases for which other effective vaccines are available. However, the mechanism underlying the development of VITT is not yet understood and may be related to the vaccine-expressed spike protein, which is specific to COVID-19 vaccines. In fields other than prophylactic vaccines, such as therapeutic cancer vaccines, adenovirus-based vaccines may have a favorable risk/benefit ratio. Further research on the mechanism of action and safety of adenovirus-based vaccines is required, and future insights may allow the use of this platform also for prophylactic vaccines.

## 3. Concluding remarks

Vaccines play a crucial role in improving human health, as evidenced by the COVID-19 pandemic. Adenovirus-based vaccines have proven to be an effective tool against infectious diseases due to the potent and protective immune responses they elicit. The work of this thesis has contributed to the knowledge on their mechanism of action and safety profile. These findings may be used for the development of new generations of immunogenic and safe adenovirus-based vaccines.

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**Appendices**

Author Contributions
Nederlandse Samenvatting
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List of Publications

# Author Contributions

## Chapter 1

SMM wrote this chapter, created the figures, and processed the feedback from SK, RCZ and FJMvK.

## Chapter 2

SMM wrote this manuscript, created the figures, and processed the feedback from all co-authors and TU.

## Chapter 3

SMM was involved in the conceptualization of the project. SMM designed, performed, and analyzed all experiments except for figure 3. SMM created all manuscript figures, wrote the manuscript, and revised it following feedback from co-authors and FJMvK.

## Chapter 4

SMM contributed to the conceptualization of the project and the design of experiments shown in figures 1, 2, 3, 7, A1, A2, A3 and A6. SMM performed the initial characterization of the circulating spike protein (figure 3) and the characterization of the interference of anti-spike antibodies in the detection of spike protein (figures 7 and A6). SMM created all figures, wrote the manuscript, and revised it based on feedback from co-authors and FJMvK.

## Chapter 5

SMM performed and analyzed the experiment regarding the circulating spike characterization (figure 3) and produced the original figure of the study design (figure A1). SMM contributed to the coordination of the rabbit study from which the samples for all main figures were obtained (figures 1-3). SMM prepared figures 1 and 2 and contributed to the editing of the manuscript. SMM revised the figures and manuscript following the feedback from co-authors and FJMvK.

## Chapter 6

SMM designed and performed the phenotyping experiment. SMM analyzed the data and created the figure. SMM wrote this chapter and revised it with feedback from SK, RCZ and FJMvK.

# Nederlandse Samenvatting

Vaccins spelen een cruciale rol in de verbetering van de menselijke gezondheid door de incidentie en mortaliteit van door voorkombare infectieziekten, zoals difterie, pokken, polio, mazelen, bof en rodehond, te verminderen. Vaccins bieden bescherming tegen specifieke pathogenen door een immuunrespons op te wekken in de gastheer. Er zijn verschillende platformen beschikbaar voor de productie van vaccins, waaronder geïnactiveerde of verzwakte pathogenen of toxines, virale vectoren (zoals adenovirus of gemodificeerd vacciniavirus Ankara), subunitvaccins en genetische vaccins (bv. mRNA-vaccins).

Op adenovirus-gebaseerde vaccins coderen voor één of meerdere ziektespecifieke transgenen om immuniteit tegen het doelwit te induceren. Adenovirale vectoren hebben bepaalde kenmerken waardoor ze uitstekende vaccinplatforms zijn, zoals de inductie van transgenespecifieke immuniteit, grote verpakkingscapaciteit (tot 35 kbp transgene sequentie) en breed tropisme. Op het gebied van profylactische vaccins is het gebruik van adenovirale vectoren beperkt tot niet-replicerende vectoren. Deze vectoren worden gemanipuleerd door genetische modificaties van het virus om een niet-replicerende virion te produceren die een transgen van belang kan dragen. Adenovirus-gebaseerde vaccins wekken sterke immuunreacties op tegen het transgen dat ze dragen en deze immuunreacties zijn afhankelijk van vroege immuunreacties na vaccinatie.

In dit proefschrift bestuderen we vroege gebeurtenissen na vaccinatie met adenovirus om hun rol in het vormen van adaptieve immuunreacties en de ontwikkeling van bijwerkingen te begrijpen.

In **Hoofdstuk 2** werden de vroege gebeurtenissen na vaccinatie met adenovirus onderzocht (cellulaire toegang, transgene expressie en aangeboren immuunresponsen), met focus op verschillen in adenovirustype. Bovendien werd de rol van vroege gebeurtenissen in de ontwikkeling van transgen-specifieke adaptieve immuunresponsen samengevat.

Een van de factoren die een directe invloed kan hebben op adaptieve immuunreacties tegen het transgen is de transgene expressie zelf. Deze kan verschillen tussen adenovirustypen met betrekking tot grootte en distributie. In **Hoofdstuk 3** karakteriseerden we de omvang en duur van transgene expressie na een enkele intramusculaire toediening van een adenovirus 26-vector (Ad26) in muizen en evalueerden we de verschillen met adenovirus 5-vectoren (Ad5). Daarnaast hebben

we inzicht gegeven in het specifieke effect van transgene expressie op adaptieve immuunresponsen na intramusculaire vaccinatie met Ad26 bij muizen.

Elk gelicentieerd vaccin kan bijwerkingen veroorzaken, maar de meeste daarvan zijn mild of matig en verdwijnen binnen een paar dagen na vaccinatie. De massale COVID-19 vaccinatiecampagnes leidden tot de detectie van zeldzame bijwerkingen bij alle vaccinplatformen die niet waren gedetecteerd in klinische studies vanwege hun lage incidentie. Enkele van de zeldzame bijwerkingen die werden vastgesteld na COVID-19 vaccinatie zijn onder andere het Guillain-Barré syndroom, ontstekingsziekten van het hart en door vaccinatie veroorzaakte immuuntrombotische trombocytopenie (VITT). VITT is een zeldzame bijwerking geïnduceerd door adenovirus-gebaseerde COVID-19 vaccinatie, gekenmerkt door trombocytopenie en trombose, vaak op atypische anatomische locaties, en de aanwezigheid van antilichamen tegen platelet factor 4 (PF4). VITT is gemeld na vaccinatie met adenovirus (Ad26.COV2.S en ChAdOx1 nCoV-19) en, sporadisch, na COVID-19 vaccinatie met andere vaccinplatformen. Vanwege de veiligheidsimplicaties van deze zeldzame maar ernstige bijwerking en de mogelijke betrokkenheid van vroege gebeurtenissen na vaccinatie met adenovirus bij de ontwikkeling ervan, werden in dit proefschrift enkele mogelijke factoren onderzocht die betrokken zijn bij de ontwikkeling van VITT, zoals het gevaccineerde spike-eiwit en/of de adenovirale vaccincomponenten (**Hoofdstukken 4 en 5**).

In **Hoofdstuk 4** werd de bijdrage van het transgene eiwit (spike) aan de ontwikkeling van VITT na intramusculaire toediening van Ad26.COV2.S geëvalueerd. We onderzochten de biodistributie, kinetiek en samenstelling van het spike-eiwit na intramusculaire toediening van Ad26.COV2.S in preklinische modellen en klinische monsters als potentiële drijfveren van VITT. Op basis van de bevindingen in dit hoofdstuk werd geen directe associatie van spike met de ontwikkeling van VITT vastgesteld. Het kan echter niet worden uitgesloten dat spike bijdraagt aan de pathogenese van VITT in de context van Ad26 vaccinatie en in combinatie met andere beïnvloedende factoren, zoals eerdere infecties, genetische aanleg en reeds bestaande gezondheidsproblemen.

In **Hoofdstuk 5** onderzochten we of intraveneuze toediening (als model voor accidentele systemische blootstelling) van Ad26.COV2.S geassocieerd was met de ontwikkeling van VITT in preklinische modellen door het evalueren van klinische pathologieparameters, histopathologische bevindingen en systemische blootstelling aan spike-eiwit in vergelijking met intramusculaire toediening bij konijnen. We toonden aan dat intraveneuze toediening goed werd verdragen en dat er geen significante verschillen werden waargenomen in vergelijking met de intramusculaire route. Ad26.COV2.S induceerde echter significant hogere niveaus van C-Reactief proteïne op dag 1 na intramusculaire vaccinatie in vergelijking met intramusculaire vaccinatie.

**Hoofdstuk 6** bestaat uit een samenvattende discussie van de resultaten beschreven in dit proefschrift, de introductie van een nieuw muismodel om doelcellen na vaccinatie te bestuderen en de toekomstperspectieven van op adenovirus gebaseerde vaccins.

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# About the Author

Sonia Márquez Martínez was born on the 2nd of April 1995 in Seville, Spain. In 2013 she finished high school with high honors and began a Bachelor's program in Basic and Experimental Biomedicine at the University of Seville. During the second year of her studies, she joined the microbiology department as an intern student. She was awarded an Erasmus+ grant to carry out an internship at Prof. Dr. K. Maloy's lab at Oxford University, where she studied inflammatory bowel disease. Sonia obtained her Bachelor's degree in 2017 and pursued a Master's program in Infection and Immunity at the University of Amsterdam. During the program, she completed two internships and a literature thesis. Her first internship took place at the University Medical Center in Amsterdam, supervised by Prof. Dr. R.W. Sanders and Dr. M. van Gils. The project focused on the optimization of the first encounter between naïve B cells and HIV vaccine immunogens. Sonia completed her second internship at Cambridge University with an Erasmus+ grant. There, she studied the immune evasion of vaccinia virus under the supervision of Prof. Dr. G. Smith and Dr. A. Altenburg. Her literature thesis on the potential threat of influenza viruses was supervised by Prof. Dr. C. Russell, and she was awarded the Master of Science degree in 2019. In December 2019, Sonia began her PhD position at Janssen Vaccines and Prevention and Utrecht University. This PhD position was part of the Marie Skłodowska-Curie Innovative Training Network 'INITIATE' for early-stage researchers funded by the European Commission under the H2020 program. Sonia was supervised by Dr. R. Zahn and Dr. S. Khan at Janssen, and Prof. Dr. F.J.M. van Kuppeveld at Utrecht University. The results of this research are described in this thesis and have been published in various international scientific journals.

# Publications

Khan S, **Marquez-Martinez S**, Erkens T, de Wilde A, Costes LMM, Vinken P, De Jonghe S, Roosen W, Talia C, Chamanza R, et al.: Intravenous Administration of Ad26.COV2.S Does Not Induce Thrombocytopenia or Thrombotic Events or Affect SARS-CoV-2 Spike Protein Bioavailability in Blood Compared with Intramuscular Vaccination in Rabbits. *Vaccines (Basel)* 2023, 11.

**Marquez-Martinez S**, Vijayan A, Khan S, Zahn R: Cell entry and innate sensing shape adaptive immune responses to adenovirus-based vaccines. *Curr Opin Immunol* 2023, 80:102282.

Keszei Z, Vendrell XM, Soultsioti M, Molina PR, Wirth L, Weiss H, Alexandridi M, Aloise C, Susma B, Guy C, Loverre A, **Martinez SM,** Mazej J, Spahn A, Sularea M, Hiscott J, Hoogen BVD: 100 days of solitude: The spring of COVID-19 through the eyes of 15 young virologists of the INITIATE program. *Virus Res.* 2020 Oct 2;287:198093.

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