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# Vaccination Potential Of Adenoviral Vectors Displaying Heterologous Epitopes In Their Capsid Proteins

Aleksandra Anchim

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CBMS Cancérologie : biologie - médecine - santé

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par

**Aleksandra Anchim**

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***Vaccination potential of adenoviral vectors displaying  
heterologous epitopes in their capsid proteins***

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**Thèse présentée et soutenue à Villejuif, le 29/03/2016**

**Composition du Jury :**

Uriel Hazan	Professeur des universités, ENS Cachan	Président
Jennifer Richardson	Directeur de Recherche CNRS, ENVA Maisons-Alfort	Rapporteuse
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**Titre :** *Potentiel vaccinal d'adénovirus porteurs d'épitopes heterologues insérés dans les protéines de capsid.*

**Mots clés :** adénovirus, vaccination, HCV

**Résumé :** Les vecteurs adénoviraux ont été utilisés dans de nombreuses approches vaccinales en exprimant la protéine d'intérêt à l'aide du transgène. Néanmoins, leur efficacité est limitée par la forte séroprévalence chez l'homme des anticorps anti-Ad capables d'inhiber le transfert de gène. Afin de s'affranchir de cette étape, mon laboratoire a opté pour une stratégie d'insertion d'épitopes dans les protéines de capsid virale (epitope display).

La première partie de ma thèse a cherché à comprendre les paramètres contrôlant l'immunogénicité de l'approche epitope display. Ainsi, en utilisant différents Ad porteurs d'un épitope B dérivé de l'ovalbumine (Ova), j'ai montré que l'ablation des interactions des Ad avec leurs récepteurs (CAR, intégrines, héparane sulfate) ne modifiait pas leur capacité à induire une réponse humorale contre l'Ova. Ces résultats suggèrent que le processus d'infection virale n'est pas requis pour l'induction d'une réponse humorale par les Ad porteurs d'épitopes Ova. Parallèlement, j'ai évalué l'impact de différents systèmes senseurs de l'immunité innée sur la réponse anticorps induite par les vecteurs porteurs de l'épitope Ova. Les résultats obtenus basés sur l'utilisation de modèles de souris génétiquement déficientes pour les protéines MyD88 ou MAVS indiquent que les voies des *Toll-like receptor* et *RIG-I-like receptor* ne sont pas indispensables ou jouent des rôles redondants dans le contrôle du potentiel immunogénique des Ad porteurs d'épitope Ova.

Le second objectif de ma thèse a été d'évaluer la capacité de l'approche d'epitope display à induire des réponses cellulaires chez la souris. Des vecteurs Ad à capsid modifiée par insertion d'un épitope T dérivé de l'antigène modèle Ova ont été produits et nous avons mis en évidence que ces vecteurs induisaient des réponses cellulaires de type CD8<sup>+</sup>. Nous avons observé que (1) ces réponses immunitaires sont quantitativement plus importantes quand l'épitope T est inséré dans la protéine hexon plutôt que dans la fibre (2) et sont réduites chez les animaux immuns pour l'Ad comparativement aux animaux naïfs.

Parallèlement, nous avons voulu analyser la capacité de la stratégie d'epitope display à induire des réponses cellulaires de type cytotoxiques contre une cible thérapeutique, le virus de l'hépatite C. Différents Ad porteurs d'épitopes T dérivés de la protéine NS3 d'HCV ont été produits. Après administration des Ad à des souris transgéniques pour HLA-A2, nous avons mis en évidence l'induction de réponses cellulaires de type CD8<sup>+</sup> dirigées contre la protéine NS3 du VHC.

L'ensemble de mes travaux de thèse permet de mieux comprendre les paramètres qui contrôlent la vaccination par epitope display à l'aide d'Ad. Ils constituent une étape dans la mise au point de vaccins qui bénéficient des propriétés adjuvantes de l'Ad et présentent des effets secondaires réduits.

**Title :** *Vaccination potential of adenoviral vectors displaying heterologous epitopes in their capsid proteins.*

**Keywords :** adenovirus, vaccination, HCV, epitope display

**Abstract :** Recombinant adenoviruses (Ad) have recently been employed in a wide range of vaccination strategies. Unfortunately, highly prevalent pre-existing neutralizing antibodies reduce their ability to trigger transgene expression. To avoid the step of gene transfer a new vaccination strategy has been proposed based on the use of Ad displaying epitopes inserted into their capsid proteins.

In the first part of my thesis, we sought to better understand the molecular mechanisms dictating the vaccination potential of epitope display strategy. To that end, Ad vectors displaying B cell ovalbumin (Ova) epitope in fiber protein and ablated in interactions with their natural receptors (CAR, integrins, heparan sulfates) were produced and characterized in vitro. After their administration to C57BL/6 mice, these vectors led to unmodified anti-epitope humoral responses, suggesting that their efficacy does not depend on their ability to transduce cells. In parallel, we sought to evaluate the impact of innate immune pathways on the outcome of anti-epitope adaptive immune responses. Upon immunization of MyD88- and MAVS-deficient- mice we observed that humoral responses induced in both knock-out mice strains were as potent as in wild-type mice. This indicates that the signalization pathways of Toll-like receptors and RIG-I-like receptors are not crucial or play a redundant role in controlling the vaccination potential of Ad displaying Ova epitopes.

In the second part of my thesis, we assessed the capacity of epitope display strategy to induce cellular responses in mice. Ad vectors displaying T cell epitopes derived from the model antigen Ova were constructed, produced and characterized in vitro. Afterwards, C57BL/6 mice were immunized with Ad displaying T cell epitopes and cellular responses were assessed by measurement of cytokine production by splenocytes upon in vitro antigen restimulation or directly by quantification of epitope-specific T cells. The results indicate that (i) CD8+ cellular responses are more potent when T cell epitope is inserted into Ad hexon and not fiber protein and (ii) pre-existing anti-Ad responses limit the induction of anti-epitope cellular immunity.

Finally, we sought to evaluate the potential of epitope display strategy in mounting cellular responses directed against a therapeutic target, hepatitis C virus (HCV). Thus, Ad vectors displaying T cell epitopes from HCV protein NS3 inserted either into hexon or fiber were constructed and characterized. Remarkably, vector administration into HLA-A2 transgenic mice triggered the induction of HCV-specific CD8+ cellular responses

Altogether, our work allows better understanding of the epitope display vaccination strategy and provides the foundations for the development of Ad-based vaccines with minimized side effects and unaltered adjuvant properties.

## Abbreviations

aa : amino acid	MHC : major histocompatibility complex
Abs : antibodies	MHC-I : MHC class I molecule
Ad : adenovirus	MHC-II : MHC class II molecule
APCs : antigen presenting cells	MVA : modified vaccinia Ankara virus
CAR : coxsackie-adenovirus receptor	MyD88 : myeloid differentiation primary response 88 protein
CTL : cytotoxic CD8 <sup>+</sup> lymphocytes	nAbs : neutralizing antibodies
Dd : dodecahedron	Ova : ovalbumin
DCs : dendritic cells	PAMPs : pathogen-associated molecular patterns
FX : factor X	PRRs : pattern recognition receptors
HCV : hepatitis C virus	RIG-I : retinoic acid inducible gene I
HIV : human immunodeficiency virus	RLRs : RIG-I-like receptors
HLA : human leukocyte antigen	SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis
HSPG : heparan sulfate proteoglycans	T <sub>CM</sub> : central memory T cells
HVR : hyper variable regions	T <sub>EM</sub> : effector memory T cells
i.m. : intramuscular	Th : helper T cell
i.n. : intranasal	TLR : toll-like receptor
i.p. : intraperitoneal	VA-RNA : virus-associated RNA
i.v. : intravenous	VLP : virus-like particles
IFN : Interferon	
Ig : Immunoglobulin	
IL: Interleukin	
MAVS : mitochondrial antiviral signalling protein	



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# ***Introduction***

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# 1 Immune System and Vaccination

## 1.1 Introduction to the Immune System

Historically, the Immune System was defined as a totality of cells and molecules conferring protection against infectious diseases. However, the fact that immune responses can also be elicited by non-infectious foreign substances as well as description of various autoimmune pathologies shed a new light on the complexity of this system. A more inclusive definition was proposed, describing the Immune System as a complex set of protective mechanisms distinguishing Self from Non-self and allowing for protection against pathological microbes and toxic or allergenic proteins, defined from now on as antigens (Chaplin, 2010). Once a non-self element is present in the organism, two layers of defense are set in action: innate immunity and adaptive immunity. In the first turn innate immunity is activated, conferring immediate but non-specific protection. The main goal of this germ line-encoded layer of defense is to limit the infection during the first days, before adaptive response is established. This chapter will describe in a general manner the main elements and mechanisms of both innate and adaptive immune responses. The immune responses induced by adenovirus will be described in chapter 2.

### 1.1.1 Innate immunity

This evolutionary older defense is implicated in the first events after the antigen encounter. Innate immunity mechanisms are poorly specific and do not generate immunological memory, although the development of memory Natural Killer cells has recently been reported (reviewed in O'Sullivan *et al.*, 2015). Deficiencies in this branch of immunity are associated with recurrent infections, which underlines its importance (Janeway, 2002).

#### 1.1.1.1 Molecular components of innate immunity

Among the main components of this robust antimicrobial protection we can distinguish physical barriers, such as tight cell-cell interactions, mucosal protection found in respiratory and gastrointestinal tracts as well as various soluble proteins (complement factors, cytokines, chemokines and defensins). Most importantly, specific types of receptors, called pattern recognition receptors (PRRs) are found on the membranes and in the cytosol of various cells. Among them, Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nucleotide-binding and oligomerization domain

(NOD)-like receptors (NLRs), and C-type lectin receptors are capable of sensing structures shared by different microbes, known as pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira, 2010).

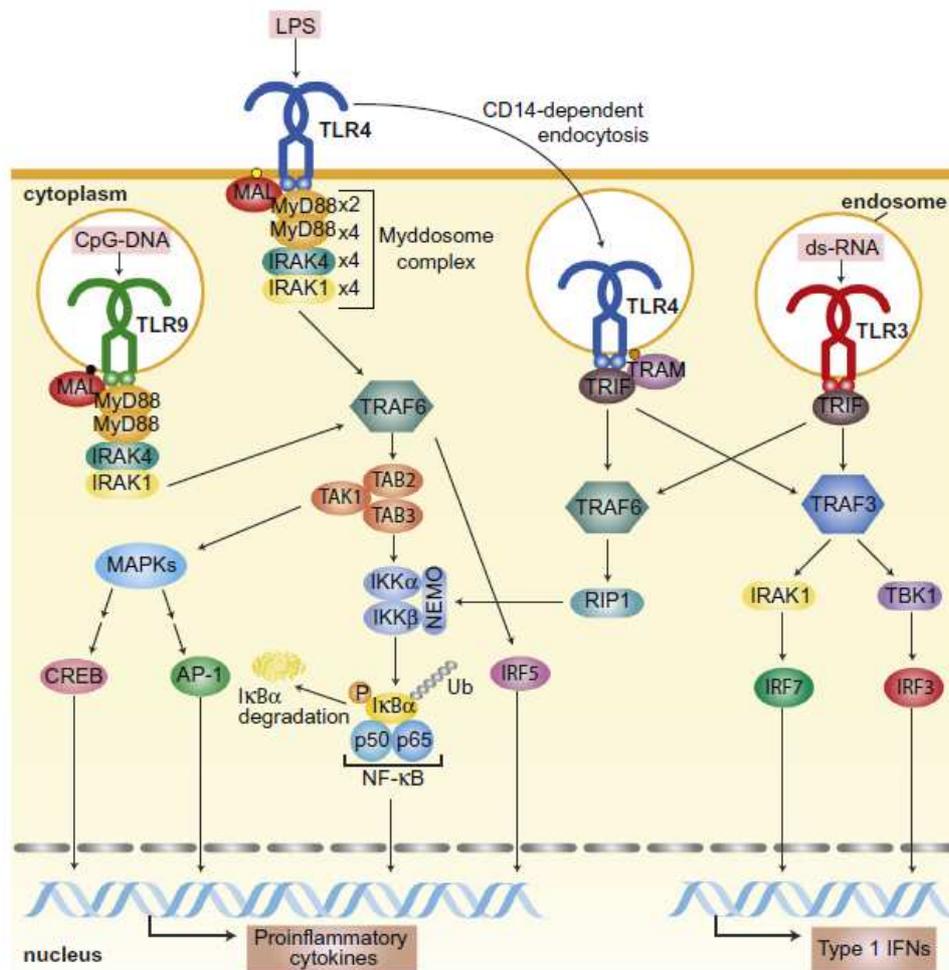
#### 1.1.1.1.1 Toll-like receptors (TLRs)

Among the PRRs of particular interest are TLRs, one of the major interferon (IFN)-inducing families of PRRs in mammals. Ten human and twelve murine TLRs have been identified until now, sensing different ligands (Table 1.1).

**Table 1.1. TLRs and their ligands.** Table summarizes the Toll-like receptors described until today, indicating their localization, ligand and ligand origin (Takeuchi and Akira, 2010).

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa

Located on the cell surface or in the endolysosomal membranes, TLRs are type-I integral membrane glycoproteins containing N-terminal leucine-rich repeats and cytoplasmic Toll/IL-1R homology (TIR) domain (Bowie and O’Neill, 2000). After ligand binding, dimerization of two identical (homodimerization) or two different (heterodimerization, e.g. TLR2:TLR6) TIR domains of TLR proteins is required to activate downstream signaling cascades. These can pass through distinct pathways, depending on the type of recruited adaptor molecules. Myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF), TIRAP/MAL, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM) also contain TIR domains, through which they are recruited to TLRs (Oshiumi et al., 2003; Yamamoto et al., 2002). The signaling pathways triggered by MyD88 and TRIF are different but both lead to the production of pro-inflammatory cytokines and type I IFNs (**Figure 1.1**).



**Figure 1.1. Overview of TLR signaling pathways.** TLR3, TLR4 and TLR9 signaling pathways are depicted as examples. Activated cell-surface TLR4 or endosomal TLR9 recruit adaptor protein MyD88, and with the participation of MAL and IRAK1/4 form structure called Myddosome. Active IRAK kinase activates TRAF6, ultimately leading to NF- $\kappa$ B, IRF5, AP-1 and CREB translocation to the nucleus and pro-inflammatory cytokine production. Alternatively, TLR3 activation, through recruitment of TRIF, induces TRAF3/6 signaling cascade. This results in activation of IRF7 and IRF3, their migration to the nucleus and production of type I IFNs or induction of cytokine production through RIP1/ NF- $\kappa$ B pathway. TLR4, by recruiting TRAM, can also follow TRIF signaling pathway (de Nardo, 2015).

Signaling through MyD88 occurs upon stimulation of all TLRs with the exception of TLR3, and in the case of TLR2 and TLR4 TIRAP/MAL is additionally needed for MyD88 bridging (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001 and 2002). In addition to TIR domain, in the structure of this adaptor protein we can also distinguish a death domain (DD) that interacts with DD of IL-1R-associated kinase 4 (IRAK-4) and IRAK-1, leading to the formation of a supramolecular organization center (SMOC) called myddosome (Kagan, 2014). IRAKs subsequently dissociates from SMOC and interacts with TNFRF-associated factor 6 (TRAF6), displaying an E3 ubiquitin protein ligase activity (Li *et al.*, 2002). TRAF6, along with ubiquitination E2 enzyme complex consisting of Ubc13 and Uev1A, catalyzes the

formation of a lysine 63-linked polyubiquitin chain on TRAF6 itself as well as on IKK- $\gamma$  / NF- $\kappa$ B essential modulator (NEMO) (Deng *et al.*, 2000). TGF- $\beta$ -activated kinase 1 (TAK1) and the TAK1 binding proteins TAB1, TAB2, and TAB3 are then recruited to TRAF6, allowing TAK1 to phosphorylate IKK- $\gamma$  and MAP kinase kinase 6 (MKK6) (Wang *et al.*, 2001). Subsequently, IKK complex (NEMO, IKK $\alpha$  and IKK $\beta$ ) phosphorylates I $\kappa$ B, withdrawing its inhibitory effect on NF- $\kappa$ B, resulting in its translocation to the nucleus and activation of pro-inflammatory cytokine genes. MAP kinase cascades, on the other hand, activate AP-1 and CREB transcription factors, also targeting cytokine genes involved in inflammation (Takeuchi and Akira, 2010). In parallel, IRF-5, another transcription protein crucial for pro-inflammatory cytokine production, moves to the nucleus (Takaoka *et al.*, 2005).

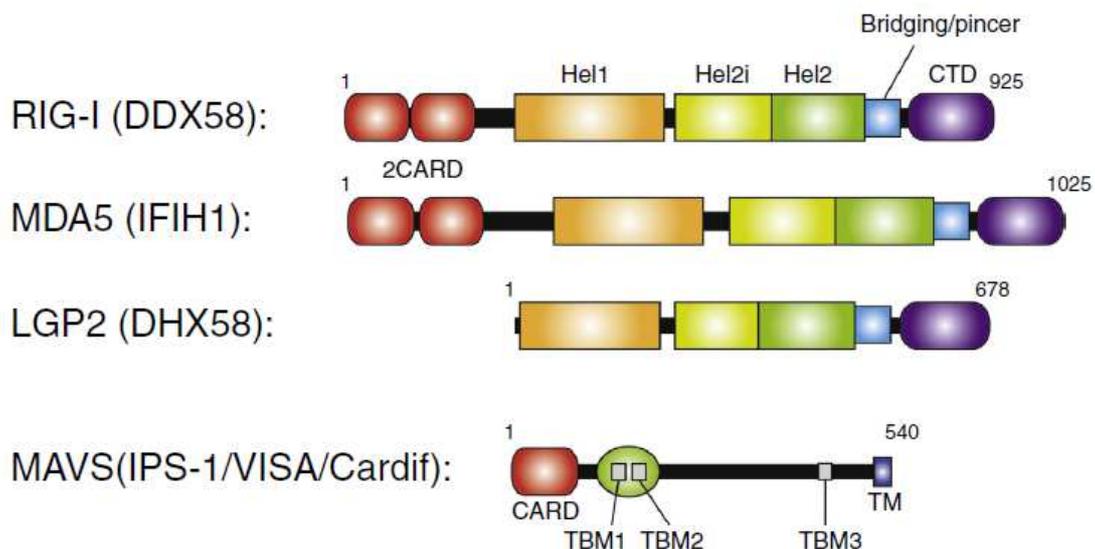
In the group of TLRs recruiting TRIF as an adaptor molecule we find TLR3 and TLR4, whose interaction with TRIF is mediated by TRAM (Fitzgerald *et al.*, 2003b, Yamamoto *et al.*, 2003). Stimulation of these PRRs induces the production of type I IFNs through TRAF family-member-associated NF- $\kappa$ B activator (TANK)-binding kinase 1 (TBK1), phosphorylating IRF-3 and IRF-7 and allowing its binding to Interferon-sensitive response element (ISRE) (Fitzgerald *et al.*, 2003a). On the other hand, through RIP1 signaling, TLRs involving TRIF are also inducing NF- $\kappa$ B activation (Meylan *et al.*, 2004). A distinct pathway is found in plasmacytoid dendritic cells (DCs) following TLR9 stimulation. It involves MyD88 and IRAK-1 proteins, and in a TBK1-independent manner results in significant IFN- $\alpha$  production (Uematsu *et al.*, 2005)

#### **1.1.1.1.2 RIG-I-like receptors (RLRs)**

Another important group of PRRs are RLRs, which are the main element of the host machinery responsible for sensing foreign RNA. RLRs family comprises retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), proteins present in the cytosol of different cell types (Yoneyama *et al.*, 2004). They sense distinct viral RNAs and through the interaction with their adaptor protein, mitochondrial antiviral signaling (MAVS) protein, also known as IPS-1, trigger the production of type-I interferons (IFN-I) and type-III interferons (IFN-III).

In the structure of RIG-I, MDA5 and LGP2 we can distinguish DExD/H-box RNA helicase domain, responsible for sensing viral RNA and a C-terminal domain (CTD) (**Figure 1.2**). Additionally, RIG-I and MAD5 possess in their N-terminus a tandem of caspase recruitment domains (2CARD), through which they can interact with MAVS. Since LGP2 does not possess CARD domain but still binds RNA with high affinity, this protein was initially described as RIG-I and MDA5 regulator

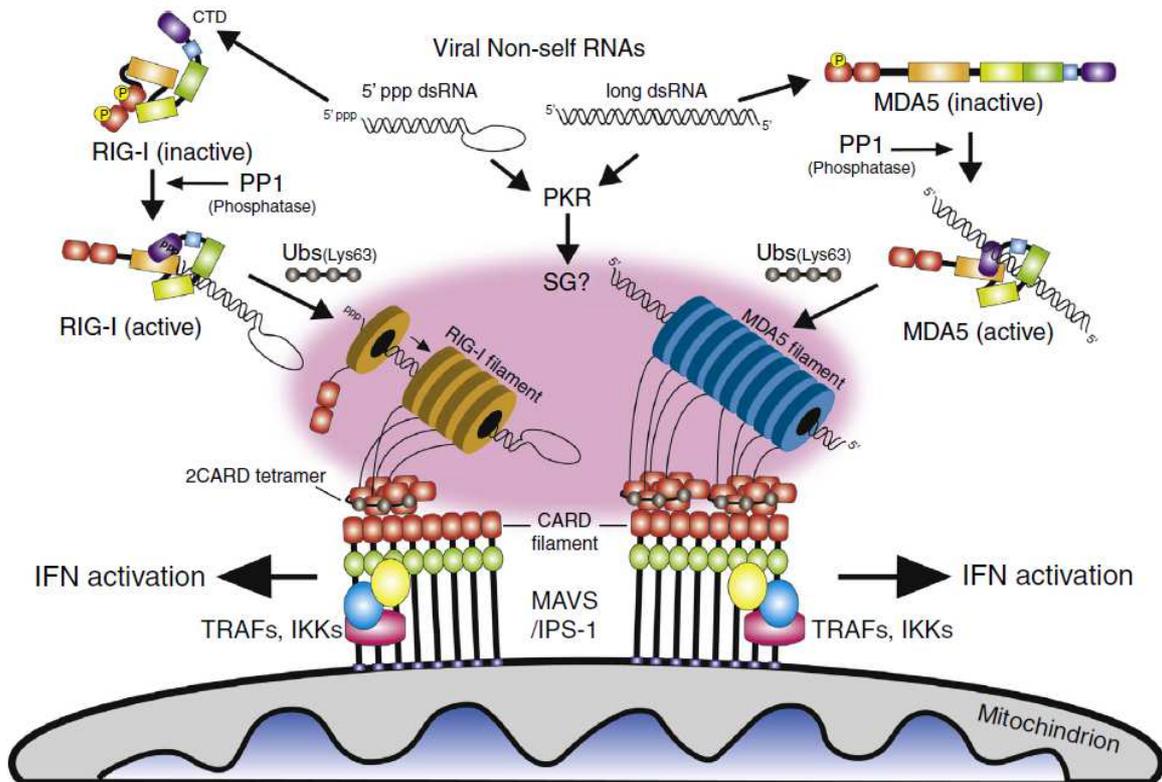
(Yoneyama *et al.*, 2005). Nevertheless, a recent study reports that it may cooperate with MDA5 during viral RNA recognition (Deddouche *et al.*, 2014). MAVS protein displays a CARD domain in N-terminus, a central region consisting of two TRAF binding motifs (TBM) TBM1 and TBM2, with additional TBM3 next to the transmembrane domain at the C-terminus. Transmembrane domain anchors MAVS in the membranes of mitochondrion, peroxisomes and ER subdomain called mitochondrion-associated membrane (MAM) (Dixit *et al.*, 2010; Horner *et al.*, 2011; Seth *et al.*, 2005).



**Figure 1.2. Structures of RLRs and MAVS.** All three RLRs contain helicase domains 1 (Hel1) and 2 (Hel2), Helicase insertion domain (Hel2i), bridging/pincer domain and C-terminal domain (CTD). Additionally, RIG-I and MDA5 have a tandem of CARD domains (2CARD). In the structure of MAVS protein we distinguish three TRAF binding motifs (TBM), a CARD domain in N-terminus and a C-termina transmembrane domain (TM) (Yoneyama *et al.*, 2015).

RIG-I senses a short double-stranded RNA which contains 5'-triphosphate (5'ppp). This RNA structure should also possess complementary end and/or a poly-U/UC rich double strand fragment (Weber *et al.*, 2013). More recent paper reports that RIG-I can also recognize viral RNA bearing 5'-diphosphate (5'pp) moiety (Goubau *et al.*, 2014). MDA5, on the other hand, recognizes long dsRNA (>1kbp). In an inactive state both RIG-I and MDA5 are phosphorylated, the former at 2CARD and CTD and the latter exclusively at 2CARD (Sun *et al.*, 2011). In virus-infected cells a series of events takes place in order to switch RIG-I and MDA5 from inhibited to activated state (**Figure 1.3**). Upon PAMP binding, RIG-I undergoes conformational changes which release 2CARD from auto-repression and allow them to interact with MAVS (Kowalinski *et al.*, 2011). Both RIG-I and MDA5 are

dephosphorylated by phosphatase 1 (PP1) (Wies *et al.*, 2013) and subsequently undergo full activation upon K-63 ubiquitination on their CTD and 2CARD domains (Peisley *et al.*, 2014).



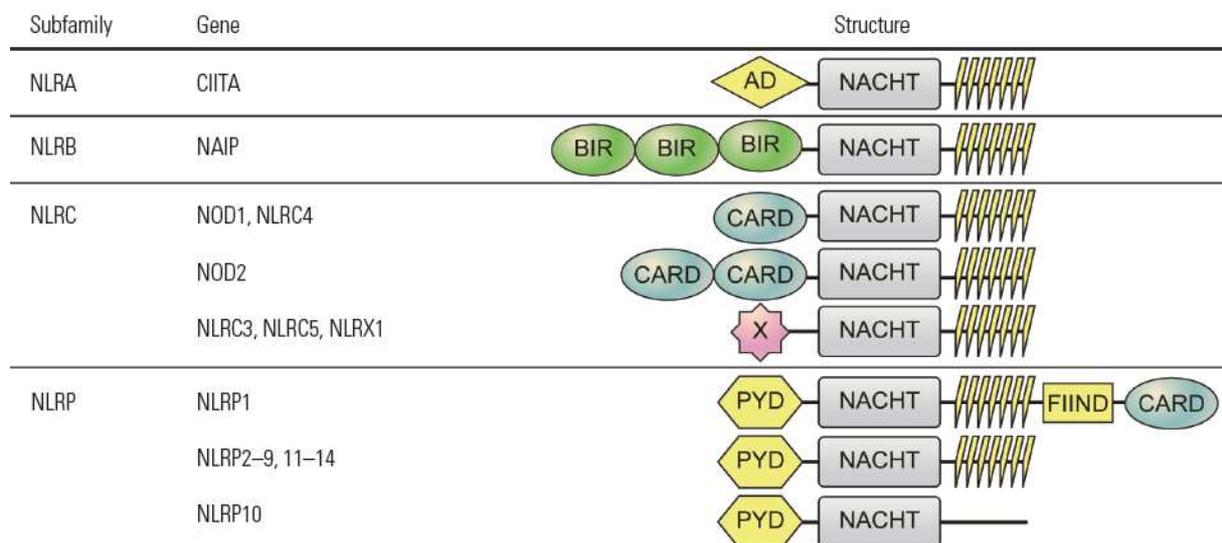
**Figure 1.3. Overview of RLRs signaling pathways.** In a resting state RIG-I and MDA5 are phosphorylated and inactive. Upon recognition of specific non-self RNA structures, PP-1 phosphatase dephosphorylates both proteins, which are then Lys63 ubiquitinated. Active RLR proteins form filamentous structures on the foreign RNA, presumably in cytosolic aggregates resembling stress granules (SG). This oligomerization generates formation of 2CARD tetramers, able to interact with aggregates of MAVS protein, an adaptor protein anchored in mitochondrion membrane. Subsequently, through TRAF and IKK signaling production of IFN type I and III is set in motion (Yoneyama *et al.*, 2015).

Recent reports demonstrate that viral infection induces in the cytoplasm formation of so-called stress granule (SG)-like aggregates (Onomoto *et al.*, 2012), and inhibition of formation of these structures interferes with the IFN-I production in infected cells. SG are thought to serve as a platform, where RLRs recognize their PAMPs. Activated RIG-I and MDA5 are able to form filamentous structures on the viral RNA, which results in the creation of tetrameric CARD structures, interacting with prion-like MAVS aggregates, anchored in the mitochondrial membranes (Hou *et al.*, 2011). Recent studies propose a model, where multimolecular aggregates of RIG-I/MAVS or MDA5/MAVS are formed, through which signaling takes place.

RLR interaction with MAVS passes through TRAF3 and TRAF6 and activates kinases of IKK family. Through their phosphorylation activity, these kinases remove NF- $\kappa$ B inhibition and in parallel activate IRF3 and IRF7. Then, the transcription factors translocate to the nucleus and induce the expression of genes encoding IFNs and pro-inflammatory cytokines.

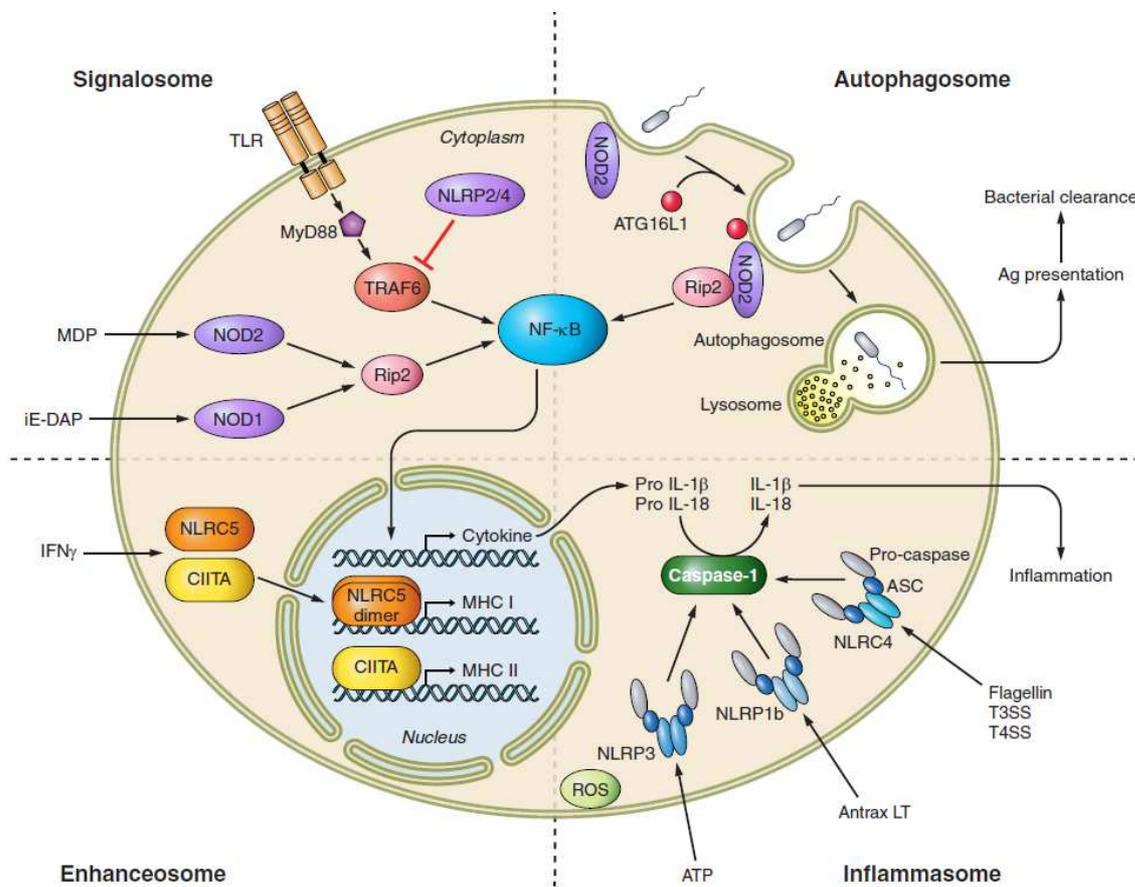
### 1.1.1.1.3 NOD-like receptors

NOD-like receptors are another family of PPRs, recognizing various PAMPs and DAMPs. In the structure of all proteins we can distinguish central NACHT domain (NAIP, CIITA, HET-E, and TP-2), implicated in dNTPase activity and oligomerization. Located at C-terminus, LRR domains are involved in sensing activators. NLR proteins depending on their N-terminal domain can be divided into four subfamilies (**Figure 1.4**). NLRA subfamily possess acid transactivation domain and includes only one member, CIITA performing a role of MHC-II transactivator. NLRB subfamily has the baculoviral inhibitory repeat-like (BIR) domain in its N-terminus and is represented by only one protein, NAIP. NLRC subgroup displays the caspase recruitment and activation domain and comprises six different members: NOD1, NOD2, NLRC3, NLRC4, NLRC5 and NLRX1. Finally, NLRP subfamily includes fourteen proteins named NLRP1 -14.



**Figure 1.4. Protein structure of NLR receptor family.** Depending on the N-terminus domain type, NLR proteins can be divided into four subfamilies. NLRA, NLRB, NLRC and NLRP subfamilies possess, respectively, AD domain, BIR domain, CARD domain and PYD domain. All proteins exhibit in their central part NATCH domain (NAIP, CIITA, HET-E, and TP-2) (Kim *et al.*, 2016).

NLRs members are implicated in various cell mechanisms (**Figure 1.5**). NLRs can interfere with signal transduction, influencing NF- $\kappa$ B activity. NLRP/4 are negatively regulating NF- $\kappa$ B activation by inhibiting TRAF6 (Fontalba *et al.*, 2007). NOD1 sense Gram-negative molecules containing D-glutamyl-meso-diaminopimelic acid (iE-DAP) moiety (Girardin *et al.*, 2003a) and NOD2 recognize muramyl dipeptide (MDP) from both Gram-negative and Gram-positive bacteria (Girardin *et al.*, 2003b). Both activate NF- $\kappa$ B by interacting with receptor interacting protein 2 (Rip2). NOD2 is also implicated in autophagy induction by recruiting ATG16L1 proteins to the site of pathogen entry (Travassos *et al.*, 2010). NLRC5 and CIITA in response to IFN- $\gamma$  increase the production of MHC-I and MHC-II molecules, respectively, enhancing induction of adaptive immunity (Chang *et al.*, 1996; Staehli *et al.*, 2012).



**Figure 1.5. Overview of NLR functions.** NLR activities can be divided in four main categories: signaling, inflammasome formation, autophagy, and transcriptional activation. NOD1 and NOD2 are positive regulators of NF- $\kappa$ B signaling, while NLRP2/4 blocks NF- $\kappa$ B activation. NOD2 is also involved in autophagy process by localizing ATG16L1 at the pathogen entry site. NLRC5 and CIITA are implicated in the transcription of MHC-I and MHC-II molecules, respectively and influence induced adaptive immunity. NLRP1, NLRP3 and NLRP4 induce inflammasome assembly, resulting in production of active form of IL-1 $\beta$  and IL-18 (Motta *et al.*, 2015).

Another important role played by NLRs is the formation of inflammasomes, multimeric protein complexes activating caspase-1 (Davis *et al.*, 2011). Active caspase-1 is crucial for processing and maturation of IL-1 $\beta$  and IL-18 pro-inflammatory cytokines. Inflammasomes are formed with the help of NLRs members which serve as a support for interaction with pro-caspase-1. Among the four identified inflammasomes types three are regulated by NLR proteins, NLRP1, NLRP3 and NLRC4, with the remaining one regulated by absent in melanoma (AIM-2) protein, a cytosolic sensor of foreign dsDNA. NLRP1 recognizes MDP and anthrax lethal factor. NLRP3, one of the best characterized members of NLRs senses different PAMPs derived from viruses (e.g. RNA), bacteria, fungi and protozoa (Allen *et al.*, 2009). It also detects endogenous components like ATP, hyaluronan, monosodium urate crystals, and  $\beta$ -amyloid. NLRC4 detects flagellin and bacteria like *P. aeruginosa* or *Shigella flexneri*. Inflammasome formation is initiated by NLRs detecting its PAMP or other activators, which induces oligomerization of NLR proteins. Bridging of the adaptor protein ASC allows to bind pro-caspase-1 and fully activate inflammasome. Recent studies report the formation of inflammasomes based on NLRP6, NLRP7, and NLR12, which could lead to inflammatory disease and tumorigenesis (Rubino *et al.*, 2012).

#### **1.1.1.2 Effector mechanisms of innate immunity**

The inflammation process, triggered mostly by macrophages, leucocytes, mast cells and, less frequently, by endothelial non-professional immune cells is one of the main elements of innate defense. The innate immune system contributes to the development of inflammation through sensing of PAMPs or DAMPs (danger-associated molecular patterns) upon, respectively, microbial infection or tissue damage (Akira *et al.*, 2006). Depending on the type of activated PRRs, different genes are upregulated, encoding pro-inflammatory cytokines (IL-1, IL-6 and tumor necrosis factor (TNF)), IFN-I, chemokines or antimicrobial proteins. This leads to increase in vascular endothelial permeability, recruitment of blood cells to the inflamed tissues and production of acute phase proteins, all aiming at pathogen or tissue damage elimination (Medzhitov, 2008). Some of the recruited blood cells, such as neutrophils or macrophages, are able to engulf encountered microbes and kill them in phagolysosomes.

Innate immunity is also implicated in the activation of acquired immunity. Microbe sensing by PRRs expressed by antigen presenting cells (APCs), such as DCs, leads to the activation of adaptive immune responses through upregulation of costimulatory molecules (CD80 and CD86) and stimulation of pro-inflammatory cytokine production. Stimulation of particular PRRs allows APC to

determine the antigen origin and the type of encountered infection, and results in skewing the lymphocyte development into the appropriate effector subpopulation (Akira *et al.*, 2006; Iwasaki and Medzhitow, 2015).

### **1.1.2 Antigen presentation**

The initial recognition of a pathogen by previously described innate immunity elements is a crucial but not always sufficient event in eliminating the threat and re-establishing homeostasis. Pivotal role in linking innate immune response with an adaptive one is played by APCs, able to display a fragment of detected antigen, called epitope, to T lymphocytes. Professional APCs include macrophages, B cells, some activated epithelial cells and most importantly DCs, playing critical role in naïve T cell activation, called priming. Antigen presentation can occur in two distinct pathways, described briefly as class-I and class-II presentation, depending on the epitope source and the type of involved major histocompatibility complex (MHC) molecule. Additionally, another process of antigen presentation was discovered, described as cross-presentation. For more detailed description of the processes presented below, see Rock *et al.*, 2004; Blum *et al.*, 2013 and Vyas *et al.*, 2008.

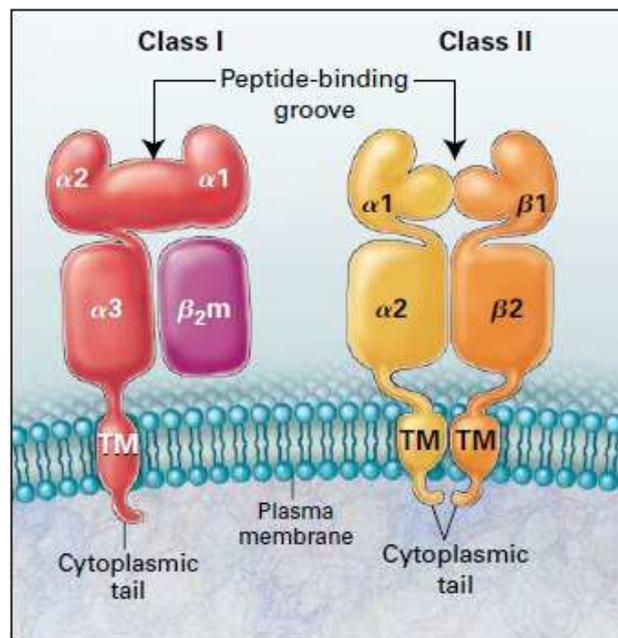
#### **1.1.2.1 Major Histocompatibility Complex**

The major histocompatibility complex (MHC) is a set of genes, encoding molecules originally identified as a major factors in graft rejection. Its role has later been broadened, as it has been discovered to be strongly involved in the process of mounting an adaptive immunity. More precisely, MHC molecules play a crucial role in presenting epitopes to T cells, which in contrast to B cells are unable to recognize native antigens.

In humans, MHC, described also as Human Leukocyte Antigen (HLA) system, is encoded on the chromosome 6 and comprises more than 200 genes. Among them the most important are those encoding HLA molecules of class I and II, involved in antigen presentation, a process described in details in the next paragraph. The region encoding HLA system is considered to be the most polymorphic in our genome. Indeed, alternative forms and variants of HLA molecules have been identified at stable frequencies in the population, and certain loci display as many as 250 alleles. This polymorphism in parallel with co-dominant expression of all single gene variants encoded in a genome ensures the ability to present a wide array of epitopes, providing protection from quickly mutating pathogens.

### 1.1.2.1.1 Class I MHC molecule

MHC class I molecules (MHC-I) are crucial in the activation of CD8<sup>+</sup> T cells. By binding class-I epitopes in their groove and subsequently interacting with TCR, they activate the specific CD8<sup>+</sup> T lymphocytes. MHC-I are expressed by many somatic cells, however their expression level differs between tissues. MHC-I molecule consists of two non-covalently linked polypeptide chains,  $\alpha$  chain and a  $\beta_2$ -microglobulin subunit ( $\beta_2m$ ) encoded outside the HLA locus. C-terminus of  $\alpha$  chain resides inside the cytoplasm and, after crossing the cell membrane,  $\alpha$  chain displays three-quarters of its length on the cell surface (**Figure 1.6**). The extracellular N-terminal fragment is organized in three domains,  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ . MHC-I molecule variability is located in  $\alpha_1$  and  $\alpha_2$ , which form a platform for peptide binding. This peptide binding groove, closed at both ends, accommodates peptides of 8-10aa in length. The conserved  $\alpha_3$  domain is responsible for binding CD8 molecule on T lymphocyte and for non-covalent interaction with  $\beta_2$ -microglobulin. The tethering of  $\alpha_3$  to  $\beta_2$ -microglobulin is stabilized by peptide anchoring to  $\alpha_1$  and  $\alpha_2$  and vice-versa, and so MHC-I reaches its full stability in the presence of all these three components.



**Figure 1.6. Structure of HLA Class I and Class II molecules.** In the structure of MHC class I molecule we distinguish  $\alpha$ -chain with two peptide-binding domains ( $\alpha_1$  and  $\alpha_2$ ) as well as  $\alpha_3$  domain, transmembrane domain (TM) and cytoplasmic tail. The beta<sub>2</sub>-microglobulin ( $\beta_2m$ ) constitutes the second chain. MHC class II molecule is formed from two chains,  $\alpha$  and  $\beta$ , both composed of two extracellular segments, transmembrane (TM) and cytoplasmic tail. Domains  $\alpha_1$  and  $\beta_1$  are forming peptide binding groove (Kein, 2000).

There are around 20 class-I genes in the HLA region. Three genes encoding  $\alpha$  chain, HLA-A, HLA-B and HLA-C were described as classical and are the most important due to their role in the adaptive response. They are present in two different copies in heterozygous individual and their codominant expression result in the presence of six different MHC-I molecules on the surface of one cell. In murine genome, HLA-A, HLA-B and HLA-C have their corresponding genes named H-2K, H-2D and H-2L.

#### **1.1.2.1.2 Class II MHC molecule**

MHC class II molecules (MHC-II) bind class-II epitopes and present them to  $CD4^+$  T cell. Their expression is limited to cells of the immune system, including DCs, B cells, activated T cells, macrophages and thymic epithelial cells.

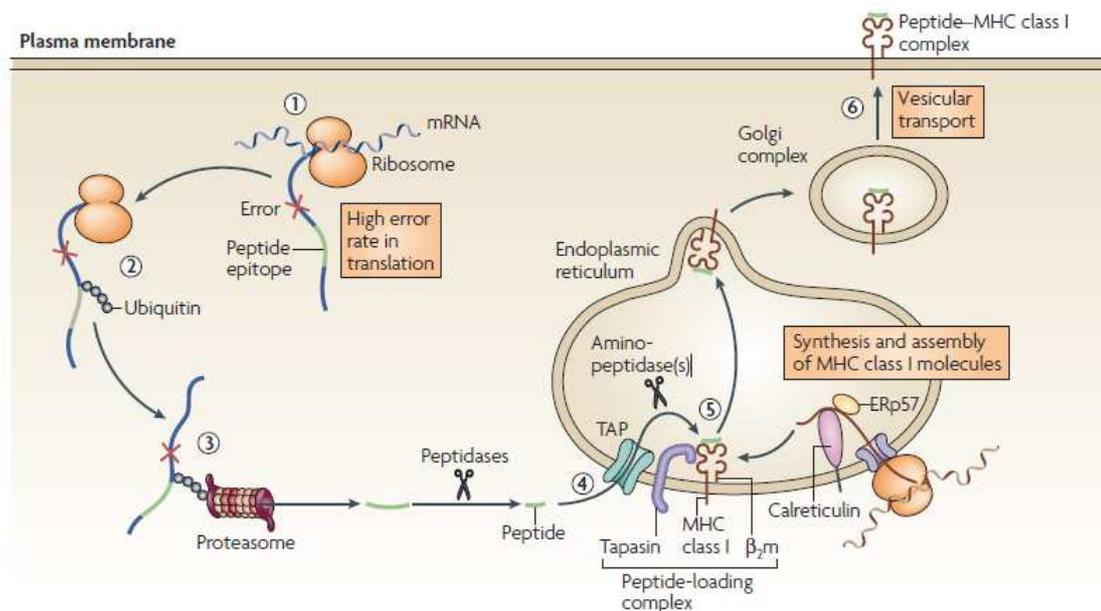
In MHC-II structure two chains can be distinguished,  $\alpha$  and  $\beta$ , both crossing the membrane with C-terminus inside the cell and with N-terminal end outside the cell surface (**Figure 1.6**). Both chains in their extracellular part are forming two domains.  $\alpha 2$  and  $\beta 2$  domains are conserved, with  $\beta 2$  involved in interaction with CD4 molecule on T cell. In contrast,  $\alpha 1$  and  $\beta 1$  domains are highly polymorphic and together form the peptide-binding groove. While peptide binding groove of MHC-I molecules is closed at both ends, the one of class-II is open and able to accommodate longer fragments, up to 30aa in length.

In humans, each parent provides its progeny with one allele of genes encoding  $\alpha$  chain (DP $\alpha$ 1, DQ $\alpha$ 1 and DR $\alpha$ 1) and of genes encoding  $\beta$  chain (DP $\beta$ 1, DQ $\beta$ 1 and DR $\beta$ 1) as well as duplicated DRB gene that can encode additional alleles (DRB3, DRB4 and DRB5). Thus each heterozygote inherits six or eight types of MHC-II. In mice, MHC-II molecules are called H-2A (I-A) and H-2E (I-E).

#### **1.1.2.2 Class-I antigen presentation**

In the frame of the class-I presentation, epitopes coming from intracellular proteins are presented on the cell surface (**Figure 1.7**). They can be of intracellular bacteria, virus or tumor origin, but may also emerge from degradation of the host intracellular proteins as part of the process of protein turnover. Short peptides, around 4-15aa are generated as the ubiquitinated proteins undergo proteasome cleavage in the cytosol. Less than 1% of these peptides is then transported into the endoplasmic reticulum (ER) lumen through Transporter associated with Antigen Processing (TAP), where it is loaded onto nascent MHC-I molecules. This step is coordinated by the so-called Peptide Loading Complex, consisting of tapasin and two housekeeping proteins found in ER, calreticulin and

ERp57. Upon peptide loading onto the MHC-I, its extremities can be additionally trimmed by amino-peptidases in order to fit within the binding groove. These events trigger its detachment from TAP and enables complex migration from ER through Golgi apparatus to the cell surface, where it can be recognized by specific CD8<sup>+</sup> T cell.

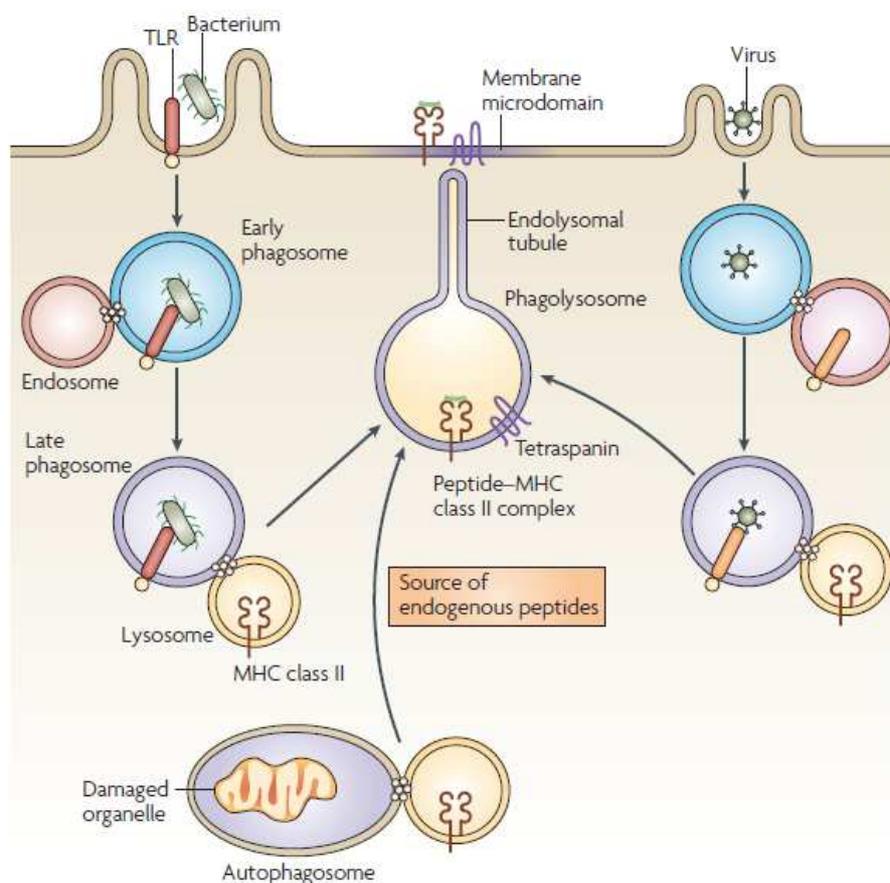


**Figure 1.7. Loading and trafficking of MHC class I molecules to the cell surface.** Presentation of the antigen on the MHC-I molecule occurs in six major steps. 1) Antigens are generated, e.g. as a result of translation error. 2) Ubiquitination process marks proteins for degradation. 3) Ubiquitinated proteins are cleaved in proteasome. 4) Longer fragments are additionally cleaved by peptidases and generated peptides are transported to the ER endoplasmic reticulum (ER) lumen by the transported associated with antigen processing (TAP) complex. 5) Peptide is loaded on the nascent MHC-I molecule with the help of tapasin, ERp57 and Calreticulin. 6) MHC-I with loaded peptide through Golgi complex are transported to the cell surface (Vyas *et al.*, 2008).

### 1.1.2.3 Class-II antigen presentation

MHC-II antigen presentation pathway focuses on scanning extracellular space and allows for presentation of peptides coming from e.g., extracellular parasites, dead cells or tumor cells. Antigen is uptaken by APCs and directed into the vesicular pathway consisting of progressively more acidic and proteolytically-active compartments: early endosomes, late endosomes and lysosomes. Internalized proteins are enzymatically degraded and digested into a set of peptides (**Figure 1.8**).

MHC-II molecules are synthesized in the ER, and trafficked with their binding site blocked by invariant chain ( $I_i$ ) to the endosomes, where they co-localize with digested extracellular proteins. Fusion of lysosome and MHC-II-containing vesicle results in degradation of  $I_i$ , release of residual amino acid chain called class II-associated invariant-chain peptide (CLIP) and peptide loading into the molecule cleft. The final length of trimmed peptide varies between 10 and 30aa. The proposed mechanism of MHC-II:peptide complex transport to the cell surface is thought to involve the transformation of compartments containing MHC-II into tubular structures, reaching site of  $CD4^+$  T cell interaction at the APC cell surface, where it can be found in clusters with tetraspanin-rich microdomains or lipid rafts (Hiltbold *et al.*, 2003).

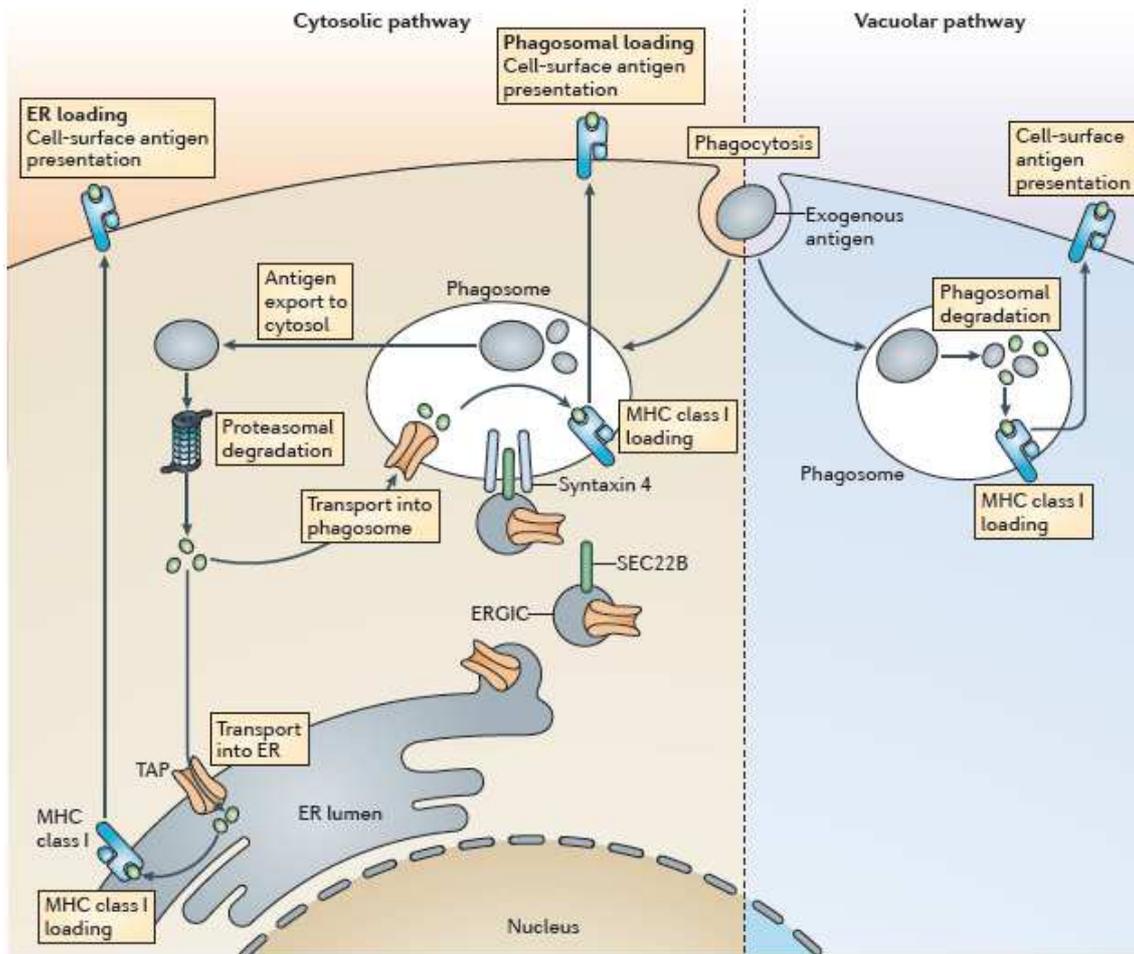


**Figure 1.8. Loading and trafficking of MHC class II molecules to the cell surface.** Upon pathogen engulfment, TLR becomes activated, dictating the nature of phagosome maturation. Phagosomes fuse with lysosomes which contain MHC-II. Proteases found in lysosomes cleave pathogen proteins, and generated peptides are then loaded into the MHC-II groove. Autophagosomes containing e.g. damaged organelle can also fuse with lysosomes to provide them with endogenous peptides for MHC class II presentation. MHC-II molecules with loaded peptide, along with other lysosomal proteins, such as tetraspanins, are transported to the cell surface through the endolysosomal tubules (Vyas *et al.*, 2008).

#### 1.1.2.4 Cross-presentation

MHC-I presentation serves as the reporter of intracellular environment, while MHC-II senses antigens present in the extracellular milieu. However, APCs are also able to present on MHC-I molecules peptides derived from exogenously acquired antigens. This process, called cross-presentation, was first described almost 40 years ago (Bevan, 1976). Important and dangerous events, such as viral infection or malignant transformation, occurring in non-professional immune cells need to be surveyed and detected by adaptive responses. Cross-presentation process ensures stimulation of CD8<sup>+</sup> T cells responding to pathogens which do not directly infect DCs. Although different non-professional immune cells, such as epithelial cells (Bagai *et al.*, 2005) were shown to be able to perform this process, the most potent cross-presenting cells *in vivo* are DCs (Jung *et al.*, 2002).

The exact mechanism of how exogenous antigens can enter the MHC-I maturation and peptide-loading pathway remains an intensively investigated subject. Many possible pathways of cross-presentation have been proposed (**Figure 1.9**). One of them, called “cytosolic”, suggests antigen processing in the endocytic vesicles and/or in the cytosol upon leaving the vesicular pathway, allowing generated peptides to gain access to the MHC-I in the ER. Alternatively, TAP and MHC-I loading complex would be recruited to phagosomes or endosomes and peptide loading could take place directly in these endocytic compartments (Guermónprez *et al.*, 2003, Houde *et al.*, 2003). This is ensured by interaction of ER–Golgi intermediate compartment (ERGIC) associated protein, SEC22B with Syntaxin4 found on phagosomes. In a “vacuolar” scenario, on the other hand, both antigen processing and loading on MHC-I molecule occur in endocytic compartments. Nevertheless, the exact molecular regulations of cross-presentation remains to be fully elucidated.



**Figure 1.9. Intracellular pathways for cross-presentation in dendritic cells.** In the cytosolic model phagocytosed, exogenous antigens can be released into the cytosol, making them available for the proteasome degradation. Subsequently generated peptides may enter classical MHC-I antigen presentation pathway and be loaded on MHC-I molecules in the endoplasmic reticulum (ER). Alternatively, peptides can re-enter the phagosome in order to be loaded on MHC-I molecules. Protein called SNARE SEC22B, found in the ER–Golgi intermediate compartment (ERGIC) through its interaction with syntaxin 4 present on phagosomes, coordinates bridging the ER subcomponents containing transporter associated with antigen processing (TAP) to the phagosome. In the vacuolar pathway antigen does not enter the cytosol, instead it is degraded in the phagosome where it is then loaded on MHC-I molecule (Joffre, 2012).

### 1.1.3 Adaptive immunity

The adaptive response corresponds to a sophisticated and highly specific set of biological mechanisms, involving humoral and cellular responses. The mechanisms of adaptive immunity, in

contrast to innate immune responses, are highly specialized and able to provide immunological memory.

### 1.1.3.1 Cellular responses

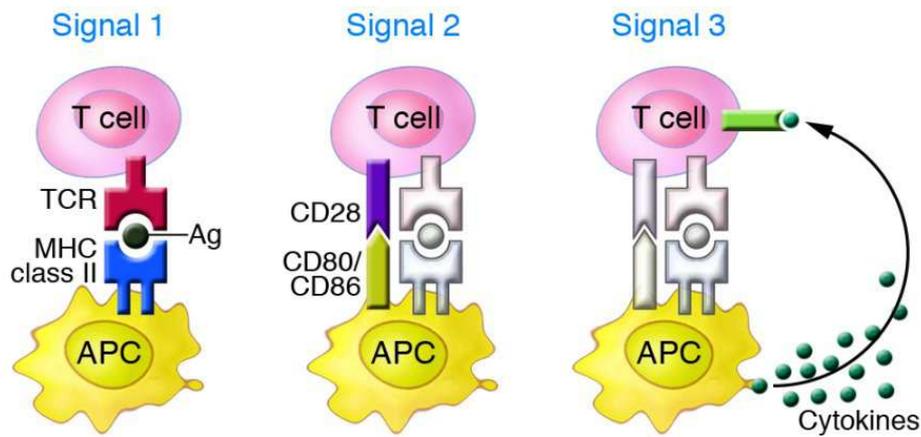
The cellular responses assure protections against pathogens residing and replicating within phagocytic or non-phagocytic cells. The most important cells are T lymphocytes.

#### 1.1.3.1.1 Development and activation of T lymphocytes

T cells originate from bone marrow but mature fully in the thymus. This primary lymphoid organ is a place where  $\alpha\beta$  T cells undergo positive selection, allowing for survival of MHC-binding lymphocytes. Subsequently, cells that avidly recognize self-structures are eliminated in the course of negative selection to prevent auto-immunity. After these checkpoints, lineage commitment takes place, directing the maturing T cells in two major subsets,  $CD4^+$  and  $CD8^+$ . The less frequent subpopulation of  $\gamma\delta$  T cells which derives from the same bone marrow precursor but matures in thymus as a separate lineage will not be discussed here.

Naïve T cells migrate to the secondary lymphoid organs, where upon encountering mature, epitope-presenting APCs they become activated. A few crucial elements are needed in order to fully activate T lymphocytes and induce their clonal expansion (**Figure 1.10**). The first one depends on T cell receptor (TCR) binding to a peptide-MHC molecule complex (MHC-I for  $CD8^+$  T cells and MHC-II for  $CD4^+$  T cells). A second signal comes from co-stimulatory signaling, assured e.g. by interaction of T cell CD28 receptor with B7-1 (CD80) and B7-2 (CD86), whose expression on DCs up-regulated upon response to PAMPs. This interaction between T cell and DC is localized to a specific zone called the immunological synapse, created by the surfaces of both cells and clustering all molecules involved in T cell activation (Huppa and Davis, 2003). The cytokines produced by DCs are the third important signal, providing stimulation for differentiation of epitope-specific clones. This results in rapid amplification of cells which will either become effectors, eliminated after antigen eradication, or will persist in the organism as a long lasting memory cells.

Worthy of note, the pivotal role of co-stimulatory molecules in cellular response induction and the influence of PRR signaling on the level of their expression highlights the need for a suitable adjuvant addition during vaccine development.



**Figure 1.10. T cell activation signals.** The first signal is the presentation of the peptide loaded on the MHC molecule and its recognition by the antigen-specific T cell receptor (TCR). The second signal is provided by interaction of co-stimulatory molecules present on the surface of APCs and T cells, e.g; such as CD28 and CD80/86. The last signal is provided by APC through the secretion of specific cytokines (Gutcher *et al.*, 2007).

#### 1.1.3.1.2 Effector functions of T-lymphocytes

- Cytotoxic CD8+ lymphocytes (CTL)

This subset is especially important in fighting intracellular pathogens and eliminating tumor cells. CTL are recognizing TCR-specific class-I epitopes, displayed on the surface of the target cells on MHC-I molecules. This leads to the production of effector cytokines by CTLs, among which the most important is IFN- $\gamma$ , playing a pivotal role in fighting intracellular microbes. Recognition of specific peptide-MHC-I complex results in the formation of a junction between the CTL and target cell. Through this membrane fusion cytotoxic molecules stored in CTL cytoplasmic granules, such as perforin and granzymes, are transported into target cells and trigger an apoptotic process. Apoptosis can be also induced by binding of FasL, expressed on the activated CTL to the Fas death receptor, expressed by many cells. After delivering the “lethal hit” the CTL is able to detach from the dying cell and act on another target expressing the same peptide-MHC-I complex.

- Helper CD4<sup>+</sup> T-lymphocytes

Helper T lymphocytes ( $T_h$ ) are important cells performing auxiliary functions and reinforcing performance of both humoral and cellular immunity. The type of stimuli presented in the early stages of immune response defines the subset in which CD4<sup>+</sup> T lymphocytes will differentiate:  $T_h1$ ,  $T_h2$ ,  $Th17$ ,  $T_{FH}$  or Treg (**Figure 1.11**). The distinction between these subsets is made based on their capabilities to produce cytokines (Magombedze *et al.*, 2013).

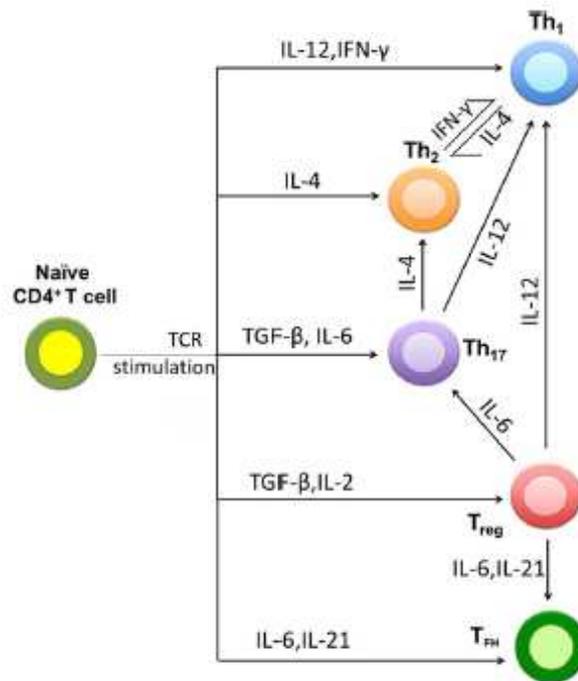
$T_H1$  cells are involved in fighting the microbes residing inside the phagosomes of the phagocytes. Since many types of pathogens developed mechanisms allowing them to persist and replicate inside these vesicles, innate immunity is not always sufficient to destroy them.  $T_H1$  cells, through their hallmark cytokines IFN- $\gamma$  and TNF- $\alpha$ , are able to activate the phagocytes and enhance microbe killing through stimulation of the synthesis of reactive oxygen intermediates and nitric oxide. They induce Ab switching into IgG2a and IgG2b class of antibodies (described below), involved in the phagocytosis and complement activation. Additionally, when the antigen does not stimulate potent innate immune responses (low level of co-stimulatory molecule expression),  $T_H1$  cells might participate in CTL activation by providing the “second signal” through cytokine secretion or CD40-CD40L interaction with APC. This is particularly important in mounting immune response towards tumor cells or cells displaying viral antigens. In order to skew T cell differentiation into  $T_H1$  subset, APCs or other surrounding cells need to produce IFN- $\gamma$  and IL-12 cytokines (**Figure 1.11**).

Helminthic parasites are eliminated with the assistance of  $T_H2$  cells. This subpopulation develops upon production of IL-4 by APCs, and in turn produces IL-4, IL-5 and IL-13. This branch of protection is developed when the antigens causes persistent T cell stimulation but does not induce strong inflammation or macrophage activation.  $T_H2$  cells promote eosinophil/mast-cell mediated immune reactions and stimulate B cell switching to the production of IgG1 and IgE antibodies, able to opsonize the helminths.  $T_H2$  axis also participates in immune responses to allergens.

Naïve  $CD4^+$  T cells differentiate into IL-17 and IL-22 producing Th17 subset in the presence of IL-6 and transforming growth factor (TGF)- $\beta$ . The main function of Th17 T cells to fight extracellular bacteria and fungi, mainly by neutrophil recruitment. Strong evidence also implicates Th17 lineage involvement in several autoimmune disorders.

IL-6 and IL-21 favor the differentiation of follicular helper T cells ( $T_{FH}$ ). This T cell subset plays a pivotal role in humoral immunity by providing help in germinal center formation, differentiation of B cells into plasma and memory cells, and contributes to establishing long-lived memory humoral responses.

Finally, under the influence of TGF- $\beta$  and IL-2 regulatory T lymphocytes (Treg) can develop from naïve  $CD4^+$  T cells (Magombedze *et al.*, 2013). By producing anti-inflammatory cytokines (TGF- $\beta$  and IL-10), they limit the magnitude of immune responses and contribute to immune homeostasis maintenance.



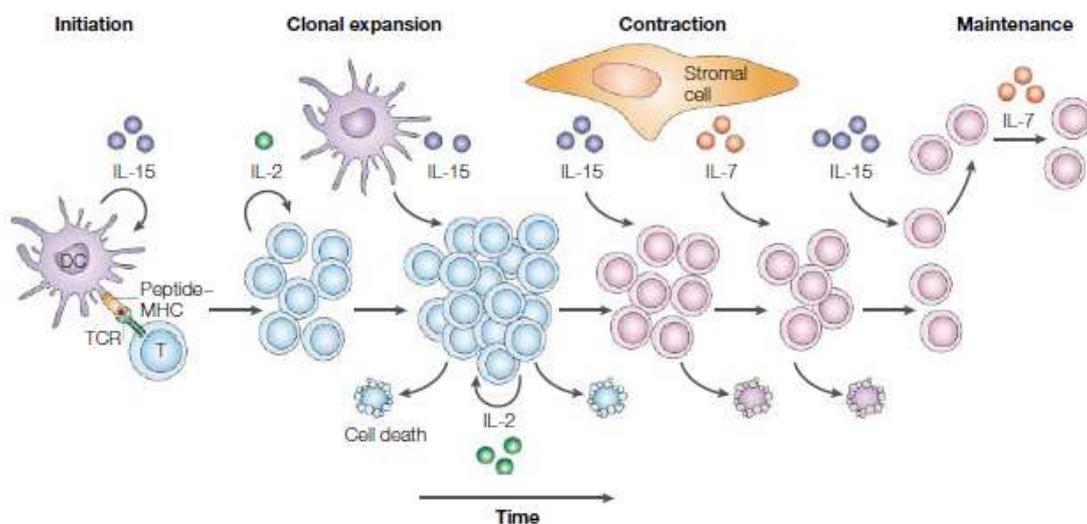
**Figure 1.11. Pathways of CD4<sup>+</sup> T lymphocyte lineage development.** Naïve CD4<sup>+</sup> T cells, depending on the type of cytokines present in the environment, can differentiate into various subsets. Cell plasticity allows generated CD4<sup>+</sup> to convert from one type into another (Magombedze *et al.*, 2013).

### 1.1.3.1.3 Memory T cells

As soon as the threat is eradicated from the organism, the majority of effector cells undergo apoptosis in the so-called contraction phase. A small part of the responding cells, however, is able to persist and become long-lived memory cells (**Figure 1.12**) (Gasper *et al.*, 2015). Thanks to the IL-7 and/or IL-15 cytokines, memory cells are homeostatically maintained, poised to respond rapidly to secondary infection (Schluns *et al.*, 2003). The population of memory T cells is heterogenous, as it comprises CD4<sup>+</sup> and CD8<sup>+</sup> T cells on different stages of differentiation. Depending on their function and homing preferences they can be subdivided into T central memory cells (T<sub>CM</sub>) and T effector memory cells (T<sub>EM</sub>). In mice these two populations can be distinguished by different levels of CD62L and CD127 expression, with the CD127<sup>+</sup> CD62L<sup>+</sup> phenotype corresponding to T<sub>CM</sub> and T<sub>EM</sub> characterized by CD127<sup>+</sup> CD62L<sup>-</sup>. Additionally, T<sub>EM</sub> express CD44 on their surface which allows these cells to be retained in the extravascular sites where they are more prone to encounter an antigen. Another molecule allowing to distinguish between T<sub>CM</sub> and T<sub>EM</sub> is C-C chemokine receptor type 7 (CCR7). It is expressed at high levels on the surface of T<sub>CM</sub>, mediating their homing to the secondary lymphoid organs where they reside and function as a source of effector cells. They possess

intrinsic proliferative capacity that is activated upon antigen challenge but exhibit limited effector functions.  $T_{EM}$  are lacking CCR7 molecule, and thus can be found in peripheral tissues, ready to produce cytokines such as IFN- $\gamma$  upon antigen stimulation. Having low proliferation rate, they are considered to be responsible for a quick initial response, but the full eradication of secondary antigen exposure relies most probably on  $T_{CM}$ . Thus, both populations provide important foundation for vaccine-induced immunity.

More recently, another subset of memory T cells has been discovered, named tissue-resident memory T cells ( $T_{RM}$ ) (for a review see Schenkel and Masopus, 2016). This lineage is found in non-lymphoid tissues but not in peripheral blood and it is thought to derive from effector T cells that remained poised in the peripheral tissues.  $T_{RM}$  are  $CD62L^- CD127^+$  and often display increased expression of CD103 and CD69, allowing for their retention in tissues. However, the phenotype of  $T_{RM}$  differs depending on their location and thus the tissues of residence play an important role in shaping  $T_{RM}$  signature, governing further changes in expression levels of surface molecules. The mechanisms of  $T_{RM}$  establishment and maintenance are not clear and remain to be fully investigated, but most probably, they are tissue-dependent.



**Figure 1.12. Cytokine effect on T cell proliferation and memory cell generation.** Different cytokines are crucial for T cell survival and development at different stages of the immune response. During T cell activation, interleukin-15 (IL-15) is important in order to activate dendritic-cells (DCs). Upon priming, T cell clonal expansion is moderated by the presence of IL-2 and this process can be additionally enhanced by IL-15. In the late stage of clonal expansion, during the contraction phase, IL-2 can also induce T cell death. At this stage, however, IL-15 and IL-7 might prevent death of a part of T cell population which will generate memory population. Survival of those memory T cells is enabled by low level of their proliferation, ensured by IL-15. IL-7 is also implicated in sustaining the T cell memory by favoring its survival (Schluns *et al.*, 2003).

### **1.1.3.2 Humoral responses**

The physiological functions of this branch of the adaptive response are mediated by antibodies (Abs). Humoral immunity is responsible mainly for eliminating extracellular microbes. Nevertheless, viruses before entering host cell or after being released from infected cell can also be neutralized along with produced virulent factors.

#### **1.1.3.2.1 Maturation and activation of B-lymphocytes**

B-lymphocytes originate from bone marrow and then populate peripheral lymphoid tissues. In a resting state, before encountering the antigen, they express on their surface immunoglobulin (Ig) IgM and IgD, performing the function of B cell receptors (BCRs). These molecules recognize specific portions of antigen, called B cell epitopes which vary significantly in length and can either be linear or conformational (Batista and Harwood, 2010). B cell stimulation by CD4<sup>+</sup> T lymphocyte is required for mounting humoral response against antigens of protein origins, which are therefore described as T-dependent or thymus-dependent antigens. After engulfing this type of antigen, B cell functions as APC, displaying the peptides on its MHC-II to T<sub>h</sub> cell. On the contrary, multivalent antigens such as polysaccharides and lipids do not require T cell stimulation, as cross-linking of BCR is sufficient to activate B lymphocytes. Analogically, we describe these antigens as T-independent. Upon B cell activation plasma cells are generated, secreting huge amounts of Abs which correspond to BCRs deprived of membrane-anchoring domain.

#### **1.1.3.2.2 Properties and effector functions of antibodies**

Abs are proteins secreted by activated B cells, called plasma cells. In their Y-shaped structure we can distinguish Fc constant region, involved in various Ab-mediated immune responses and Fab region, extremely variable and responsible for antigen binding. Each immunoglobulin (Ig) is build up from two heavy chains and two light chains and all of them consisted of constant and variable part. The constant part of heavy chains are encoded in five different variants in the genome, and its type defines the Ig class, i.e. IgM, IgD, IgG, IgA and IgE. Naïve B cells express BCR in the form of IgM and IgD only. In case of T-dependent antigens, CD4<sup>+</sup> T cells interact with B cells through CD40-CD40L axis and induce isotype class swiching. The type of heavy chain constant region determines the biological function of Ig and depends strictly on the type of cytokine secreted by helper T cells. IgM are secreted in primary response and are able to activate complement. During secondary responses the

most abundant Ab are IgG. They participate in phagocyte responses and are the only Ab which could cross placenta. IgG can be further divided in sub-classes, in mice named IgG1, IgG2a, IgG2b, IgG2c and IgG3 and in human described as IgG1, IgG2, IgG3 and IgG4 . IgA are associated with mucosal immunity while IgE participate in allergic reaction and immunity against helminths. In mice, in the context of  $T_h1$  stimulation, IgG2a and IgG3 Abs are mostly detected, while  $T_h2$  dictated switching to IgG1 and IgE, which complements effector functions of these responses. Antibody affinity maturation is also assured by helper T lymphocytes, more precisely by  $T_{FH}$  subset. In this important process, B cells undergo somatic hypermutations in their Ig genes and subsequently enter clonal selection phase, where with the help of follicular dendritic cells and  $T_{FH}$  cells B cell clones displaying the biggest affinity for the epitope will survive and expand.

#### **1.1.3.2.3 Memory B lymphocytes**

As a result of clonal expansion, some progeny of activated B cells acquire the ability to survive a long time. These memory B cells reside in lymphoid organs or recirculate between spleen and lymph nodes and are capable of mounting rapid and strong responses during subsequent antigen encounter. This process is  $CD4^+$  T cell dependent, as T-independent antigens do not induce immune memory. Owing to this memory B cell population, the secondary humoral response surpasses the primary one qualitatively and quantitatively.

## 1.2 Principle and purpose of vaccination

Vaccination is considered to be the biggest success in the history of Public Health. During the last century it has resulted in increased control over many major infectious diseases, such as diphtheria, tetanus, yellow fever, pertussis, flu, measles, mumps, rubella, typhoid and rabies. Most remarkably, vaccine against smallpox led to the eradication of this virus, and in the case of poliomyelitis the disease occurrence dropped by 99% due to the discovery of two effective vaccines (Salk and Salk; 1984; Sabin, 1991).

The principle concept of vaccination comprises induction of a protective immune memory and neutralizing Abs against the disease-causing agent without inducing the disease. This protection should result in fast and effective response during the actual pathogen challenge, preventing the infection or, in case of cancer vaccine, counteracting tumor growth. An ideal vaccine should be effective, safe, easy to produce and able to provide protection for a population with different, immunogenetically heterogeneous individuals. To meet this expectation various techniques of vaccines design were applied, roughly divided into a classic vaccines and new generation vaccines, described in this section.

### 1.2.1 Classical vaccines

First attempts to induce protective immunity were based on administration of infected material to healthy individuals, a method known as “variolation”. This technique was used mostly in China, Middle East and Africa. The term “vaccination”, on the other hand, dates back to the end of XVIII century, when Edward Jenner observed that milkmaids exposed to cowpox were protected from smallpox disease (Plotkin, 2011). At that time little was known about the mechanisms of immune responses or the nature of infectious agents, limiting the progress in the area of vaccinology for more than a century after Jenner’s achievement. Brilliant works of researchers like Louis Pasteur or Robert Koch, who at the turn of the XIX and XX centuries started identifying the pathology-causing microorganisms, contributed significantly to further development in the area of vaccinology (**Figure 1.13**)



### **1.2.1.1 Live attenuated vaccines**

The development of cell culture methods allowing for pathogen propagation *ex vivo* gave rise to a new strategy of vaccine formulation. Iterative rounds of microbe propagation *in vitro* rendered them non-pathogenic, presumably due to mutations accumulated in their genome. Live microorganisms upon administration provided long antigenic stimulation, giving sufficient time for establishment of strong adaptive responses. The excellent immunogenicity of this type of vaccination was burdened with their reduced safety, as administration of attenuated vaccines is poised by the risk of pathogen reversion to its virulent form.

Live attenuated vaccines led to the formulation of e.g., yellow fever and tuberculosis vaccine in 1930s, measles, mumps, and rubella vaccines in 1960s (Hilleman, 1968) and during the following decade gave rise to the protection against zoster virus, Japanese encephalitis virus and tick-borne encephalitis virus. Attenuated vaccines led to the biggest successes in the vaccination field i.e. the eradication of smallpox and significant reduction of poliomyelitis prevalence (Sabin vaccine).

### **1.2.1.2 Inactivated vaccines**

This vaccination strategy developed in 1890s, when typhoid and cholera bacilli vaccines became available (Plotkin, 2014). Microorganisms were inactivated with physical methods, such as heat and UV or with the use of chemicals. It was crucial not to destroy protein structure and integrity in the process of inactivation, as this would lead to a loss of antigenicity. Blocking microbe ability to efficiently infect host cell, replicate, produce their antigens etc., resulted in reduced immunogenicity vis-à-vis live attenuated vaccines. As a result, repeated administrations were required to trigger good protection level. Nonetheless, alterations in pathogen virulence assured better safety profile.

Inactivation was applied for the development of vaccines against *Bordetella pertussis*, Influenza virus, *Rickettsia*, Poliovirus (Salk vaccine), Rabies virus or Hepatitis A virus.

## **1.2.2 New approaches of vaccination**

Conventional vaccination demonstrated its efficacy for many decades and deserves full acknowledgement for huge contribution to the Public Health. Nevertheless, a few drawbacks were associated with its usage, such as unnecessary antigenic load resulting potentially in reactogenic or allergenic responses. The development of various laboratory techniques along with the rise of

“Reverse vaccinology” concept allowed for high-throughput screening of potential vaccine candidates (Donati and Rappuoli, 2013). It became possible to dissect the pathogen and select and administer only the specific target of natural anti-pathogen immune responses. In order to assure an efficient protection against a given infectious agent, it was crucial to first identify the most relevant immunity components counteracting this pathogen. High affinity Abs are essential to neutralize many extracellular pathogens and their toxins. Eradication of intracellular microbes and cancer cells, on the other hand, relies mostly on the cytotoxic activity of CD8<sup>+</sup> T cells. Understanding the direct mechanisms implicated in threat eradication facilitated the choice of optimal antigens that should be included in vaccine formulation. This could be the entire protein or polysaccharide (subunit vaccine), or just a peptide of interest containing a particular epitope (peptide vaccine). Other modern vaccination techniques are based on DNA plasmids (DNA vaccine) or viral/bacterial vectors serving as vaccine platforms (vectored recombinant vaccines). The main outlines and features of these new techniques of immunization are described below.

#### **1.2.2.1 Subunit vaccines**

The better understanding of anti-pathogen/anti-cancer immune responses and antigen structure allowed to determine the most immunogenic part of pathogens. This, along with the advances in the protein production and purification methods, enabled fractioning of desired antigen subcomponent from the remaining, unwanted material. Selected proteins or polysaccharides could alone provide a sufficient stimulation for mounting adaptive immune response. Decades of research precede the development of first, successful subunit vaccine. During influenza vaccine development solvents were used to disrupt the viral lipid envelope. More complex methods of eliminating biological impurities were introduced during pneumococci or pertussis vaccine design (Edwards and Decker, 2008). By eliminating the unwanted components of a pathogen, vaccines with reduced reactogenicity and improved safety profile were developed. The first vaccine against Hepatitis B virus (HBV) was formulated by purifying HBV surface antigen, HBsAg, from the plasma of infected individuals (Hilleman, 1983). Capsular polysaccharides from *Haemophilus influenzae*, meningococci and pneumococci were turned into conjugated subunit vaccines upon linking them to carrier proteins (Fitzwater *et al.*, 2012; Tan *et al.*, 2010). This improved significantly polysaccharide immunogenicity, since their ability stimulate B cells was enhanced by T cell recognition of class-II epitopes in the sequence of the carrier protein. Also, helper CD4<sup>+</sup> cells provided the stimulation pivotal in memory B cell development.

With the advances in genetic engineering it became possible to design recombinant subunit vaccines. Rather than purifying the desired antigen component, the gene of interest is introduced into bacterial, yeast, insect or mammalian cells and after large scale amplification can be purified in huge quantity. The most important example of this process is recombinant subunit vaccine against Hepatitis B virus. The surface antigen HBsAg is obtained in high yield production in yeast or mammalian cells. HBV vaccine combines three main progresses in vaccinology: first viral subunit vaccine, first vaccine preventing human cancer and first recombinant-expressed vaccine (Hilleman, 2000). Another successful achievement with this strategy is vaccine for Human Papilloma Virus (HPV), based on L1 coat protein able to assemble as virus-like particles (VLP), resembling capsid structure and deprived of any genetic material (Zhou *et al.*, 1991).

Of note, since these types of vaccines are based on highly purified molecules oftenly deprived of natural PAMPs, they require addition of suitable adjuvants in order to elicit an adaptive immune responses.

**Table 1.2. Examples of vaccine vectors.** Examples of various bacteria- and virus-based vectors are listed in the table, along with their main advantages and disadvantages (Liu, 2010).

Viruses		
Poxviruses	Licensed veterinary vaccine vector	Possible issue of pre-existing immunity for vaccinia (smallpox) vaccinees
	MVA known clinical safety	Probably will need prime, increasing complexity and cost
	Room for large gene insert	
	Various strains available	
Adenovirus	Historic oral adenovirus vaccine	Pre-existing immunity to certain human strains may not preclude use as vectors, but results of HIV STEP trial need to be understood re: safety for high-titered vaccinees relative to subsequent risk of HIV infection if exposed
	Strong immune responses	Human Ad strains oncogenic in animals
	Many strains available	
Adeno-associated virus	Apparent efficacy as vector in small gene therapy trial for eye disease <sup>a</sup>	Disputed risk of integration
	Safety shown in small gene-Rx trials	Limited gene insert capacity
Alphaviruses	Virus particles or DNA replicons	Modest gene capacity
	High expression	
	No risk of integration because RNA particle can target DCs	
Herpes virus	Broad tropism including DCs	Neurotropism may be safety issue
		Possible pre-existing immunity
Measles virus	Mucosal delivery possible	Possible pre-existing immunity
	RNA virus (no integration issue)	
Vesicular stomatitis virus	Broad tropism including DCs	Neurotropism <sup>b</sup> may be safety issue
	High expression	
Bacteria		
Salmonella/Shigella	Oral delivery	Concern about heterologous gene if on plasmid
	Infect M cells (gut antigen uptake cells)	Potential stability of antigen gene if on plasmid
BCG	Extensive safety of BCG vaccine	Bacterial (versus mammalian) posttranslational modifications
Listeria	Efficient mechanisms for CTL induction	Concerns re: vector virulence in immunocompromised

### 1.2.2.2 Recombinant vaccines

Recombinant vaccines rely on genetic engineering methods, allowing to tailor and produce the antigen of interest. With the use of sophisticated techniques of molecular cloning or directed mutagenesis it became possible to suppress pathogen virulence in a more durable manner. After deleting or inactivating genes responsible for microbe pathogenicity, reverting to the virulent form upon *in vivo* administration became virtually impossible. This resulted in the development of vaccines with significantly improved safety compared to corresponding live attenuated vaccines.

These attenuated viruses or bacteria could serve also as vectors carrying genetic information for a heterologous antigen. By introducing genes of interest into vector genomes, the antigenic protein is produced by a recombinant bacteria or, in case of viral vehicle, expression takes place inside the infected cells. Viral recombinant vaccines are most often based on poxvirus, adenovirus, retrovirus or adeno-associated virus. Poxviruses were among the first viruses to be genetically altered. One member of poxvirus family, Vaccinia virus, was modified to encode rabies virus glycoprotein and has been used for immunization of wild animals for almost 20 years (Hanlon *et al.*, 1998). Until today, progress in this vaccination field gave rise to vectors of various origin, encoding antigens from pathogens like HIV, dengue virus, *Plasmodium falciparum* or *Mycobacterium tuberculosis*. Clinical trials using adenoviruses as a vaccine platform will be described in 2.2.3. Even though they are less popular than the viral ones, bacterial vectors were also successfully used. They are based mostly on *Shigella*, *Salmonella* or *Listeria* (Liu, 2010). Another example is BCG, used as a vaccine against tuberculosis but also as a carrier of a heterologous antigen. Examples of vaccine vectors are listed in **Table 1.2**.

The appealing advantage of recombinant vaccines is the induction of innate immunity by PAMPs associated with the vector. This may allow to circumvent the addition of a supplementary adjuvant. However, few key points still need to be considered while applying this method of vaccination. One of them is the safety of used vectors, especially those based on retroviruses which can integrate into host genome, causing mutations. Another burning issue is immunity to the vaccine vector. Both pre-existing immunity and anti-vector response developed after the first administration hamper transgene expression and result in reduced efficacy of immunization. Methods to overcome anti-adenovirus immunity will be detailed in Chapter 2 of this introduction.

### 1.2.2.3 DNA vaccines

The observation that injection of DNA plasmid containing genes of interest results in their transcription and translation to proteins carried subsequently to APC in the bone marrow, laid a groundwork for development of DNA vaccines (Wolff *et al.*, 1990). Although the first results showed rather poor Abs production, later improvements in the DNA delivery method and addition of adjuvants significantly increased its efficacy (Donnelly *et al.*, 2005; Flingai, 2013). One of the firstly developed delivery techniques was DNA-coated golden particles, administered with the use of gene gun (Tang *et al.*, 1992). Later, electroporation turned out to be the most impactful enhancement in the area of DNA delivery. Applied electric current resulted in more efficient DNA capture by the cells and induced local inflammation, both contributing to stronger immune reaction. Various additional components were also introduced, enhancing the generated immune response. One strategy includes co-administration of immunomodulatory proteins, either encoded in a separate plasmid or as a gene co-expressed with the antigen on the same plasmid. These genes might be coding for pro-inflammatory cytokines (IL-12), IFN- $\gamma$ , or molecules mediating recruitment and activation of APCs (Xiang *et al.*, 1995; Sin *et al.*, 1998). Another strategy introduces genes of co-stimulatory and adhesion molecules, such as CD80 and CD86, in order to improve T cell activation (Tsuji *et al.*, 1997; Kim *et al.*, 1999).

DNA plasmids have a capacity to trigger Abs production and to prime T cell responses. DNA vaccine mimics the process of natural viral infection by delivering its genes to the host cell and leading to expression of encoded proteins. In case of direct DCs transfection, expressed class-I epitopes are presented on MHC-I molecule and prime CD8<sup>+</sup> cells (classical pathway of class-I antigen presentation). Alternatively, in the process of cross-presentation DCs can present CD8<sup>+</sup> epitopes obtained from extracellular milieu upon engulfing apoptotic or necrotic transfected cells or capturing secreted antigen protein. Transfection of both professional and non-professional immune cells result in release of the antigen, making it available for B cell detection (Flingai, 2013).

Within the past decade three DNA vaccines have been licensed for veterinary use: for treatment of West Nile virus in horses (Davis *et al.*, 2001), hematopoietic necrosis virus in salmon (Garver *et al.*, 2005) and melanoma in dogs (Bergman *et al.*, 2006). Human clinical trials were performed with HIV DNA vaccine adjuvanted with IL-12 and cellular responses were detected (Kalams *et al.*, 2012). DNA vaccination seems to be the most promising candidate for the priming step in heterologous prime-boost strategies. Despite all these advances, until today there is no DNA vaccine licensed for human use. Continuous refinements, however, bode well for this vaccination strategy.

#### 1.2.2.4 Peptide vaccines

Epitopes are specific fragments of macromolecules recognized by T cells or B cells and they serve as the main targets for adaptive immune responses. The blossom of “Reverse Vaccinology” provided an effective tools to identify, produce and purify these precise elements, reducing the antigen complexity to a single peptide fragment. Fragments which are selected during the vaccine formulation are usually around 10-30aa in length and comprise linear epitopes, whose immunogenicity does not depend on a three-dimensional structure of the protein. “*Epitope display*”, strategy of vaccination I evaluated during my thesis also belongs to this group of vaccines. It will be fully described in Chapter 3.

Selection of the epitope is the first and most important step in the formulation of a peptide vaccine, and depending on the epitope type different aspects needs to be considered. In case of B cell epitope, its 3D accessibility is crucial and its integrity needs to be preserved by the choice of an accurate delivery method. Ideally, its sequence should also comprise CD4<sup>+</sup> epitope, providing T helper stimulation, pivotal for affinity maturation and induction of memory B cells. As for the T cell epitope, efficient delivery to APCs should be considered. Also, the specificity of MHC molecules needs to be stressed, as e.g. peptide presented by HLA-A2 allele of MHC-I molecule might not be displayed in the context of HLA-A1 and vice-versa. Thus, prevalence of different MHC molecule alleles among individuals needs to be carefully considered in order to target various individuals in an immunogenetically heterogeneous population. Finally, both B cell and T cell epitopes should be highly conserved in the pathogen sequence. This is emphasized especially in the case of highly variable pathogens like HCV or HIV where existence of numerous virus genotypes, differing significantly in their sequence, accentuates the significance of the identification of a common epitope.

Various approaches employing *in silico* methods, experimental methods or a mix of both are undertaken during epitope search. One of the most powerful methods is algorithm motif prediction. With bioinformatics tools it became possible to propose potential epitopes based on parameters like peptide translocation to ER (MHC-I), cleavage in lysosomal compartments (MHC-II), peptide binding to MHC molecules or T cell receptor recognition. Methods allowing for epitope detection rely on X-ray crystallography and mass spectrometry (Hager-Braun and Tomer, 2005), use of phage display library (Panina-Bordignon *et al.*, 1989) and on databases regrouping known T cell epitopes with supplementary information about their MHC binding, HLA restriction, etc. Another frequently used approach is overlapping peptide library derived from the protein of interest. Pools of peptides (9-20aa in length) are generated and overlap to ensure presence of each possible epitope. This allows for the discovery of both CD4<sup>+</sup> and CD8<sup>+</sup> epitopes in the context of several alleles of MHC

molecules. Also, in the frame of Immunoproteomic, it became possible to directly identify MHC class I-presented epitopes extracted from infected cells. An impediment of this strategy is the difficulty of separating the infection/cancer-related peptides from the non-relevant ones, constituting the majority of the isolated set.

After carefully selecting the epitopes a suitable delivery method has to be chosen. Purified peptide is poorly immunogenic and needs to be administered in specially adapted form. Among the proposed strategies the most popular is embedding the peptide in an emulsion-based adjuvant. Stable emulsion obtained by vigorous mixing of water and oil phase ensures slow antigen release and prolonged stimulation. The most well-known example is Freund's complete adjuvant, containing killed mycobacteria. Other option is delivering peptides in phospholipid bilayer structures called liposomes. Their composition can be tailored in order to facilitate uptake by APCs, and adjuvants can be encapsulated along with the antigen (Joshi *et al.*, 2012). Studies showed induction of both humoral and cellular responses with these formulations (Nagata *et al.*, 2007; Kohyama *et al.*, 2009). Virosomes, liposomes carrying influenza envelope proteins have been developed and also provide increased antigen uptake by APC and immune cell activation. The use of Immunostimulatory complexes (ISCOMs) has been reported, composed of antigen, cholesterol, phospholipid and saponin. Polymeric particles like albumin or collagen can also serve as a support.

In the context of this work, peptide delivery by viral particles is of the biggest interest. Viral vectors have been already mentioned in the context of recombinant vaccines, where they were modified to encode the antigen of interest in their genome. With the tools of genetic engineering, it became possible to introduce epitope-encoding sequence inside the genes coding for capsid proteins. This resulted in recombinant viral vectors, displaying heterologous peptides on their capsid surface. The good immunogenicity of the viral vector performs an adjuvant role. Ease of manipulation is another quality of this method of vaccination. In our study we chose adenovirus as an epitope carrier due to its appealing features described in Chapter 2. Nevertheless, other viruses are also commonly used as a vaccine platform for heterologous peptides. Among them we can list AAVs (Manzano-Szalai *et al.*, 2014), influenza virus (Langley *et al.*, 2010) and human rhinovirus (Smith *et al.*, 1998).

Many peptide vaccines are currently being developed to protect against HIV (Liu *et al.*, 2007) malaria (Epstein *et al.*, 2007), HCV (Kolesanov *et al.*, 2013), influenza (Stanekova *et al.*, 2011), HPV (Solares *et al.*, 2011) or different types of cancer, like pancreatic or lung cancer (Bernhardt *et al.*, 2006; Brunsvig *et al.*, 2011) (**Table 1.3**). However, no peptide vaccine has reached the market yet.

**Table 1.3. Worldwide clinical trials of peptide vaccines from Li *et al.*, 2014.**

Peptide Vaccines Under Development		Clinical Indications for Candidate Peptide Vaccines Under Development
No. of Phase I Studies	270	Anticancer studies, Malaria, Falciparum Malaria, Anti-Plasmodium vivax, Influenza, Alzheimer's disease, Insulin dependent diabetes mellitus, Hand foot and mouth disease, anti HIV, HCV, HBV, CMV, Diabetes Mellitus, Type One, Cat allergy, Allergy
No. of Phase II Studies	224	Anticancer studies, anti HIV, HCV, HBV, CMV, Pneumococcal, genital Herpes—Herpes Simplex Type II, Tuberculosis, Diabetes, Diabetes Mellitus, Type One, Cat allergy, Ragweed allergy, Grass allergy, Ashtma, House dust mites - Rhinoconjunctivitis, maximum studies—anticancer
No. of Phase III Studies	12	Anti-cancer studies
No. of Phase IV Studies	NIL	No peptide vaccine reached market yet

### 1.2.2.5 Adjuvants

In the classical vaccination strategy the whole pathogen is administrated in order to induce protection. This provided organism with a set of various epitopes and PAMPs, usually sufficient to induce effective stimulation. Modern vaccines, based on highly selected and purified proteins or peptides offer better safety profile but can lead to insufficient stimulation of innate immunity, thus resulting in reduced adaptive immune responses. In order to overcome this problem additional components called adjuvants were introduced during vaccine formulation (**Figure 1.14**).

Adjuvants are crucial factors in mounting an effective immune protection, performing their function through various mechanisms. They can serve as the delivery system, enhancing the antigen uptake by APC and subsequently ensure its progressive release, slower clearance and better exposure to the Immune System. Adjuvants may also interfere at the different levels of adaptive response activation by affecting the PRRs stimulation, antigen presentation on MHC molecules or recognition of co-stimulatory signal (Leroux-Roels *et al.*, 2010). They contribute to broad Ab response, increasing its magnitude and functionality. By leading to more potent immune responses they allow for reduction of injection number in vaccination schedule. Adjuvants are of great importance in the vaccines for the elderly, who display reduced T cell populations. Additionally, as previously mentioned in chapter 1.1.1.2, the type of stimulated PRRs shapes adaptive response by directing naïve T cells into different maturation lineages. This gives an opportunity to control the nature of induced adaptive responses by selecting proper adjuvants. Noteworthy, in the vaccination regimens performed during my thesis, no additional adjuvants were required nor added, thanks to the remarkable Ad immunogenicity. This property will be explained in paragraph 2.2.2.

Historically, the most extensively used adjuvants were based on aluminium salts. For more than 80 years they were included in various vaccine formulations, albeit the exact mechanism of their mode of action remained elusive. Recent studies reported that antigens absorbed on the aluminium salts are better presented and more efficiently internalized by APCs (Morefield *et al.*, 2005). Also, aluminium salts have been shown to activate inflammasome complex components (Lambrecht *et al.*, 2009). One of their limitation is enhancement of Th<sub>2</sub> response and weak effect on Th<sub>1</sub>, limiting their performance in vaccines against viral pathogens or cancer. Emulsions, briefly mentioned in previous paragraph, are another choice of adjuvants. Complete Freund adjuvant, based on water-in-oil emulsion with embedded mycobacterium particles was too reactogenic to be used in humans. Refinement of used materials resulted in safer combinations, acceptable in therapeutic vaccines formulation. Oil-in-water emulsions have also been developed, with MF59, AS03 and AF03 being one of the most frequently selected. MF59 is included e.g. in seasonal influenza vaccine and has been shown to enhance the immune response in elderly (Podda, 2001). By inducing chemokine production it attracts monocytes and neutrophils to the immunization site, where they can take up the antigen (O'Hagan *et al.*, 2012). Liposomes and virosomes, also mentioned in 1.2.2.4, are another type of frequently used adjuvants. They improve induction of immune response by increasing the level of antigen presentation (Ambrosch *et al.*, 2007). Another type of delivery vehicles mentioned in 1.2.2.4, ISCOMs, also has adjuvantic properties (Pearse, 2005).

Among the novel adjuvants TLRs agonists are a promising example. MPL, TLR-4 antagonist based on LPS, is one of the leading immunostimulants, included e.g; in the Cervarix<sup>®</sup> HPV vaccine (from GSK). TLR9 ligands, such as CpG oligodeoxynucleotide (ODN) are also currently used. Different example of immunomodulatory molecules are plant-derived saponins which originally were used only in veterinary vaccination. After reducing their reactogenicity thanks to the improved purify they are now used in human vaccine trials. It has been showed that addition of MPL to purified saponin from *Quillaja saponaria* (QS21) helps to mount strong humoral and cellular immune responses against hepatitis B surface antigen (Leroux-Roels *et al.*, 2015).

Recent data shows that choice of both antigen and adjuvant are equally important in a successful vaccine design. Adjuvant performance was shown to depend on administration route. Also, different formulations of the same adjuvant may induce substantially different immune responses. By carefully selecting and/or combining different adjuvants, enhanced immunity can be induced, improving the vaccination outcome.

## 2 Adenovirus and their use in vaccination

### 2.1 Adenovirus

Ad are a non-enveloped, double stranded DNA viruses isolated for the first time over 50 years ago from human adenoid cells (Rowe, 1953). Since their discovery adenoviruses have been detected in various animals, and depending on the host type they have been classified into four groups: Mastadenovirus (mammals), Atadenovirus (ruminants and reptiles), Aviadenovirus (birds), Siadenovirus (frogs and fish) (Davison *et al.*, 2003). Adenovirus infecting humans belong to the Mastadenovirus group and can be further divided into seven species named A-G. Almost 70 human serotypes have been distinguished until today (Ghebremedhin; 2014), with serotype 2 and 5 from group C being the most intensively studied and used in gene therapy and vaccination studies. All of the serotypes can cause rather mild pathologies, such as cold or diarrhea but in rare cases Ad infection can lead to more severe disorders like epidemic keratoconjunctivitis or respiratory infections (Monto, 1992).

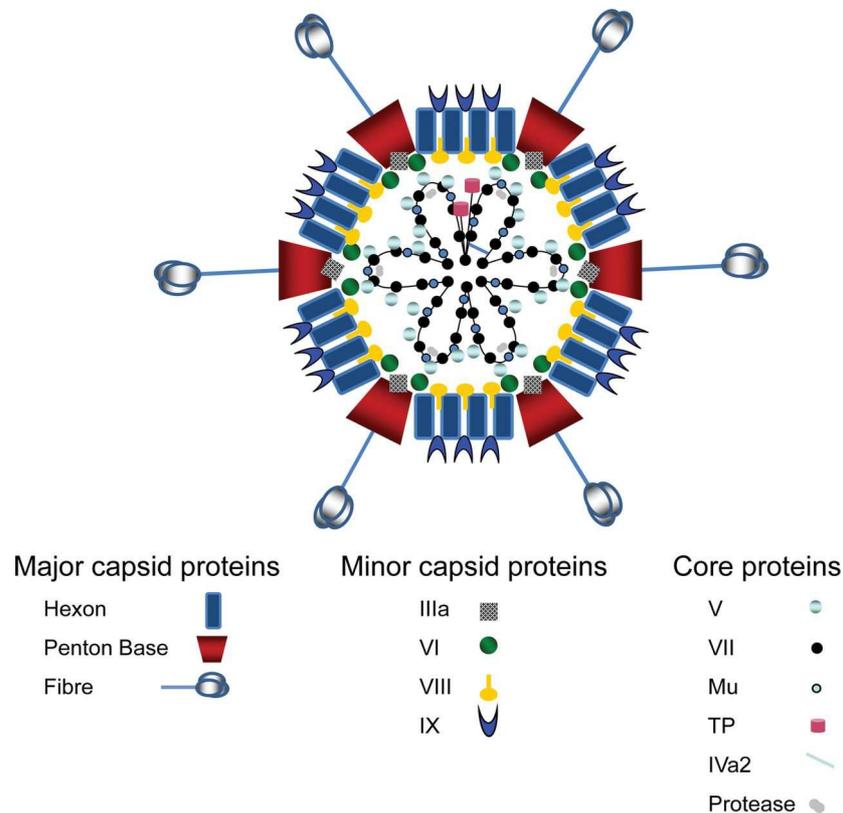
#### 2.1.1 Proteins and capsid structure

Ad capsid, the protein shell enclosing the viral genome, has diameter of ~95 nm and it is composed of thirteen structural proteins, identified in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Maizel *et al.*, 1968; Ginsberg *et al.*, 1966). Various experiments such as stoichiometric analysis (van Oostrum and Burnett, 1985) or electron microscopy (Horne *et al.*, 1959; Valentine and Pereira, 1965) permitted to look closer at the geometry and architecture of Ad particle. The trimeric hexon protein, present in a total copy of 240 units organized in 20 triangular facets, is the most abundant element of the icosahedrally shaped capsid. Pentameric penton base in non-covalent interaction with protruding trimeric fiber generates a complex called penton, located at all 12 vertices of the capsid. Remaining minor components such as proteins IIIa, VI, VIII and IX are stabilizing the architecture of the viral particle, acting as a “molecular glue” (**figure 2.1**).

The viral capsid encloses a viral core, a tightly packed viral DNA in complex with DNA binding viral proteins: core proteins, protein VII, protein X also described as  $\mu$ , the maturation protease AVP and the terminal protein (TP) (Mangel and San Martin, 2014). Protein VII is responsible for DNA condensation. Although the exact organization of the viral core is not clear, some studies reported that it creates nucleosome-like beads which allow to pack the DNA inside the capsid (Vayda *et al.*, 1983). TP protein by fixing the 5' DNA extremities protects them from degradation (Rekosh *et*

*al.*, 1977). Protein V connects viral core with the capsid by interacting with penton proteins and, through IIIA and VI proteins, with hexon proteins.

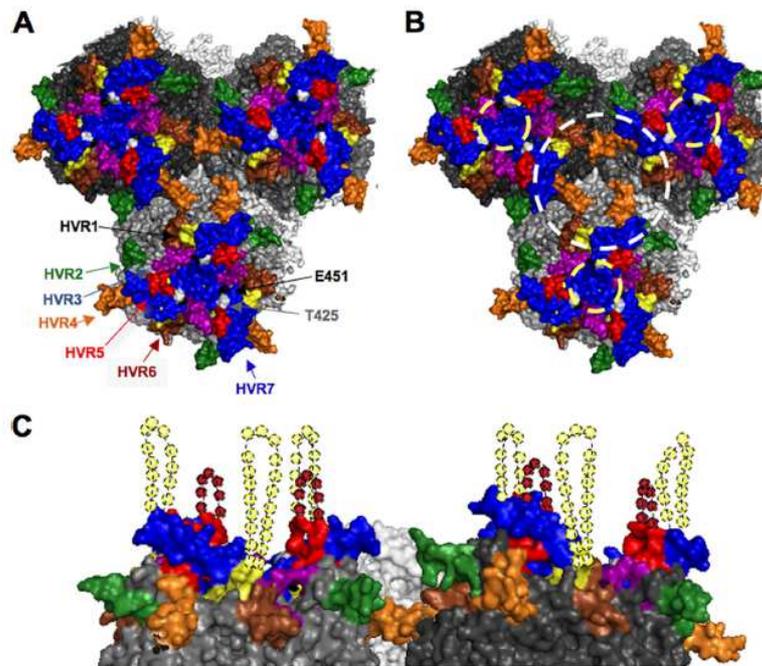
Of note, the majority of studies investigating the structure of the Ad capsid focused on viral particles of serotype 2 and 5, and thus descriptions of the capsid proteins found in these chapter is based on these two serotypes.



**Figure 2.1. General structure of Ad virus particle.** The schema shows thirteen structural proteins present in Ad viral particle. The capsid organization was based on electron microscopy and crystallography studies. The capsid is mainly built from hexon, penton base and fibre proteins. The remaining minor capsid proteins, i.e. IIIa, VI, VIII and IX enhance its stability. Inside the capsid, the viral core consists of dsDNA genome associated with proteins V, VII, Mu, TP, IVa2 as well as viral protease (Russel *et al.*, 2009).

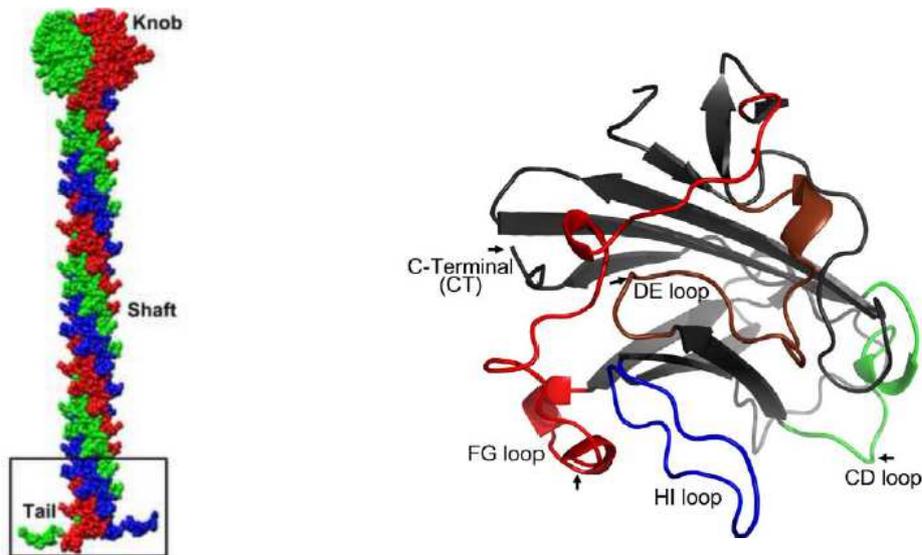
During the last century, advances in technology resulted in a blossom of structural biology techniques. Ad hexon was not only the first animal virus protein to be crystallized (Pereira *et al.*, 1968) but also the longest polypeptide analyzed by X-ray diffraction (Roberts *et al.*, 1986). Thanks to protein crystallography the atomic structures of the fiber knob (Xia *et al.*, 1994), fiber shaft (van Raaij *et al.*, 1999) as well as penton base (Zubieta *et al.*, 2005) were uncovered. All these efforts gave rise to the full, high resolution atomic structure of Ad (Liu *et al.*, 2010; Reddy *et al.*, 2010).

In hexon structure we can distinguish the evolutionarily conserved core of the molecule which is crucial for the capsid formation and weakly conserved loops exposed at the top of the molecule (**Figure 2.2**). Among these sequences, seven Hyper Variable Regions (HVR) were originally described, later reclassified to nine (Rux *et al.*, 2003). They contain serotype-specific residues (Crawford-Miksza and Schnurr, 1996), still remaining the most amenable sites for engineered modifications).



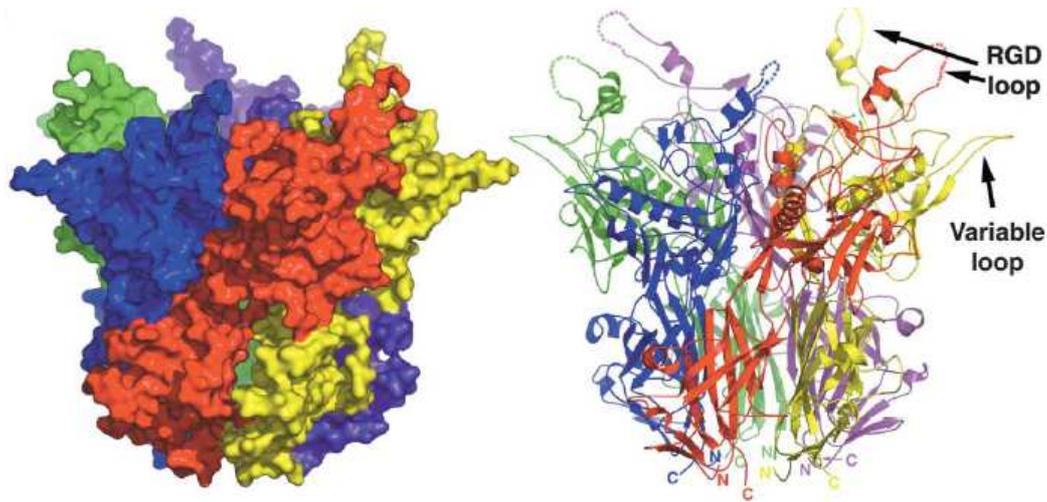
**Figure 2.2. Top-down (A and B) and side (C) view of trimeric hexon protein X-ray structure.** A and B) Depiction of trimeric hexon structure, with HVRs in the following colours: yellow HVR1, green HVR2, light blue HVR3, orange HVR4, red HVR5, brown HVR6, blue HVR7. C) Side view of trimeric hexon, dashed loops represent speculated localization of HVR2 and HVR5 loops (Khare *et al.*, 2012).

The trimeric fiber protein can be divided in three domains. Its conserved base, through the N-terminal flexible tail is anchoring the protein to the penton base. A shaft domain contains repeated conserved motifs of 15-22aa (Green *et al.*, 1983). Differences in the repetition number dictates the fiber length and varies between the serotypes. The KKTK motif found in the fiber shaft allows for the interaction with heparan sulfate proteoglycans (HSPG) (Dehecchi *et al.*, 2000). A distal knob interacts with coxackie-adenovirus receptor (CAR) and displays flexible loops on its surface (**Figure 2.3**). Among them, HI loop was most extensively investigated. Studies report that it is not playing a role in intrinsic molecular interactions and thus is a promising site for genetic modifications (Xia *et al.*, 1994).



**Figure 2.3. General structure of trimeric fiber and detailed structure of fiber knob.** On the left, a fiber model displaying a globular knob domain, a long shaft domain and three N-terminal tails. Three subunits are represented by different colors: red, green and blue. On the right, model of fiber knob with the positions of C-terminus and various loops, represented by different colors: blue HI loop, green CD loop, brown DE loop and red FG loop (Liu *et al.*, 2011; Sharma *et al.*, 2013).

The penton base together with the fiber protein creates a penton protein placed in all twelve vertices of the viral particle. The penton base is formed by five identical monomers. In the structure of each monomer we can distinguish two domains (**Figure 2.4**). First, the jelly-roll barrel domain is conferring stability and site of interaction with the fiber protein (Cusack, 2005). Second, the distal domain is an irregular fold, comprising RGD loops and variable loops exposed to the capsid surface. RGD loops comprises RGD motifs (arginine-glycine-aspartate) responsible for  $\alpha_v$  integrin binding during cell entry (Zubieta *et al.*, 2005). Variable loops, on the other hand, as the name indicates, vary between different serotypes and their function remains to be determined. Interestingly, pentons of certain serotypes are able to auto-assemble by twelve units into so-called dodecahedrons (Norrby, 1966), structures capable of entering the host cell (Fender *et al.*, 1997).



**Figure 2.4. Structure of penton base protein.** On the left, depiction of the pentameric structure of the penton base, with the monomers represented by different colors. On the right, ribbon diagram of penton base with the depiction of RGD loop and variable loop localization (Zubieta *et al.*, 2005).

## 2.1.2 Adenovirus life cycle

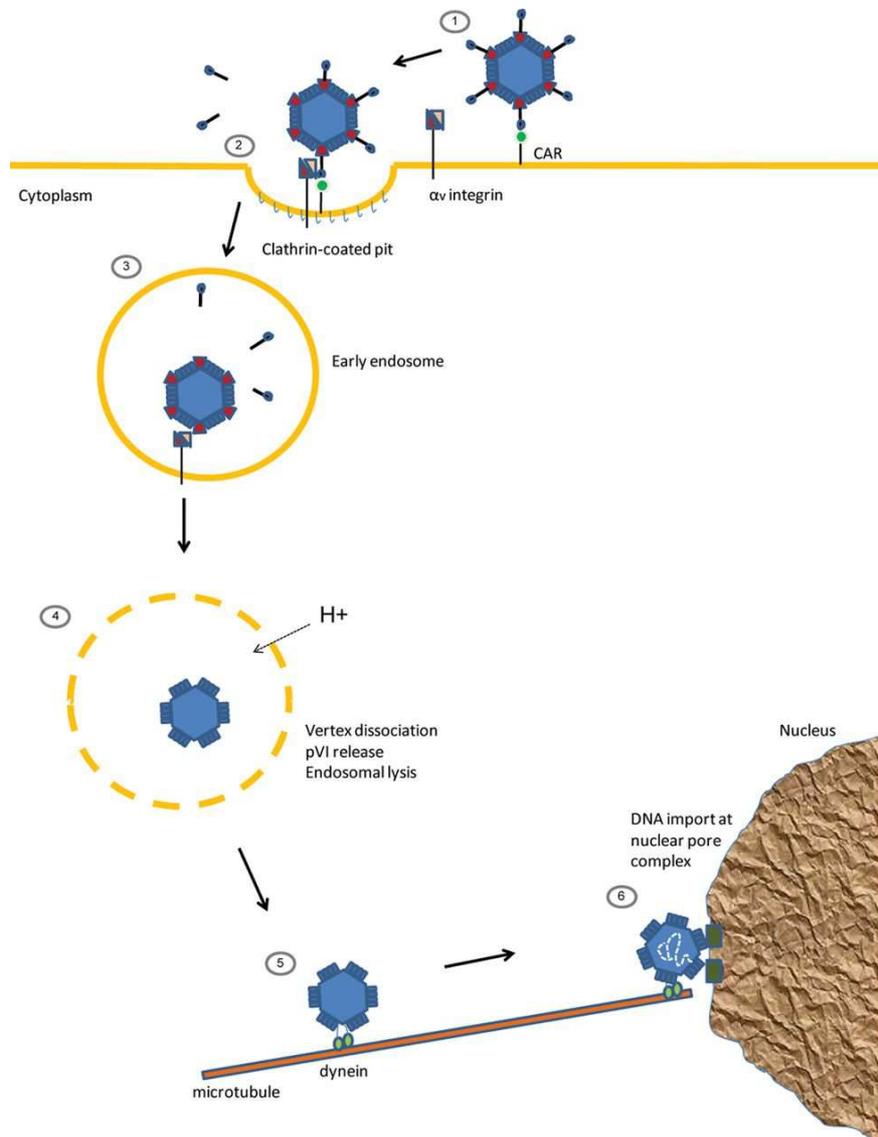
### 2.1.2.1 Interaction with receptors

Ad cell entry process differs between the various serotypes as they interact with different cell-surface molecules. Additionally, some discrepancies can be observed in the cell entry *in vitro* and *in vivo*. In this part I will describe Ad5 infectivity process which was observed in *in vitro* studies. For the detailed vector biodistribution *in vivo*, please refer to chapter 2.2.2.

Ad entrance into the host cell is initiated by interaction of fiber knob with CAR, anchoring the viral particle to the cell surface (Bergelson *et al.*, 1997; Freimuth *et al.*, 1999) (**Figure 2.5**). Loops AB, CD, DG and FG from adjacent fiber monomers are involved in CAR binding and one fiber knob can interact with three CAR molecules, increasing significantly the affinity of this interaction. More precisely, residues Ser408 and Pro409 in the AB loop, Tyr477 in the DG loop and Leu485 in  $\beta$ -strand F, have been described as the pivotal fragments involved in CAR interaction (Kirby *et al.*, 2000). CAR receptor is predominantly expressed in epithelial cells lining the body cavities and more precisely in their tight junctions (Raschperger *et al.*, 2006). It was discovered that the infected cells have a tendency to over-produce fiber protein, which upon release from the infected cell binds CAR and induces tight junction opening (Walters *et al.*, 2002).

As Ad attaches to the cell surface through the interaction with CAR, RGD motif in penton base is available to bind cell-surface  $\alpha\beta_3$  integrins (Wickham *et al.*, 1993). This causes integrin

clustering and results in initiation of an “outside-in” signaling (Qin *et al.*, 2004). Through activation of PI3 kinase remodeling of cytoskeleton takes place, resulting in clathrin-dependent Ad5 cell entry. Although this entrance pathway is the main mechanism used by Ad5, it has been also shown that this virus can access the cell through macropinocytosis (Meier *et al.*, 2002).



**Figure 2.5. Ad5 entry pathway.** After initial cell attachment through fiber knob interaction with CAR and subsequent integrin binding by RGD motif in penton base (1), Ad enters the cell in a clathrin-dependent manner (2). This interaction together with the low pH in the early endosome favours capsid disassembly (3 and 4). The naked viral particle enters the cytoplasm and thanks to the dynein is transported towards the nucleus along the microtubules (5). Viral DNA is transported inside the nucleus through the nuclear pore complex (6) (Nemerow *et al.*, 2009).

Other important molecules are heparan sulfate proteoglycans (HSPG). These ubiquitously expressed molecules are found in increased concentration on hepatocytes. Ad5, presumably through a KKTK motif present in the fiber shaft, can use HSPG to more efficiently enter CAR-negative cells (Smith *et al.*, 2003). More recent reports entail that Ad5 particle coated with coagulation factor X (FX) can be bridged to HSPG and efficiently enter hepatocytes (Waddington *et al.*, 2008), a point that will be discussed in chapter 2.2.

Once internalized into the endosomal pathway, viral particles undergo structure disruption. This is initiated through change of the penton base conformation upon interaction with integrins, resulting in loosening of its interaction with hexon protein and in fiber detachment. The low pH of endosomal environment is another key factor favoring capsid disassembly as it causes exposition of protein VI, implicated in endosome escape (Wiethoff *et al.*, 2005).

In the cytoplasm, interaction of Ad5 hexon protein with dynein catalyzes the “naked” virus movement towards the nucleus (Bremner *et al.*, 2009), using microtubules as a molecular track (Warren *et al.*, 2007) (**Figure 2.5**). Upon reaching microtubule-organizing center (MTOC) the virus detaches from microtubules and hexon protein and pIX interact with nuclear pore complex (NPC). This leads to complete disruption of the viral particle and ejection of viral DNA into the nucleus (Strunze *et al.*, 2011).

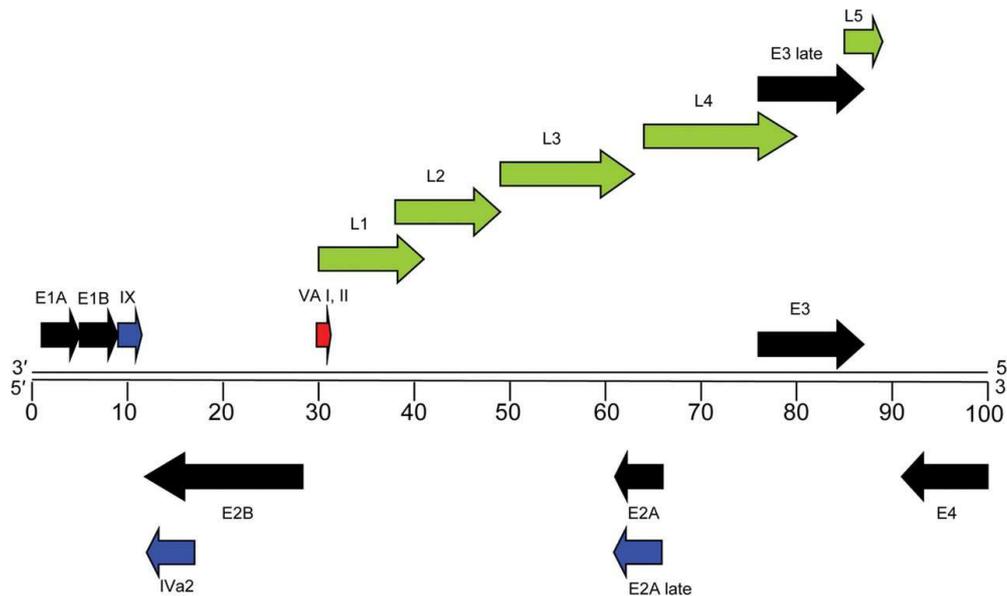
#### 2.1.2.2 Genome

Ad viral genome reaches the nucleus in the complex with viral protein VII. The role of this interaction remains uncertain, but one of the proposed roles is the protection of viral DNA from degradation by DNA repair mechanisms (Karen and Hearing, 2011). Another possibility is the pVII implication in the regulation of viral genome replication and expression (Chen *et al.*, 2007).

Cellular proteins such as Template activating Factor-I $\beta$  (TAF-I $\beta$ ) or members of the TAF-II and TAF-III complex have been shown to interact with pVII/DNA complex and influence its remodelling (Haruki *et al.*, 2003; Kawase *et al.*, 1996; Okuwaki *et al.*, 2001). More recently it was also demonstrated that cellular histones can bind to Ad genome (Komatsu *et al.*, 2011). In addition, bridging of H3 histone to Ad DNA through chaperone protein HIRA is influencing the expression level (Ross *et al.*, 2011).

Ad genome in the form of 30-35 kb double stranded DNA is divided in six segments comprising early genes (E1a, E1b, E2a, E2b, E3, and E4), two intermediate genes coding for IX and IVa2 proteins and five segments encoding late products (L1-L5). Additionally, two genes encoding

virus-associated RNAs I and II (VAI and VAI RNAs) are present in Ad genome. They serve as inhibitors of host protein synthesis in Ad-infected cells (**Figure 2.6**).



**Figure 2.6. Organization of Ad genome.** The rightward transcription results in expression of genes encoding E1A, E1B, IX, L1-5 late proteins, virus associated RNA (VA-RNA) and E3 units. The transcription from the complementary strand provides E4, E2A, E2B and IVa2 transcripts. Early proteins are marked with black arrows, blue arrows indicate intermediate transcription units while late messengers are depicted in green. Virus VA-RNAs I and II are marked as red arrows (Hall *et al.*, 2010).

Proteins encoded by region E1, E2 and E4 are playing essential roles during virus replication and expression of late genes. E1A pushes infected cells into S phase of cell cycle and activates transcription of remaining viral genes (Berk, 1986). This early protein modifies the function of key regulatory proteins like the chromatin remodeling protein p400 or retinoblastoma (Rb). By interacting with p400, E1A protein induces the formation of Myc/p400 complexes on chromatin, resulting in activation of Myc-target genes (Chakraborty and Tansey, 2009). In parallel, through its interaction with Rb-binding domain CR1 and CR2, E1A leads to the liberation of E2F transcription factor and to activation of genes involved in DNA replication and nucleotide metabolism, crucial in S phase (Berk, 2005). E1B region encodes proteins E1B-19K and E1B-55K, which display anti-apoptotic activities (Berk, 2005). E1B-55K by inhibiting p53 ensures cell entry to G1/S phase (Grand *et al.*, 1999). E1B-19K is able to sequester pro-apoptotic proteins BAK and BAX to prevent cell death. E2 region codes for viral polymerase, DNA binding protein (DBP) stabilizing the single-stranded DNA intermediates and preterminal protein (pTP) fixed on ITRs found on the genome extremities. E3 products exhibit immunomodulatory functions, e.g. E3/19K protein blocks expression

of newly synthesized MHC molecules on the cell surface by retaining them in ER (Windheim *et al.*, 2004). E4 region contains at least seven open reading frames, which products provide coordination of viral RNA metabolism. Some of the E4-encoded proteins, such as E4orf1, E4orf3 or E4orf6, plays a role in induction or enhancement of cell transformation. Interestingly, it has been discovered that E4orf4 could inhibit lysis of adenovirus infected cells by CTLs (Kaplan *et al.*, 1999). In the following steps two proteins of viral capsid are produced, IX and IVa2. Finally, expression of late genes assures production of the remaining capsid proteins as well as additional proteins, coordinating and facilitating new capsid assembly and maturation.

### **2.1.2.3 New virion assembly and release**

Newly produced viral proteins needs to return from the cytoplasm back to the nucleus in order to encapsidate replicated copies of viral genome. In case of penton base and fiber this event is assured by the presence of nuclear localization sequences (NLS) and after self-assembly which takes place in cytoplasm, proteins are entering the nucleus (Fender *et al.*, 1997). While inside the nucleus, these two proteins assemble into a penton protein. Chaperon protein L4-100K participates in hexon trimerisation in the cytoplasm and their transport to the nucleus (Hong *et al.*, 2005). While inside the nucleus, all capsid components are able to assemble with the help of L1 52K and 55K proteins (Hasson *et al.*, 1992) and a chaperone protein L4 33K (Fessler *et al.*, 1999). This structure called pro-capsid is then able to encapsidate viral DNA containing  $\Psi$  sequence. Finally, viral protease (L3-23K) by cleaving the polypeptides of minor capsid proteins ensures final structure of viral particle.

Accumulation of new virions in a non-fully elucidated mechanism induces host cell lysis. Most probably this step is depending on L3 protein (Chen *et al.*, 1993) as well as on Ad death protein (ADP) (Tollefson *et al.*, 1996). Ad-induced cell death is of non-apoptotic type, resembling programmed necrosis (Abou El Hassan *et al.*, 2004). Of note, autophagy was also proposed as a mechanism of Ad-induced cell death (Jiang *et al.*, 2008).

## 2.2 Adenoviral vectors: their advantages and limits

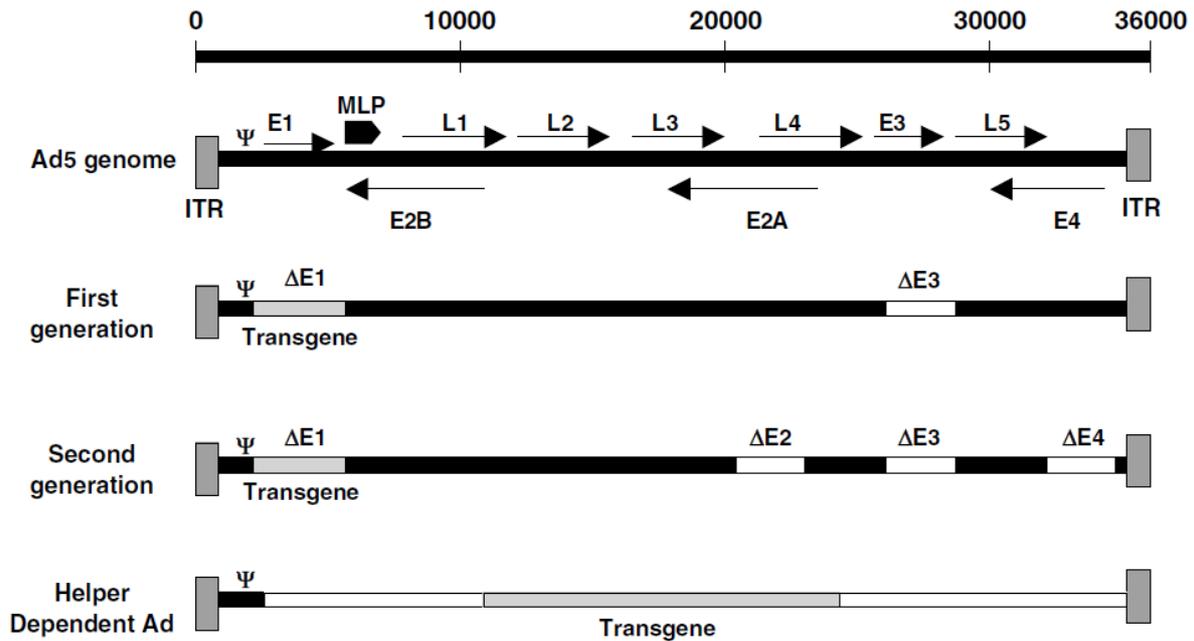
Using Ad5, different vectors were obtained. This part of the manuscript will first describe the construction of different generations of Ad viral vectors. Subsequently, two main shortcomings of their applications will be discussed: *in vivo* tropism as well as their high immunogenicity.

### 2.2.1 Development of adenoviral vectors

Nowadays, adenoviral vectors are the more frequently used platforms in a vaccination studies. This choice is dictated by many advantages they offer, facilitating their construction and usage. Firstly, their biology is well investigated. Ad have a transgene capacity of up to 36kbp and are able to efficiently transduce the cells regardless of the cell cycle stage. They are non-pathogenic, and their episomal nature allows to the insertional mutagenesis. As for the vaccine formulation and scale-up production, Ad vectors can be easily produced at high titer ( $10^{11}$ - $10^{13}$  vp/ml) and their non-enveloped nature allows for a formulation of stable lyophilized preparation.

The first generation of Ad vectors was established by deleting E1 (Ad $\Delta$ E1) region, thus allowing to insert heterologous DNA fragment of 5,5 kb (Bett *et al.*, 1993) and at the same time rendering the vector replication-defective (Danthinne and Imperiale, 2000) (**Figure 2.7**). As a result, production of these vectors could take place only in complementing cell lines providing the missing E1 products. Interestingly, low level of viral gene expression from these vectors can also be observed in non-replicating cell lines due to partial complementation by host cell factors. This leads to the presence of small amounts of viral protein inside the cell and on the cell surface in the form of peptides bound to the MHC molecules, resulting in priming ant-Ad cellular responses (Yang *et al.*, 1994). Moreover, the potential threat of this production technique is the emergence of replication competent Ad (rAd) vectors, which have integrated E1 gene in their genome during the production process through homologous recombination (Lochmuller *et al.*, 1994).

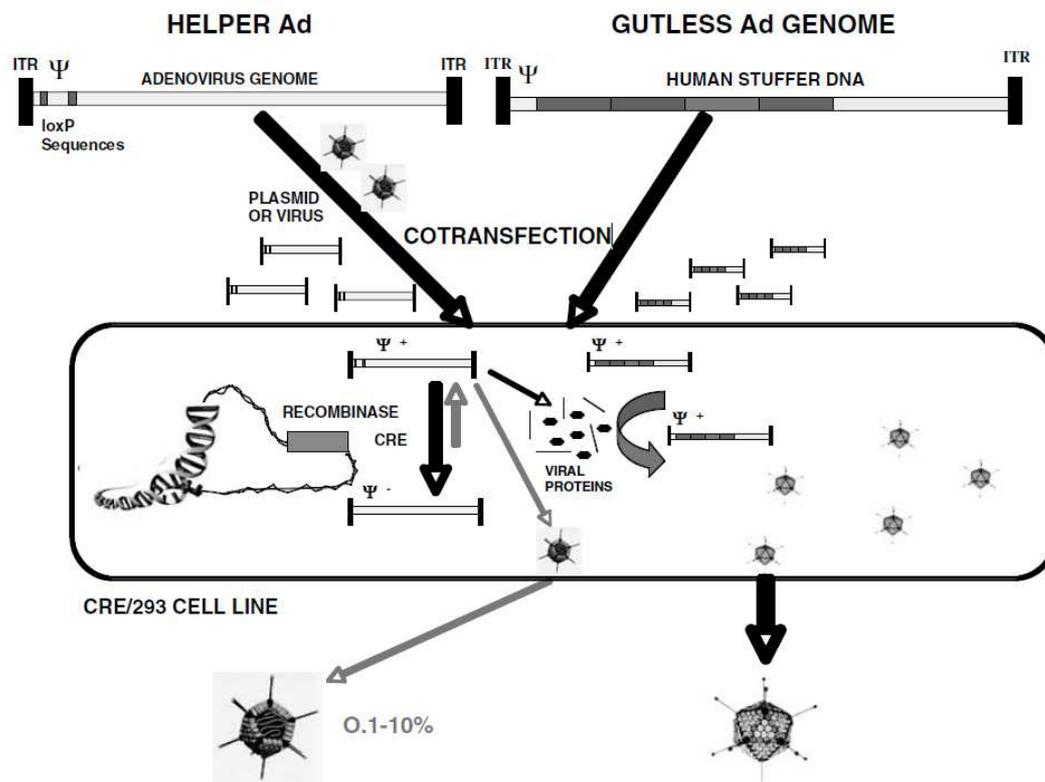
In order to additionally expand the cloning capacity, the first generation vectors were also deleted in E3 segment (Ad $\Delta$ E1 $\Delta$ E3), which permitted to increase the cloning capacity up to 7,5 kb (Amalfitano *et al.*, 1998) (**Figure 2.7**). Even though the majority of work with adenoviruses continues to use Ad $\Delta$ E1 $\Delta$ E3 vectors, the need for a bigger transgene insertion gave rise to the next generation of Ad vectors deleted additionally in regions E2 and/or E4 and able to accommodate up to 10 kb. Similarly to E1, E2 and E4 proteins are also mandatory for the replication process and need to be provided *in-trans* during the vector production (Lusky *et al.*, 1998). Of note, the concomitant deletion of E1, E2 and E4 genes reduces the risk of rAd production.



**Figure 2.7. Different generations of Ad vectors.** Genomes of first and second generation Ad as well as helper-dependent Ad are depicted under the schema of wild type Ad5 genome. E1–E4 represents early transcripts and late transcripts are marked as L1–L5. MLP: major late promoter;  $\Psi$ : packaging signal (Alba, 2005).

As the last generation vectors, helper-dependent vectors were obtained by deleting all the viral genes and by placing the transgene between 5' and 3' ITR and encapsidation sequence  $\Psi$  (Figure 2.7). These “gutless” Ad have a cloning capacity of 36 kb and due to the significantly weaker immune system stimulation, resulting from the absence of viral genes, show better stability in various tissues. There is a relatively tight restriction on the length of DNA which can be packaged into virions in order to preserve vector stability and growth (Bett *et al.*, 1993; Parks *et al.*, 1997). Since inserts are rarely reaching 36kbp, additional nucleotide fragments, called stuffer DNA should be introduced in order to reach the stability threshold. Coding regions, repetitive sequences or hot spots for recombination should be avoided while choosing a stuffer DNA sequence. Gutless Ad vectors are completely derived of viral genes, and for the efficient production cells needs to be co-infected with a so-called helper virus, providing viral proteins essential for the replication process and new virion production. This concomitant infection underlines the need for optimization of the purification process in order to avoid contamination by the helper construct (Alba *et al.*, 2005). One of the strategies comprises weakening the efficiency of helper vector genome packaging, either by introducing mutations in the encapsidation sequence or by increasing the total genome length. Another approach uses helper vector with encapsidation sequence flanked with the loxP sites and cell line expressing Cre

recombinase (293Cre) (**Figure 2.8**). After transduction of these cells, the packaging sequence from helper vector genome is removed, preventing packaging into newly formed virions.



**Figure 2.8. Gutless Ad vector production using the loxP-Cre system.** Two constructs are needed for the production of gutless vector. The genome of gutless Ad contains transgene, stuffer DNA and encapsidation sequence  $\Psi$  placed between its inverted terminal repeats (ITR). Helper Ad construct is containing the whole wild-type Ad genome and the encapsidation sequence  $\Psi$  flanked with loxP sequences. Upon co-transfection of the Cre/293 cells, both genomes are amplified and viral proteins are produced. Cre recombinase removes  $\Psi$  sequence from helper Ad DNA to prevent its packaging. This strategy allows to produce gutless vector with the high purity (90-99.9%) (Alba, 2015).

Regardless of many advantages they provide, Ad vectors are confronted with some hurdles that limit their potential. Two main obstacles, their large biodistribution and their strong immunogenicity will be discussed in the following sections.

## 2.2.2 Tropism and biodistribution of Ad vectors

Vector dissemination to the non-targeted cells is especially problematic in gene therapy and cancer therapy, where specific tissues/cells need to be reached. Non-specific sequestration may also limit vector exposure to the immune system, altering the vaccination efficacy. Thus, the efficacy

of viral vector depends on its capacity to reach targeted cells or tissues. As the level of expression of cell-entry receptors and co-factors usually varies between tissues, we can identify tissues prone to virus infection and those who are non-permissive. Based on this, the virus tropism can be defined. The biodistribution described in this section is focusing on the studies involving Ad5-derived vectors.

### **2.2.2.1 Influence of administration mode**

*In vivo* Ad5 biodistribution depends on the site of administration. There are several ways of administering Ad vectors, among them intravenous (i.v.), intraperitoneal (i.p.) and intramuscular (i.m.) are the most frequently used. Upon i.v. injection virus dissemination is rather nonspecific, due to the ubiquitous expression of CAR,  $\alpha\beta3$  integrins and HSPGs. Nevertheless, there are certain organs which seem to be predominantly targeted by this virus. Results of qPCR report that 90 minutes after the injection of  $5 \times 10^{10}$  vp *via* the tail vein of C57BL/6 mice, significantly increased level of virus genome was detected in spleen, ovary and especially in liver, with the viral DNA content in this organ increased 110 times vs. lung and kidney (Alemany and Curiel, 2001). Transgene expression was also evaluated in mice 72h after the i.v. administration of  $10^9$ – $5 \times 10^9$  PFU, and revealed virus presence in lung, kidney, bladder, heart and urethra with significantly stronger presence in the liver (Wood *et al.*, 1999). Another study assessed transgene expression in various tissues seven days after injection of  $10^8$  PFU through the mouse tail vein, and the results, similarly to the previous analyses disclosed strong liver cells transduction (Kosuga *et al.*, 2000). In the case of i.p injection, 48 hours after mice were injected with  $5 \times 10^{10}$  vp, Ad vector display a more homogenous virus distribution than in the case of i.v. administration. The strongest transgene activity was detected in liver and spleen, followed by kidneys and ovaries, and finally the brain, heart and lung (Kanerva *et al.*, 2002). Intramuscular injection (i.m.) is another administration method, frequently used in clinics. Rabbits injected i.m. with  $2 \times 10^{10}$  vp of Ad vector were exhibiting transgene activity in their liver, lung, testis and spleen, and viral DNA was detected in the liver, lungs and spleen (Vajanto *et al.*, 2002).

### **2.2.2.2 Liver tropism**

Results of all biodistribution studies point at the liver as the main organ implicated in Ad sequestration, with the strongest liver tropism observed upon i.v. injection. The virus preference for this organ can be partially explained by its good vascularization. Adenovirus is first captured by liver-residing macrophages, called Kupffer Cells (KC), and liver sinusoidal endothelial cells (LSEC) lining the hepatic sinusoid. It has been shown that Scavenger Receptors (SR), expressed at high level

on the KC surface, are involved in Ad uptake (Xu *et al.*, 2008). It has been suggested that HVR regions of Ad hexon might be involved in interaction with SR, since their shielding ablated this binding (Khare *et al.*, 2013). Nevertheless, this is not an exclusive mechanism, since mice with genetic deficiency in these cells display similar levels of Ad sequestration. Role of CAR and FX in KC entry was excluded in various experiments (Smith *et al.*, 2008; Xu *et al.*, 2008). Mechanism of Ad entry to the LSEC is less elucidated, but most probably also relies on SR interaction (Pluddemann *et al.*, 2007). Due to Ad entry into the KC and LSEC, hepatocyte transduction is not linearly correlated with the amount of administered Ad vector. When administered at low dose, Ad vector is not able to reach hepatocytes as it is efficiently uptaken and removed by KC. At a high dose, KC and LSEC cells become saturated and the vector is able to transduce hepatocytes.

### **2.2.2.3 CAR receptor and integrins**

Transgenic mice with increased expression of CAR receptor are more susceptible for Ad infection, especially at the level of tissues which normally are CAR-deficient, as skeletal muscle or lymphocytes, suggesting its role in shaping *in vivo* Ad biodistribution (Nalbantoglu *et al.*, 2001; Hurez *et al.*, 2002). However, as shown by numerous studies, single ablation of CAR binding does not limit vector infectivity *in vivo*, indicating that other factors are also playing a role in cell entry (Alemany and Curiel, 2001). Similarly, blocking exclusively the integrins binding is not sufficient to reduce Ad infectivity, as mice deficient in integrin  $\beta 5$  remains susceptible to Ad (Huang *et al.*, 2000). Studies investigating the outcome of combining both mutations in –CAR and –integrins interaction gave variable results. One team showed that after i.v. injection of  $10^{11}$ vp of doubly detargeted vector levels of virus transgene in liver, lung, spleen and heart were significantly reduced (Einfeld *et al.*, 2001). Similar results were obtained by the same team while administrating Ad intraperitoneally, showing that the doubly mutated vector is less captured by non-targeted tissues and persists longer in a blood stream (Akiyama *et al.*, 2004). Contradictory observations were obtained by different group, where doubly mutated vector did not show reduced genome accumulation or transgene expression in the liver (Martin *et al.*, 2003).

### **2.2.2.4 Heparan sulfate proteoglycans**

Another factor influencing strong liver uptake is the increased expression of HSPGs on hepatocyte surface, which, as mentioned in 2.1.2.1., are one of primary Ad receptors. Binding of KKTK motif present in fiber shaft with HSPGs seems to be dictating Ad liver tropism, as inhibition of

this interaction results in a reduction in hepatocyte transduction (Smith *et al.*, 2003). Ad5 vectors with fiber from serotype 19 or 37, and thus unable to bind CAR nor HSPGs, showed reduced transgene expression in the liver 1 hour and 5 days after the administration (Denby *et al.*, 2004). My laboratory reported that pseudotyping Ad5 vector with Ad3 fiber results in reduced transgene expression in liver (Vigne *et al.*, 2003). Further analysis showed that this property is mediated by Ad3 fiber shaft and could be explained by a better KC uptake of this vector (Raddi, in revision). However, another study using Ad5 pseudotyped with a shaft from serotypes 31 or 41, also lacking KKTK motif, reported unaltered liver sequestration (DiPaolo *et al.*, 2007). A triple mutated vector, where alterations of fiber knob and RGD motif in penton base were combined with ablation of KKTK motif and HSPGs interaction (through substitution with Ad35 fiber shaft) showed a very significant reduction in transgene activity in liver and lung after i.v. injection of  $3 \times 10^{10}$  vp (Koizumi *et al.*, 2003). The same vector showed similar organ sequestration pattern upon i.p. administration of  $10^{11}$  vp (Koizumi *et al.*, 2006). These results question the role of HSPG in hepatocyte transduction and suggest involvement of other factors.

#### **2.2.2.5 Blood factors**

Extensive research revealed that host blood factors are heavily influencing Ad transduction in liver. It has been shown, using an *ex vivo* perfusion model that coagulation factor IX (FIX) and C4-binding protein belonging to the complement system can interact with Ad fiber knob and mediate virus uptake in liver through HSPGs and low-density lipoprotein (LDL) receptor-related protein. Interestingly, this hepatocyte uptake is not dependent on CAR interaction (Shayakhmetov *et al.*, 2005). Another study analyzed a more broaden spectrum of vitamin K-dependent coagulation zymogens, including protein C, prothrombin (FII), FVII, FIX FX, FXI, and FXII (Parker *et al.*, 2006). Among them, FX appeared to have the biggest impact on hepatic tropism. Indeed, mice depleted in coagulation factors were able to restore a full level of transgene expression in liver only in the presence of FX. Based on cryo-microscopy results it has been proposed that FX through its GLA domain is able to interact with hexon protein. By substituting all HVRs of Ad5 with those from serotype 48 it has been shown that these variable domains are involved in interaction with GLA domain of FX, suggesting the differences in FX binding between various Ad serotypes (Waddington *et al.*, 2008). More precise analyses based on directed mutagenesis revealed that FX binds HVR5 and HVR7 of Ad hexon (Vigant *et al.*, 2008; Alba *et al.*, 2009), and single mutation T425A in HVR7 can efficiently disrupt Ad-FX interaction (Doronin *et al.*, 2012). While bound to Ad hexon, FX is able to interact with HSPGs, however, this interaction is restrained only to HSPG possessing N- and O- linked

sulfate group. These are found predominantly on hepatocyte surface which explains Ad5 liver tropism despite HSPG ubiquitous expression (Bradshaw *et al.*, 2009).

The discovery that FX is involved in bridging the Ad to the liver HSPGs through interaction with hexon resulted in series of studies investigating the outcomes of this interaction ablation on Ad biodistribution. Ad vector mutated in hexon, unable to interact with FX showed markedly decreased liver uptake upon i.v. injection of both low ( $1 \times 10^{10}$  vp) (Alba *et al.*, 2010) and high dose ( $1 \times 10^{11}$  vp) (Vigant *et al.*, 2008; Alba *et al.*, 2010). Interestingly, vectors ablated in interaction with FX were still efficiently uptaken by KC, and depletion of these cells resulted in high vector accumulation in spleen. Thus, for the vaccination studies it might be thus interesting to disable the interaction of Ad with KC in order to target spleen more efficiently. At a high dose, this detargeted vector induced strong chemokine and cytokine production and more vigorous Th1 responses (Alba *et al.*, 2010). The same team evaluated a vector combining ablation in both hexon-FX and penton-integrin interactions (Bradshaw *et al.*, 2012). They observed that additional mutation in RGD motif resulted in attenuated spleen uptake and reduced inflammatory responses. Contradictory results were obtained in mice lacking Ext1, an enzyme required for heparan sulfate biosynthesis, resulting in 8-fold decrease in hepatocyte HSPG level. Surprisingly, Ad vector of native tropism as well as Ad vector ablated in interaction with CAR were still able to efficiently transduce the liver of these animals, suggesting an alternative pathway of hepatocyte entry, not involving HSPGs nor CAR (Zaiss *et al.*, 2016). This sheds a new light on molecular mechanisms of *in vivo* cell entry of Ad vector and underlines the need for further examinations in order to fully understand Ad behavior *in vivo*.

### **2.2.3 Immunogenicity of Ad vector**

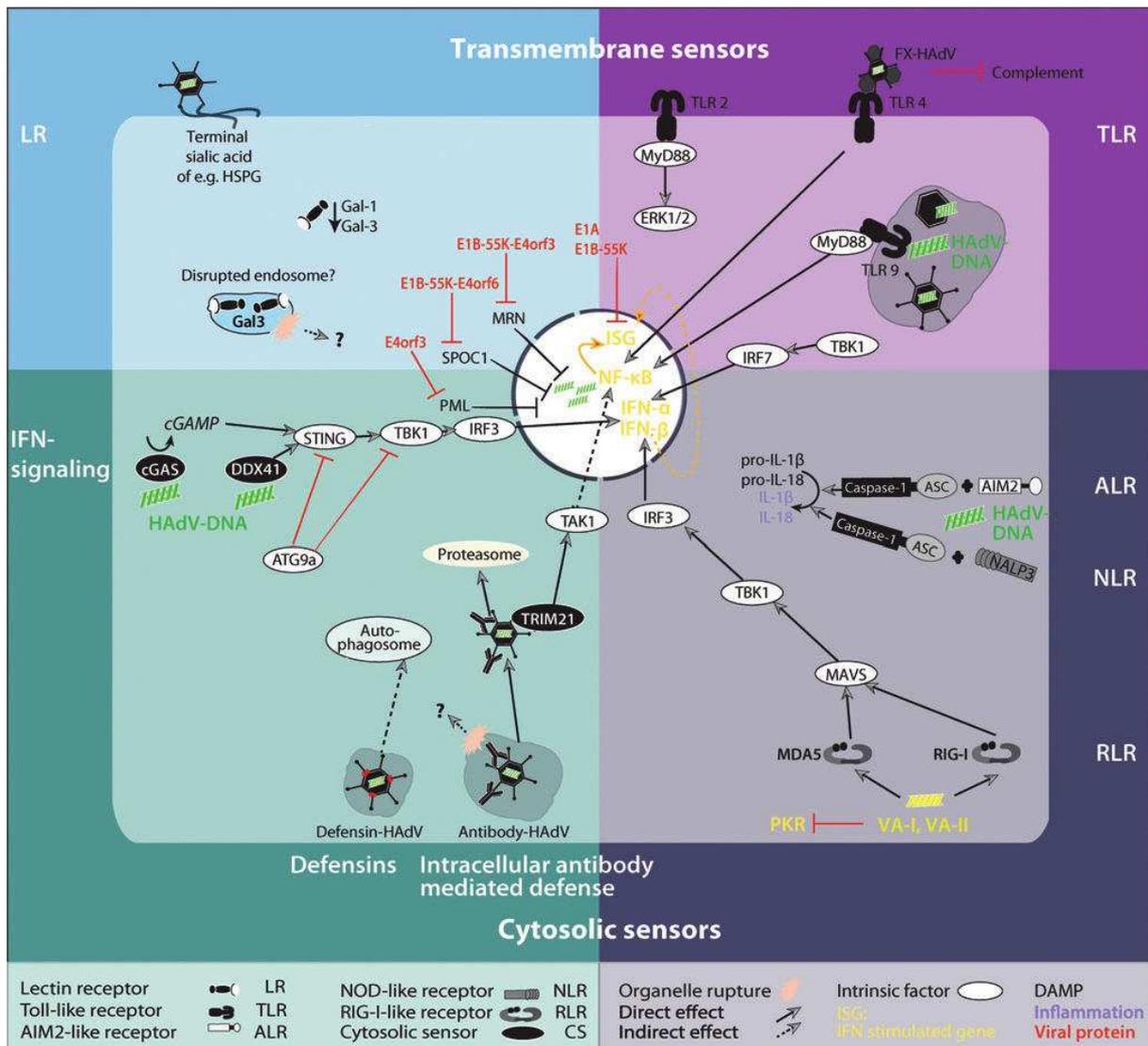
#### **2.2.3.1 Innate immune responses to Ad vector**

Adenovirus hallmark is the potency to induce robust innate and adaptive immune responses. Intrinsic immunogenicity of viral capsid was unveiled by McCoy *et al.*, who showed that innate responses induced by Ad vectors were transcription-independent (McCoy *et al.*, 1995). Strong production of pro-inflammatory cytokines and chemokines was linked to virus interaction with its cellular receptors as well as to its recognition by cytosolic, membranous and secreted PRRs (**Figure 2.9**).

Before entering the cell, virus interactions with CAR and integrins set in motion intracellular MAP kinases and PI3 kinase signaling pathways, leading to the production of IL-1, IL-6, IL-12, TNF- $\alpha$  and type-I interferons (IFN-I) (Philpott *et al.*, 2004; Tamanini *et al.*, 2006). Additionally, Ad activates

cell-surface located TLR2 (Appledorn *et al.*, 2008). More recently, role of FX-coated Ad as a TLR4 activator has been proposed, as vector ablated in FX binding failed to activate TLR4/TRAF6/NF- $\kappa$ B pathway *in vivo* (Doronin *et al.*, 2012). However, these observations obtained in mice model were not confirmed using human mononuclear phagocytes (Echhotz *et al.*, 2015), and thus the role of FX remains unclear. C-type lectins receptors (LR), such as cell surface sialic-acid binding immunoglobulin-like lectins (Siglecs) and cytosolic galectins have also been shown to recognize Ad (Rademacher *et al.*, 2012). LRs directly bind sugars of the Ad capsid and crosstalk between LR and TLR signaling has been observed, indicating that LRs enhance the innate immune responses against Ad (Kawai and Akira, 2011). Upon internalization into the endosomes, viral particle activates TLR9 (Appledorn *et al.*, 2008), resulting in IFN-I responses and subsequently, after escape into the cytoplasm, becomes recognized by AIM-2 like receptors (ALRs) and NOD-like receptors (NLRs) leading to inflammasome formation and pro-IL-1 $\beta$  cleavage (Barlan *et al.*, 2011; Stein and Falck-Pedersen, 2012). Through cytosolic machineries sensing foreign nucleic acids, viral DNA and virus-associated RNAs lead to, respectively, DDX41 /cGAS-dependent (Nociari *et al.*, 2007; Lam *et al.*, 2014) and RIG-I dependent (Minalitani, *et al.*, 2011) IRF3 activation and IFN-I production. All these branches of innate immunity sensing conjointly lead to the “cytokine storm”, which has been shown to interfere with transgene transcription from the viral vector (Sung *et al.*, 2001).

Extracellular factors are known to influence Ad innate immunity. Defensins, antimicrobial peptides secreted mostly by neutrophils are able to bind to Ad and interfere with the exposure of lytic protein VI from the capsid (Snijder *et al.*, 2013). As previously mention, Ad coating with FX not only was proposed to stimulate TLR4, but also thought to protect virus from the attack of natural Abs and the complement (Xu *et al.*, 2013). Ad in complex with Abs is known to activate the classical pathway of complement and be captured and cleared by KC more efficiently (Xu *et al.*, 2008). By using different strains of immunodeficient mice it has been shown that the presence of natural IgM Abs plays a pivotal role in hepatocyte infection by Ad ablated in FX binding. *In vitro* studies disclosed that FX binding to Ad hexon prevents the natural IgM Abs from depositing the complement proteins on Ad surface by (Xu *et al.*, 2013). Another interesting mechanism involving Abs is the activation of cytosolic Fc receptor TRIM21 by Ad-Abs immune complexes (McEwan *et al.*, 2013). Upon immune complex uptake, recognition of Fc part of Ab by TRIM21 triggers a signaling cascade, which independently of TLRs and RLRs pathways results in the activation of transcription factors such as NF- $\kappa$ B, AP-1, IRF3, IRF5 and IRF7 and subsequent production of pro-inflammatory cytokines. Interestingly, this was observed for both IgM and IgG, indicating that this mechanism plays a role during both primary and secondary Ad infection. This intracellular Ab sensing should be taken under account while designing Ad vector for vaccination purposes in pre-immune individuals (McEwan *et al.*, 2013).



**Figure 2.9. Innate immune response induced by Ad.** Adenovirus can activate various pathways of innate immunity, such as Toll-like receptors (TLRs), AIM2-like receptors (ALRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), lectin receptors LR as well as cGAS/DDX41/STING interferon signaling pathway. Additional factors have been described to influence the innate Ad sensing, such as coating with FX, intracellular antibodies or defensins. Virus early proteins (in red) as well as Virus associated RNA (in yellow) can antagonize induction of innate immunity (Hendrickx *et al.*, 2014).

### 2.2.3.2 Humoral immune response to Ad vector

Adenovirus induces strong Ab titers directed against major capsid proteins: hexon, fiber and penton (Gahéry-Ségard *et al.*, 1998; Hong *et al.*, 2003). The majority of neutralizing Abs was shown to target HVRs of hexon, and substitution of the entire hexon or solely HVRs with those from another serotype allowed the chimeric vector to escape the neutralization (Sumida *et al.*, 2005; Roberts *et al.*, 2006; Bradley *et al.*, 2012). Nevertheless, a subdominant population of fiber

knob-directed Abs was proved to be relevant, as Ad5 vector pseudotyped with both HVRs and fiber knob coming from heterologous serotype evaded pre-existing Ad5 immunity more efficiently than vector with alteration in HVRs only (Gahéry-Ségard *et al.*, 1998; Bradley *et al.*, 2012). Still, anti-fiber Abs on their own are not sufficient for vector neutralization, as sera from mice immunized with Ad7 failed to neutralize Ad5 vector pseudotyped with Ad7 fiber (Gall *et al.*, 1996). Interestingly, it has been observed that while anti-fiber Abs subpopulation is quite modest during the first injection (Gall *et al.*, 1996), it is more easily detected after subsequent vector administrations (Gahéry-Ségard *et al.*, 1998; Sumida *et al.*, 2005). The authors also suggested that anti-fiber Abs might act synergistically with those directed against penton (Gahéry-Ségard *et al.*, 1998).

Neutralizing Abs are targeting regions which vary significantly between the different serotypes. As a consequence, induced neutralizing Abs are not cross-reactive. However, non-neutralizing, cross-reactive Abs induced after repeated Ad-administration can contribute to vector elimination by mechanisms like antibody dependent cellular cytotoxicity (ADCC), complement activation or opsonisation resulting in better Ad uptake by macrophages (Moffatt *et al.*, 2000; Pichla-Gollon *et al.*, 2009). Another aspect of Abs binding to Ad was described in previous section and involves activation of pro-inflammatory cytokine production through cytosolic Fc receptor, TRIM21.

### **2.2.3.3 Cellular immune responses to Ad vector**

Both early and late gene products are targets of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses (Yang *et al.*, 2003; Onion *et al.*, 2007). Various T cell epitopes have been identified in Ad capsid proteins, with hexon being the immunodominant T cell target. The HLA-DP4-restricted, CD4<sup>+</sup> T cell epitope H910-924 was mapped to the C-terminus of hexon sequence and described to be highly conserved among various Ad serotypes and recognized by up to 75% of healthy adults (Olive *et al.* 2002; Tang *et al.*, 2004; Onion *et al.*, 2007). Human cytotoxic CD8<sup>+</sup> T cells are also targeting hexon C-terminus, where various cross-reactive class I epitopes have been localized, some of them overlapping H910-924 epitope mentioned above (Leen *et al.*, 2004, Tang *et al.*, 2006). Cytotoxic responses against fiber and penton base were also observed in humans, although turned out to be much less potent and prevalent in the healthy individuals (Tang *et al.*, 2006). Taken together these results underpin the observation that the most immunogenic entity of Ad is the highly conserved, distal, 100aa long sequence of hexon protein. In contrast to human cellular responses, cellular anti-Ad responses in mice were less extensively investigated. Nevertheless, in C57BL/6 mice (H-2<sup>b</sup> haplotype), the immunodominant class-I epitope was located in DNA binding protein 43 (Dbp43) (Mc Kelvey *et al.*, 2004).

Cellular branch of pre-existing anti-Ad adaptive immunity in human might be more prevalent than the humoral one, as one of the studies showed that while only 28% of analyzed individuals displayed anti-Ad Abs, all of them had detectable level of anti-Ad T cell responses (Calcedo *et al.*, 2009). These observations were confirmed by another team which in the evaluated group detected 64% of individuals possessing anti-Ad T cell responses, while only 55% of them had neutralizing anti-Ad Abs (Chirmule *et al.*, 1999). The widely distributed Ad-specific T cells are also cross-reactive, as they target conserved regions. Isolated human Ad-specific CD4<sup>+</sup> T cells were monofunctional, produced IL-2, IFN- $\gamma$  or TNF $\alpha$  and expressed memory markers. On the contrary, CD8<sup>+</sup> T cells were polyfunctional and displayed effector memory cell phenotype (Hutnick *et al.*, 2010).

## **2.3 Ad vectors in vaccination**

Ad5 vectors are the most frequently used vectors. Importantly, their intrinsic adjuvant features provide valuable innate immune system stimulation, crucial in vaccine design. Nevertheless, neutralizing anti-Ad5 Abs are detected in 50-90% of a general population (Holterman *et al.*, 2004) which precludes efficient gene transfer upon first vector administration. Additionally, strong humoral immunity as well as specific cellular responses (Tatsis *et al.*, 2007; Tatsis *et al.*, 2009) are induced after priming with Ad vector, thus impeding effective boosting with a homologous vector. The strong anti-vector immune responses elicited by Ad5 hamper expression of the transgene and reduce efficacy of this type of vaccination (described herein as classical vaccination) by limiting the quantity and duration of antigen availability (Schrimbeck *et al.*, 2008). In order to overcome limitations of pre-existing Ad immunity different strategies of Ad-based vaccination were developed.

### **2.3.1 Strategies to overcome anti-Ad immunity**

#### **2.3.1.1 Alternative Ad serotypes**

One of the strategies to overcome anti-Ad immunity consists in shifting to different, less prevalent human Ad serotypes or non-human Ad. Among the most frequently used is serotype 35, evaluated as a vaccine platform against HIV-1 (Keefer *et al.*, 2012) or malaria (Shott *et al.*, 2008; Ouedraogo *et al.*, 2011). Another often selected serotypes is Ad26, tested e.g. for a HIV-1 vaccine development (Baden *et al.*, 2013), serotype Ad4, also encoding antigen from HIV-1 (Jeff *et al.*, 2013) or Ad6 used in HCV vaccination (Chmielewska *et al.*, 2014). Different appealing solutions proposed the use of vaccine vectors based on non-human Ad, such as simian, canine, bovine or porcine Ad (Bangardi, 2006). A strong interest was drawn to chimpanzee Ad (AdC), among which we distinguish 9 serotypes with AdC68 being the most extensively used, usually as a HIV-1 vaccine (Fitzgerald *et al.*, 2003c; Pinto *et al.*, 2003; Reyes-Sandoval *et al.*, 2004). Although efficient upon first injection, these vectors are still triggering strong anti-vector immunity that hampers gene transfer upon homologous re-administration.

#### **2.3.1.2 Heterologous prime-boost strategy**

Human non-Ad5 serotypes were also tested for their performance in a heterologous prime-boost regimen. Serotypes 11, 35 and 49 were investigated for their ability to induce anti-transgene immune responses in an Ad5 pre-immunized background and Ad35-Ad49 regimen

turned out to be more potent than rAd35-rAd5 or rAd35-rAd11 regimens (Thorner *et al.*, 2006). The combination of serotypes 11 and 35 were also used in a mixed prime-boost schedule to mount immune response against SIV (Lemckert *et al.*, 2005). Similarly, Ad26 and Ad35 were tested for their combined efficacy in induction of adaptive immune response against SIV (Balandya *et al.*, 2014) and Ebola (Geisbert *et al.*, 2011), and in both cases robust anti-transgene cellular responses were detected. Heterologous prime-boost vaccination, however, requires construction of multiple vectors and assessment of their efficacy and safety, which is not only costly but also time consuming.

### **2.3.1.3 Pseudotyping Ad capsid**

As an alternative to the heterologous prime-boost vaccination, Ad5-based vectors with genetically engineered capsid were developed. Capitalizing on the knowledge of the Ad capsid protein structure, specific capsid alterations were made and by genetic engineering allowed to obtain novel, more optimized vectors. Evaluation of Ad5 chimera bearing the fiber from Ad7 did not provide the capacity to escape Ad5 immunity, confirming that the main target of neutralizing Abs is hexon (Gall *et al.*, 1996). The high conservation of hexon core allows for it interchanging between various Ad serotypes and for engineering the chimeric viruses. Ad5 pseudotyped with entire Ad6 hexon have been rescued and showed to be capable of evading anti-Ad5 responses (Youil *et al.*, 2002). Based on the fact that hexon loops comprising HVRs were shown to contain the main serotype-specific epitopes, Roy *et al.* substituted Ad5 L1-L4 hexon loops with corresponding sequences from Ad12. The vectors efficiently escaped pre-existing anti-Ad5 immunity (Roy *et al.*, 1998). Similarly, introducing L1-L2 loops from Ad2 in Ad5 background also significantly increased gene transfer of a chimeric vector in an Ad5-immune background (Gall *et al.*, 1998). Nevertheless, these pseudotyped vectors were quite difficult to produce, and so an alternative strategy emerged, consisting in substituting exclusively hexon HVRs. Vectors based on Ad5 with all HVRs substituted with those from Ad48 efficiently escaped anti-Ad5 immunity, inducing cellular and humoral anti-transgene responses (Roberts *et al.*, 2006). One study claimed that removing solely HVR5 is enough to induce significantly stronger cell-mediated anti-transgene responses in mice with pre-existing anti-Ad immunity (Abe *et al.*, 2009).

### **2.3.1.4 Shielding Ad vectors**

Another solution for counteracting pre-existing immunity in the classical vaccination approach with Ad vectors is based on biochemical modification of viral capsid (Kreppel and

Kochanek, 2008). Among the most popular modifications is PEGylation, consisting in coating viruses with PEG polymers which can mask Ad epitopes. Additionally, PEGylation ablates the native Ad tropism and has been shown to reduce transduction of non-target tissues *in vivo* (O’Riordan and Song, 2008). These properties depend strongly on the PEG molecular weight. While small molecules may confer immune protection, bigger polymers are required to block CAR and integrin-mediated transduction in mice (Hofnerr *et al.*, 2008). Another molecule successfully used for Ad capsid coating is poly(N-[2-hydroxypropyl]methacrylamide) (HPMA). HPMA coating on Ad provides protection from neutralizing Abs and complement and mediates a prolonged blood persistence of Ad particles in mice (Kim *et al.*, 2012).

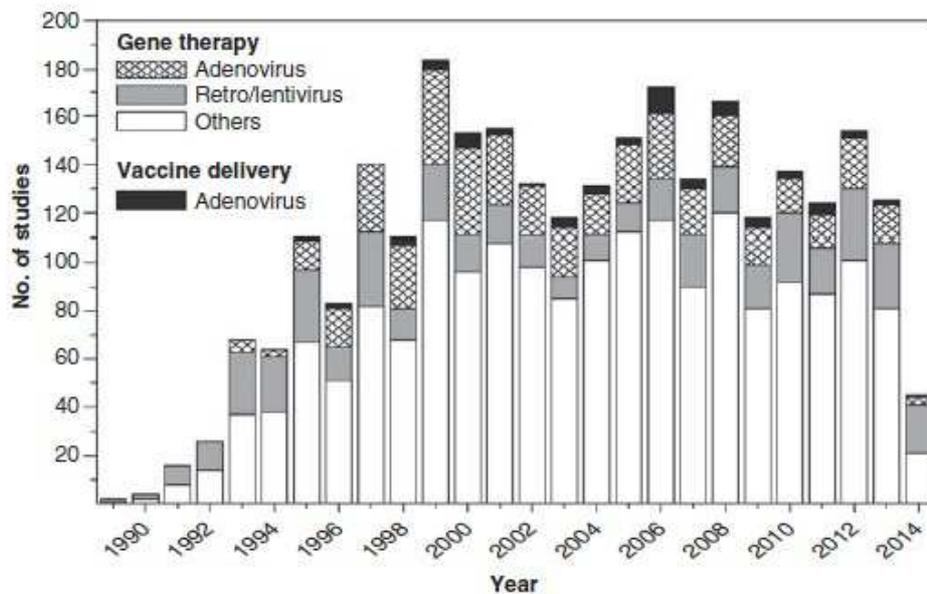
#### **2.3.1.5 Administration mode**

The mode of vector administration is another important aspect to consider while trying to avoid anti-Ad immunity. One study compared anti-transgene adaptive responses in mice upon intramuscular (i.m.), intranasal (i.n.) and oral vector administration (Croyle *et al.*, 2008). While in naïve mice i.m. injection was triggering the most potent adaptive responses, the pre-existing Ad immunity did not hamper vaccination efficacy only in the case of i.n. administration. The frequency of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells was reduced in Ad-immune animals vaccinated i.m. and orally by 80% and by 15%, respectively. This may be partially due to the lower levels of anti-Ad Abs in mucosal sites than in the systemic circulation (Croyle *et al.*, 2008). Another group also evaluated the performance of oral immunization with Ad vector and found that pre-existing anti-Ad immunity did not impair the capacity of mounting adaptive responses which displayed mixed Th<sub>1</sub>-Th<sub>2</sub> phenotype (Xiang *et al.*, 2003). While systemic delivery induces mostly systemic immunity, with little or no effect on mucosal sites, mucosal administration is capable of eliciting both systemic and mucosal immune responses which makes it an appealing mode of vector delivery (Neutra and Kozlowski, 2006). This is especially attractive in the vaccination against pathogens having a tropism for mucosal sites, such as those spread through aerosols or sexual contact.

#### **2.3.2 Clinical trials in vaccination with Ad**

Several Ad-based vaccine vectors were evaluated in animal models. Promising results obtained in these studies led to the clinical development of some vaccine candidates, notably against HIV, hepatitis C, malaria, H1N1 and H5N1 influenza A or tuberculosis. Ongoing trials are currently in different phases of clinical development, with the most advanced stages for HIV and malaria vaccine

candidates (**figure 2.10** and **figure 2.11**). As for the end of January 2016, website of the U.S. National Institutes of Health ([www.ClinicalTrials.gov](http://www.ClinicalTrials.gov)) shows eighty-four search results with the keywords “Adenovirus Vector” and “Vaccination” and thirteen among them are currently recruiting participants. Thirty-nine studies are focusing on HIV vaccination, twenty-one are investigating Ebola vaccine, thirteen are analyzing anti-malaria immunization and five trials are evaluating HCV vaccine candidates.

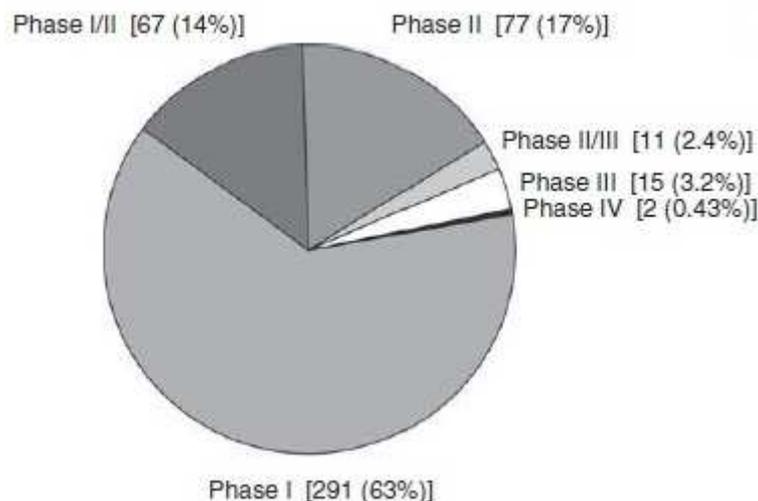


**Figure 2.4. Use of viral vectors in clinical trials.** The figure shows the number of gene therapy and vaccine clinical trials using adenovirus-, retrovirus- or other virus-derived vectors for the last 2 decades (Appaiahgari & Vratı; 2015).

The most popular clinical trial using Ad vector, called STEP, was investigating the potential of replication-defective Ad5 vector expressing various HIV proteins (*gag*, *pol* and *nef*) (Buchbinder *et al.*, 2008). At the beginning of 2004, around 3000 volunteers in various countries received the vector mixture. Despite repeated intramuscular injections of high virus dose ( $10^{11}$  viral particles per injection), the formulation was well tolerated. The vaccine proved to be immunogenic, as 75% of participants displayed anti-HIV T cell responses as measured by IFN- $\gamma$  ELISPOT assay. However, the observation that vaccinated volunteers displayed higher rates of HIV infection than the placebo-receiving group led to the premature termination of the study in 2007. It has been underlined that individuals with an increased rate of HIV infection were mostly men having sexual intercourse with uncircumcised men and/or those who had elevated titers of pre-existing Abs to Ad. One of the possible explanations of this phenomenon indicates that pre-existing anti-Ad Abs influenced antigen presentation, causing a shift from Th<sub>1</sub> to Th<sub>2</sub> type of cellular response and thus led

to the reduction of protective anti-HIV CD8<sup>+</sup> T cells. However, this seems to be unlikely, since Ad binding, non-neutralizing, cross-reactive Abs were detected in all the participants of the study, and there is no reason to assume that only neutralizing Abs influence the antigen presentation (Haut and Ertl, 2009). Hence, it remains ambiguous whether anti-Ad Abs indeed played a role in increased HIV infection rate and if so, what was the exact mechanism of this interrelationship.

Another possible mechanism of the observed enhanced HIV infection is the vector-mediated activation of Ad5-specific CD4<sup>+</sup> T cells that became ideal targets for HIV infection (Buchbinder *et al.*, 2008). This theory was also proposed in other studies, where administration of a pneumococcal-based vaccine or an influenza virus-based vaccine in HIV-1-infected patients showed that vaccination can lead to a temporary increase in viral load (Brichacek *et al.*, 1996; Staprans *et al.*, 1995). However, other clinical trials using different vaccine platforms, also inducing CD4<sup>+</sup> T cells such as poxvirus vector, did not increase susceptibility to HIV-1 infection. Interestingly, effectors T cells induced by these vaccines rapidly contract to form central memory cells while Ad, due to its persistence, stimulates a prolonged effector phase. This protracted presence of effector cells was proposed to be a possible explanation for the increase in HIV-1 acquisition rates observed in the STEP trial.



**Figure 2.5. Clinical development of Ad-based vectors.** Pie chart indicates the number of clinical trials of different stages using Ad vectors. (Appaiahgari & Vrati; 2015).

Another study developing HIV vaccine with Ad vectors, called HVTN, reached phase-II of clinical development. It consisted of priming with six DNA sequences encoding different HIV proteins (Gag, Pol, Nef and Env proteins), followed by an i.m. administration of 10<sup>10</sup>vp of Ad5 vectors

expressing the same HIV proteins. The study was suspended in 2013 due to the lack of efficacy in reducing the HIV infection rate or in alleviating the HIV infections (Hammer *et al.*, 2013).

Ad vectors based on less prevalent serotypes and xenotypes are used in a phase-I trial in the development of a HCV vaccine. HAd6- and ChAd3-based vaccine candidates encoding viral NS3 protein were administered i.m. in a dose varying from  $5 \times 10^8$  to  $7.5 \times 10^{10}$  vp in different prime-boost combinations. Induction of strong, long-lasting and polyfunctional anti-HCV CD4<sup>+</sup> and CD8<sup>+</sup> T cells responses was reported. Induced T cells were both of central and effector memory type and retained polyfunctionality and proliferative capacity (Barnes *et al.*, 2012). Malaria is another pathogen targeted with the use of Ad vectors. Phase-IIa of Controlled Human Malaria Infection trial evaluated ChAd63 vectors expressing various *Plasmodium malariae* proteins, administered i.m. at a dose of  $5 \times 10^{10}$  vp. Unfortunately, this study reported that the induced humoral and cellular responses failed to confer any protection (Sheehy *et al.*, 2012). Another example of successful Ad application in vaccination field comes from the development of tuberculosis vaccine. An Ad35 vector expressing an antigen of *Mycobacterium tuberculosis* (AERAS-402), reached a phase-I study, where healthy volunteers, immunized with BCG in their infancy were given one i.m. injection ( $3.2 \times 10^9$  vp). The vaccine was found to be safe, well tolerated in all the participants and capable of inducing adaptive immune responses. Interestingly, the vaccine was able to boost CD4<sup>+</sup> and CD8<sup>+</sup> cellular responses in the group previously immunized with BCG. Of note, Ad5 pre-existing immunity did not affect the vaccination efficacy (Abel *et al.*, 2010).

Overall, despite STEP study, Ad vectors are considered to be relatively safe. In spite of some significant discoveries highlighting their vaccination potential, further improvements need to be introduced to additionally increase their vaccination performance, especially in patients displaying pre-existing anti-Ad Abs. Among these various strategies allowing to circumvent anti-Ad5 immunity, *Epitope Display* approach seems to be taking the lead, and will be described in the Chapter 3.

### 3 Vaccination based on epitope display on adenovirus capsid

#### EPITOPE DISPLAY ON ADENOVIRAL CAPSID AS A NEW PROMISING STRATEGY OF VACCINATION

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#### 3.1 Abstract

Adenoviruses (Ad), originally vectorised for the purposes of gene therapy are becoming a very popular tool in the field of vaccination studies. Their highly immunogenic properties constitute a serious drawback to their application as gene delivery vehicles, due to the rapid elimination of transduced cells by host humoral and cellular responses. In far contrast, this ability to stimulate strong innate and adaptive immunity makes them an appealing platform for vaccination development. High level of transgene expression, capacity to infect dividing and non-dividing cells, lack of viral DNA integration into the host genome, relatively safe profile and ease of manipulation, these are only a few of the Ad features favoring their choice as a suitable immunization vehicles. Furthermore, their capacity to infect dendritic cells (DC) and to induce pro-inflammatory cytokine production results in eliciting a potent adaptive immunity and justifies why Ad are so heavily used in the vaccination field.

Unfortunately, the presence of pre-existing anti-Ad serotype 5 (Ad5) immunity in a majority of population (50-90%) is hampering Ad5-mediated gene transfer. A few strategies have been proposed in order to overcome this limitation, such as virus capsid pseudotyping, replacing Ad5 with less prevalent serotypes or choosing non-human vectors.

More recently, another strategy was developed, consisting in genetically inserting epitopes of interest into the proteins of Ad capsid. This review will present the state-of-art and current perspectives of this vaccination approach, called "*epitope display*". First, simple modification in the capsid proteins, hexon, fiber, pIX or penton base will be described. Then, studies using vectors combining both *epitope display* and transgene-encoded antigen will be analyzed. We will finish by general conclusions of Ad *epitope display* vaccination performance and outline the remaining unanswered questions and the future perspectives.

## 3.2 Introduction

### 3.2.1 Adenovirus

Ad are non-enveloped, double stranded DNA viruses isolated for the first time 50 years ago from the human adenoid cells (Rowe, 1953). Since their discovery more than 55 serotypes have been distinguished, with serotype 2 and 5 being the most intensively studied and used in gene therapy and vaccination studies. Advances in structural biology techniques gave rise to the full, high resolution atomic structure of Ad (Liu *et al.*, 2010; Reddy *et al.*, 2010). The first generation of Ad vectors was established by deleting early gene E1 (Ad $\Delta$ E1) and thus rendering them replication-defective (Danthinne and Imperiale, 2000). In order to expand the capacity of transgene accommodation these vectors were additionally deleted in E3 segment (Ad $\Delta$ E1 $\Delta$ E3) which permitted to increase the cloning capacity up to 7,5 kb (Amalfitano *et al.*, 1998). Ad $\Delta$ E1 $\Delta$ E3 are remaining the most frequently used Ad vectors in vaccination studies.

### 3.2.2 Adenovirus immunogenicity

Adenovirus hallmark is the potency to induce robust innate and adaptive immune responses. Intrinsic immunogenicity of viral capsid was unveiled by McCoy *et al.*, who showed that innate responses induced by Ad vectors were transcription-independent (McCoy *et al.*, 1995). Strong production of pro-inflammatory cytokines and chemokines was linked to virus interaction with its cellular receptors, as well as recognition by cytosol, membranous and secreted Pattern Recognition Receptors (PRRs). All this branches of innate immunity sensing lead conjointly to the “cytokine storm”, which has been shown to interfere with transgene transcription from the viral vector (Sung *et al.*, 2001) and thus to limit the Ad efficiency in the classical vaccination strategy.

Both early and late gene products are targets of adaptive immunity, which includes B cell, CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses (Yang *et al.*, 2003; Onion *et al.*, 2007). Neutralizing Abs were shown to target principally HVRs of hexon (Sumida *et al.*, 2005; Roberts *et al.*, 2006; Bradley *et al.*, 2012). Nevertheless, subdominant population of fiber knob-directed Abs was also detected (Gahéry-Ségard *et al.*, 1998; Bradley *et al.*, 2012). Various T cell epitopes, both for CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes have been identified in the Ad capsid proteins, with hexon being the immunodominant T cell target. (Leen *et al.*, 2004 ; Olive *et al.* 2002; Onion *et al.*, 2007 ; Tang *et al.*, 2004; Tang *et al.*, 2006). Cytotoxic responses against fiber and penton base were also observed, although turned out to be much less potent and prevalent in the healthy individuals (Tang *et al.*, 2006).

### 3.2.3 Overcoming pre-existing Ad immunity

Strong immunogenic properties of Ad are hampering expression of vector-encoded transgene and reducing efficacy of this type of vaccination (described here as the classical vaccination) by limiting the quantity and duration of antigen availability (Schrimbeck *et al.*, 2008). Nevertheless, its natural adjuvant features provide valuable innate immune system stimulation, crucial in vaccine design. In order to overcome limitations of pre-existing Ad immunity and turn its properties in our favor, new strategies of Ad-based vaccination were developed. One of them comprises in shifting to different, less prevalent human Ad serotypes or non-human Ad (Keefer *et al.*, 2012; Shott *et al.*, 2008; Ouedraogo *et al.*, 2011; Baden *et al.*, 2013; Alexander *et al.*, 2013; Chmielewska *et al.*, 2014). Different appealing solutions propose vaccine delivery vectors based on non-human Ad, such as simian, canine, bovine or porcine serotypes (Bangari, 2006), with the biggest interest drawn to chimpanzee Ad (Fitzgerald *et al.*, 2003; Pinto *et al.*, 2003; Reyes-Sandoval *et al.*, 2004). The human non-Ad5 serotypes were also tested for their performance in a heterologous prime-boost injections (Thorner *et al.*, 2006; Lemckert *et al.*, 2005; Balandya *et al.*, 2014; Geisbert *et al.*, 2011). This strategy, however, requires construction of multiple vectors and verification of their efficacy and safety, which is not only costly but also time consuming. Thus, as a simpler alternative, Ad5-based vectors with altered capsid proteins begun to be developed. The availability of its detailed structure enabled precise alterations and allowed to engineer novel, more optimized Ad5 vectors with immunodominant fragments deleted or replaced by those from less prevalent serotypes. Exchange of whole hexon protein resulted in generation of chimeric viruses (Youil *et al.*, 2002) and Ad5 with alterations in hexon loops escaped pre-existing anti Ad5 immunity (Roy *et al.*, 1998; Gall *et al.*, 1998; Abe *et al.*, 2009).

### 3.3 Epitope display

Limited performance of classical vaccination strategy and availability of precise capsid protein alteration techniques led to the development of another solution for escaping neutralizing anti-Ad5 Abs “*Epitope display*” vaccination approach allows to bypass the gene-transfer stage of the antigen delivery by genetically inserting epitope of interest into the Ad capsid protein. Thus, epitope is available to the immune system as early as at the time of vector injection and pro-inflammatory ambiance provided by Ad5 ensures the stimulation of innate immunity and strong cytokine production. Moreover, the presence of various CD4<sup>+</sup> T cell epitopes in Ad capsid proteins could enhance the induction of adaptive immune response against the epitope, in agreement with the co-called “carrier-hapten theory”.

First application of this vaccination strategy dates to 1994, when insertion of an epitope coming from poliovirus VP1 capsid protein into the loop 1 of Ad2 hexon upon mice immunization resulted in serum able to recognize the VP1 (Crompton *et al.*, 1994). Since this first experiment many other studies chose hexon as an insertion site, tempted by its great abundance and well characterized structure of highly variable and flexible loops. Progressively, other major proteins, such as fiber or penton base along with the minor pIX protein also gained focus.

#### 3.3.1 Hexon display

Trimeric hexon protein, present in a total copy of 240 units organized in 20 triangular facets is the most abundant element of the icosahedrally shaped virion. In its structure we can distinguish the evolutionarily conserved core of the molecule, crucial for the capsid formation and weakly conserved loops exposed at the top of the molecule. Among these sequences seven Hyper Variable Regions (HVR) were originally described, later reclassified to nine (Crawford-Miksza and Schnurr, 1996, Rux *et al.*, 2004). They contain serotype-specific residues (Crawford-Miksza and Schnurr, 1995), remaining until today the most amenable sites for engineered modifications. More recently, HVR5 and HVR7 has been shown to participate in bridging Ad5 to the heparan sulfate proteoglycans through interaction with blood coagulation factor X (FX) (Vigant *et al.*, 2008; Alba *et al.*, 2008).

Comparative studies have been performed in order to detect the most accessible fragment in the hexon sequence of Ad5. One of them consisted in inserting his6 peptide into HVR2, 3, 5, 6, or HVR7. Epitope was shown to be well exposed on the native virion only when incorporated into HVR2 and HVR5, weakly accessible in the context of HVR7 and not exposed in HVR3 and HVR6 (Wu *et al.*, 2005). Ad5 HVR2 and HVR5 were also evaluated in a different study for their flexibility in accommodating size-increasing peptides, ranging from 45 to 95aa (Matthews *et al.*, 2008). HVR5

turned out to be more permissive and able to accommodate up to 65aa without losing virus viability, while HVR2 tolerated only 45aa peptide. Insert was accessible on the non-denatured virion for all rescued viruses and induced humoral responses in C57BL/6J mice, with higher IgG titer for HVR5 display. McConnell *et al.* in their study, using increasing size of epitope coming from *Bacillus anthracis* (*B. anthracis*) propose 36aa as a limiting size of Ad5 HVR5 insert in order to retain accessibility and ability to induce humoral responses in BALB/c mice. Attempts to detect cellular responses failed, which was justified by non-confirmed presence of T cell epitope within the selected antigen sequence (McConnell *et al.*, 2006).

Many studies used Ad5 HVR2 or HVR5 as an epitope insertion site for the vaccination purposes. Worgall and colleagues placed 14aa epitope of *Pseudomonas aeruginosa* (*P. aeruginosa*) outer membrane protein in the HVR5 of the hexon and upon mice vaccination detected humoral and cellular responses. High IgG titers and specific IFN- $\gamma$ -positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells conferred protection against pathogen challenge and could be boosted by second administration of the vector. Additionally, Abs titers significantly surpassed the outcome of vaccination with a homologous purified peptide immersed in Freud's adjuvant (Worgall *et al.*, 2005). Interestingly, differences in induced humoral responses were observed between three mice strains tested in this study, C57BL/6, BALB/c, and CBA. Titers of particular IgG subclasses varied significantly, shedding a light on the influence of MHC molecule allele on the induced immunity. HVR5 of Ad5 was also evaluated by our team and insertion of ovalbumin (Ova) epitope resulted in good peptide accessibility and strong humoral responses in C57BL/6 mice (Lanzi *et al.*, 2010). Abs titers could not be significantly increased with repeated vector administration, because of the induced anti-Ad Abs masking displayed epitope.

Palma and his group chose to investigate vaccination potential of HVR1-displayed epitope. To this end epitope from *Plasmodium falciparum* circumsporozoite protein was inserted into Ad5 hexon and after confirming its accessibility, injected into C57BL/6 mice. Strong humoral responses were detected and the induced Abs were capable of recognizing *P. falciparum* sporozoites *in vitro* (Palma *et al.*, 2011). Another study evaluating Ad5 HVR1 focused on vaccinating against *Trypanosoma cruzi*. Similarly, obtained results were promising, as humoral response induced in BALB/C or C57BL/6 mice after priming could be boosted in homologous injection and conferred protection in pathogen challenge test (Farrow *et al.*, 2014).

The permissiveness of HVR7-9 to peptide insertion was evaluated with the use of neutralizing B cell epitope of porcine reproductive and respiratory syndrome virus (PRRSV) GP5 protein (Hansra *et al.*, 2015). Vectors with an epitope placed in HVR8 and HVR9 could not be rescued, suggesting to us that Ad5 is unable to tolerate insertions in these sites. As for the HVR7 display, virus was viable, grew well in cell culture and the epitope was detected *in vitro* on the virion surface. No *in vivo* tests have been performed in this study.

**Table 3.1. Vaccination studies using hexon display strategy.**

Vector	Insertion site	Type of modification	Source of epitope	Evaluated insert size	Reference
Ad5	HVR1	substitution or insertion	<i>P.falciparum</i>	20 or 24aa	Palma <i>et al.</i> , 2011
Ad5	HVR1	substitution	<i>T.cruzi</i>	24aa	Farrow <i>et al.</i> , 2014
Ad5	HVR2	insertion	<i>HIV</i>	39aa	Matthews <i>et al.</i> , 2010*
Ad5	HVR2, 3, 5, 6 or 7	substitution or insertion	His6	12aa	Wu <i>et al.</i> , 2005
Ad5	HVR2 or HVR5	insertion	RGD-his6	45 to 95aa	Matthews <i>et al.</i> , 2008
Ad5	HVR1 or HVR5	substitution or insertion	<i>P. yoelii</i>	18aa	Shiratsuchi <i>et al.</i> , 2010*
Ad5	HVR5	insertion	Influenza A	9aa	Krause <i>et al.</i> , 2006*
Ad5	HVR5	substitution	<i>B.anthraxis</i>	16 to 245aa	McConnell <i>et al.</i> , 2006
Ad5	HVR5	insertion	<i>P. aeruginosa</i>	14aa	Worgall <i>et al.</i> , 2005
Ad5	HVR5	insertion	<i>P. aeruginosa</i>	14aa	Worgall <i>et al.</i> , 2007*
Ad5	HVR5	substitution	Ovalbumin	17 or 22aa	Lanzi <i>et al.</i> , 2010
Ad5	HVR7, 8 or 9	insertion	<i>PRRSV</i>	15aa	Hansra <i>et al.</i> , 2015
replicative Ad5	HVR1	insertion	<i>P.falciparum</i>	24aa	Karen <i>et al.</i> , 2014*
AdC68	HVR1 or HVR4	substitution or insertion	<i>Influenza</i>	39aa	Zhou <i>et al.</i> , 2013
Ad3	HVR1	substitution	flag, flaghis6 or flaglgshis6	8 to 19aa	Zhong <i>et al.</i> , 2012
Ad3	HVR1 + HVR2	substitution	<i>EV71</i>	15aa	Xue <i>et al.</i> , 2013
Ad5	HVR1+ HVR2 HVR1 + HVR5	substitution	<i>HIV-1</i> and his6	6 or 7aa	Gu <i>et al.</i> , 2013

\* for detailed description see paragraphs 3.3.5. & 3.3.6.

Zhou and colleagues compared vaccination performance of non-human vector, AdC68, displaying influenza virus epitope in HVR1 or HVR4 (Zhou *et al.*, 2013). *In vitro* analysis revealed significantly reduced accessibility of influenza epitope in HVR4. These results were confirmed by

assessing IgG titers upon C57BL/6 and ICR mice immunization, which were much more modest in case of the vector displaying peptide in HVR4.

Interesting approach was investigated by Zhong and co-workers. In their vaccination design multiple epitope peptide was placed into HVR1 of Ad3. Insert corresponded to flag, flaghis6 or flaglgshis6 sequence. Surprisingly, humoral response in BALB/C mice was raised against a new epitope, generated by adjacent sequences of flag, lgs and his6. As a result, Abs produced upon mice immunization with vectors displaying flaghis6 or flaglgshis6 sequences were not able to recognize GST-his6 and GST-flag fusion proteins *in vitro* (Zhong *et al.*, 2012). This experiment underlines the necessity of incorporating the epitopes separately into different regions rather than simultaneously into one hexon site.

In the following study the same team constructed and investigated multivalent Ad3 vector, bearing two different epitopes of enterovirus type 71 (EV71) in HVR1 and HVR2 (Xue *et al.*, 2013). *In vitro* as well as *in vivo* experiments indicated, that both epitopes were equally accessible and capable of inducing comparable humoral responses. Additionally, sera from BALB/C mice immunized by this bivalent vector neutralized EV71 more efficiently than sera of animals vaccinated with vectors displaying only one epitope. This suggests that accumulation of various epitopes on one viral particle could be a new promising vaccination strategy.

Different conclusions, however, can be drawn from similar study performed by Gu and his team (Gu *et al.*, 2013). HIV-1 epitope was placed in Ad5 HVR1 and, in parallel, His6 was introduced into HVR2 or HVR5. *In vitro* tests showed that all the epitopes are equally exposed on the virus surfaces. Nevertheless, upon BALB/C mice immunization significant differences were observed. Both viruses efficiently induced production of anti-HIV Abs, however His6 humoral response was detected only for the virus displaying this peptide in HVR5. These results suggest, that HVR2 in the context of another epitope placed in HVR1 may not be a suitable insertion site, as this combination could be detrimental to the *in vivo* presentation of the antigen, resulting in lack of antibody response. Whether the discrepancy between these two studies is a result of the difference in chosen epitopes or vector serotypes, remains uncertain.

Taken together, the studies discussed above are signaling that the most promising site for epitope insertion in hexon is HVR5. It seems to tolerate the longest inserts without jeopardizing the virus viability or stability and induced humoral responses are strong and protective. Other convenient sites for peptide display are HVR1, 2 and to much lesser extend 4 and 7. Tests evaluating HVR 3, 6, 8 and 9 permissiveness failed in producing the viable vectors, indicating that these regions may contribute to capsid formation and/or stability. As for the size of the displayed peptide, depending on the HVR, 45-66 aa seems to be the limiting length. The longer the inserts become, the

less infectious the rescued viruses are. One has to also be prudent while trying to insert epitopes in multiple HVRs, as this may result in their unequal accessibility.

### 3.3.2 Fiber display

Fiber protein in a non-covalent interactions with penton base protein generates a complex called penton, located in all 12 vertices of Ad capsid. It is a trimeric protein, protruding from the capsid surface, with three distinguishable structure domains. Conserved base is anchoring fiber to the penton base (Zubieta *et al.*, 2005). The prolonged shaft with the repeating conserved KKTK motif plays a role in interactions with heparan sulfates proteoglycans (Dehecchi *et al.*, 2000). Finally, distal knob is interacting with coxackie-adenovirus receptor (CAR) and displaying a flexible loops on its surface (Freimuth *et al.*, 1999). Among them, HI loop was first chosen to be modified as is not involved in CAR interactions (Xia *et al.*, 1994).

The differences in the selected epitopes across the performed studies disabled the direct correlation between epitope insertion site and vaccination potential of recombinant vector until an elegant analysis performed by Krause. By introducing identical peptide coming from influenza A virus hemagglutinin sequence into HVR5 of hexon, C terminus of pIX, HI loop in fiber protein or the RGD motif in penton base, she concluded, that the most promising target for epitope insertion is the fiber protein (Krause *et al.*, 2006). Despite *in vitro* analysis, indicating that all insertion sites resulted in almost equal epitope accessibility (with slightly higher Abs binding to hexon), upon *in vivo* immunization of three different strains of mice, C57BL/6, BALB/c, and CBA, fiber display elicited the highest IgG antibody titer. It was also the most potent in inducing IgM production in CBA mice. These results were obtained upon injecting the same amount of viral particles in each BALB/c experimental group but also when number of injected viral vehicles was adjusted in order to obtain equal epitope copy number for each group. Evaluation of anti-hemagglutinin CD4<sup>+</sup> T cell responses in CBA mice by ELISPOT assay also privileged fiber display as the most immunogenic. This observation was surprising taking into account the fact that this protein has 20 fold lower total copy number in one viral particle than the hexon protein.

Our team also demonstrated the exceptional potential of fiber display in inducing strong humoral responses in C57BL/6 mice (Lanzi *et al.*, 2010). By inserting Ova B cell epitope into the HI loop of this capsid protein we showed that after priming epitope placed in fiber is less immunogenic than hexon HVR5 display. However, upon second administration, fiber display induced higher anti-epitope IgG titers, boosted more than 300 fold in comparison to the first injection, while no increase was observed for virus with epitope inserted into hexon. In order to understand the mechanism of

remarkable boosting potential of fiber display, splenocytes, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells or serum from naïve or Ad-immunized mice were adoptively transferred into naïve recipient mice. Subsequent vector administration unmasked crucial role of anti-Ad Abs, as epitope placed in fiber elicited in this background stronger anti-Ova humoral responses than in naïve set-up. On the contrary, in the presence of anti-Ad sera, hexon display vector presented significant reduction of efficacy. We observe discrepancy between our and Krause's studies. Contrasting observations of vectors vaccination potential upon one injection could result from different antigen choice, mode of modification (insertion vs substitution) or way of immunization (i.p vs. i.m.).

**Table 3.2. Vaccination studies using fiber display strategy.**

Vector	Insertion site	Type of modification	Source of epitope	Size of insert	Reference
Ad5	HI loop	insertion	Influenza A	9aa	Krause <i>et al.</i> , 2006
Ad5	HI loop	substitution	Ovalbumin	17 or 22aa	Lanzi <i>et al.</i> , 2010
Ad5	HI loop	insertion	<i>P. yoelii</i>	18aa	Shiratsuchi <i>et al.</i> , 2010*
Ad5	CD, DE, FG or HI loop, C-terminus	insertion	<i>P.aeruginosa</i>	14aa	Sharma <i>et al.</i> , 2013

\* for detailed description see paragraph 3.3.5.

Various sites of fiber protein were evaluated for their peptide insertion permissiveness and vaccination potential. To this end, different vectors were constructed with epitope from *P.aeruginosa* OprF protein placed in loops CD, DE, FG, HI, fiber C-terminus and in hexon HVR5 (Sharma *et al.*, 2013). *In vitro* analysis showed that all fiber alterations resulted in decreased CAR-dependent infectivity, with the least reduction observed for FG and HI insertion. These two vectors also gave the most promising results during assessment of epitope accessibility on the intact virion, bypassing the hexon HVR5 display. Subsequent *in vivo* studies in BALB/c mice confirmed the vaccination capacities of FG and HI display, which elicited IgM mucosal immunity and high IgG titers, overcoming after 3 injections those induced by Ad5 vector encoding OprF protein as a transgene. Cellular CD4<sup>+</sup> and CD8<sup>+</sup> IFN- $\gamma$  responses induced by this vaccine vehicles were detectable, although

more modest than those found in mice vaccinated with transgene-encoding vector. Both branches of adaptive responses induced by epitope placed in FG and HI conferred protection against pathogen challenge.

Studies using fiber as a site of epitope display are less numerous than those involving hexon. This may be caused by 36 fold lower abundance of this protein, misleadingly suggesting weaker stimulation of immune system. However, studies evaluating vectors with heterologous epitopes placed in this capsomer highlight its remarkable vaccination potential. In all three papers quoted in this section, immune responses induced upon several injections are more potent and stronger than for hexon display. In addition, in Ad-immunized background fiber-modified vectors seem to escape pre-existing immunity more efficiently, making them a very promising tool in Ad5 vector vaccination. In all studies HI loop was successfully used as an epitope insertion site. However, FG loop turned out to be equally accessible and potent, creating an exciting possibility of inserting many epitopes in different fiber sites.

### **3.3.3 pIX display**

Protein pIX is a minor components of capsid and it is stabilizing the architecture of the viral particle, acting as a “molecular glue” (Liu *et al.*, 2010). It interacts with hexon-formed structures, called Group Of Nine (GON), which are forming each of the capsid faces. Thus, 240 copies of pIX can be found in one viral particle. Three domains have been identified in pIX structure: conserved amino-terminal, central alanine-rich (found only in the human Ad serotypes), and conserved carboxy-terminal. In fully formed Ad structure amino-termini remain embedded in the GON while carboxy-termini is exposed on the surface, which makes it a promising locus for antigen insertion (Rosa-Calatrava *et al.*, 2001).

One of the first incorporation of heterologous peptide into pIX was described by Dimitriev, where carboxy-terminal Flag epitope introduced in pIX ectodomain did not alter virus infectivity and was shown to be accessible *in vitro* (Dimitriev *et al.*, 2002). Subsequent experiments by fusing with pIX introduced into the Ad capsid the whole proteins, such as GFP (Meulenbroek *et al.*, 2004), Herpes Simplex Virus Type 1 Thymidine Kinase (Li *et al.*, 2005; Matthews *et al.*, 2006), metallothionein (Liu *et al.*, 2014) and single-chain or single-domain antibody fragment (Vellinga *et al.*, 2007; Poulin *et al.*, 2010). These studies were aiming at analyzing vector biodistribution or altering its tropism. Another interesting study inserted complement-regulatory peptide in pIX in order to reduce the Ad-related pro-inflammatory cytokine and chemokine production, which could be especially advantageous in the field of gene therapy (Seregin *et al.*, 2010). An insertion of single-chain T cell receptor directed

against the CT antigen melanoma-associated antigen (MAGE)-A1 in complex with the HLA class I molecule of haplotype HLA-A1 is a promising discovery with regard to Ad-based cancer therapy (de Vrij *et al.*, 2008).

As for the vaccination field, in the previously quoted study 9aa epitope of influenza was inserted into pIX. *In vitro* evaluation reported relatively weak peptide accessibility, completely abolished CAR-dependent virus infectivity and significantly reduced integrin-dependent cell entry (Krause *et al.*, 2006). IgG titers induced upon vaccination didn't reach the limit of detection. First successful experiment using pIX in vaccination field was performed by fusing *Yersinia pestis* V or F1 antigen to its C-terminus (Boyer *et al.*, 2010). For both antigens humoral responses were detected upon first injection into BALB/c mice and conferred protection in a pathogen challenge test. Second vector administration additionally ameliorated the efficacy of vaccination by increasing the anti-antigen Abs titers and protecting more mice from intranasal exposure to *Yersinia pestis*. Efficacy of epitope display strategy was also compared to the subunit vaccine performance, where molar equivalent of purified V or F1 proteins were administered with Alhydrogen adjuvant (aluminium hydroxide wet gel suspension). Much better results were obtained for antigen exposed on pIX, both on the level of Ig titers and pathogen challenge assay. This is in agreement with the study performed by Worgall *et al.*, in 2005, where superiority of Ad over classical Freund's adjuvant was demonstrated.

Another study fused Friend murine leukemia virus (FMLV) gp70 protein to pIX (Bayer *et al.*, 2010). As the vaccination regimens performed in this study comprised simultaneous vaccination with two different vectors, encoding or displaying various FMLV antigens, it is difficult to assess the exact contribution of each antigen form to the immunization outcome. Nevertheless, vaccination regimen including vector displaying antigen in pIX induced high binding antibody titers. The neutralizing titers, on the other hand, were markedly lower and CD4<sup>+</sup> T cell responses were only weakly induced. The pIX antigen displaying vector needed additionally a transgene antigen form in its genome to trigger efficient response, which is described in more details in section 1.5.

Prospect of inserting the whole protein in the Ad capsid ensures a few encouraging features. One of them is possibility of administering several relevant epitopes that could broaden the induced immunity. Another advantage of this approach is the fact that B cell epitope in the context of the original protein will more likely acquire proper conformation. Despite these features pIX remains less frequently selected for this vaccination strategy than hexon or fiber. Big insertion capacity (up to 65 kDa by Matthews *et al.*, 2006) of this minor Ad protein is favoring its application in gene therapy studies, where it gained more spotlight than in the context of vaccination.

**Table 3.3. Vaccination studies using pIX or penton base display strategy.**

Vector	Insertion site	Type of modification	Source of epitope	Size of insert	Reference
<i>pIX display</i>					
Ad5	C-terminus	insertion	Influenza A	9aa	Krause <i>et al.</i> , 2006
Ad5	C-terminus	terminal fusion	Ovalbumin FMLV	45kDa or 70kDa	Bayer <i>et al.</i> , 2010
Ad5	C-terminus	terminal fusion	<i>Yersinia pestis</i>	37kDa or 15 kDa	Boyer <i>et al.</i> , 2010
Ad5	C-terminus	terminal fusion	Ovalbumin	45kDa	Johrden <i>et al.</i> , 2013*
<i>penton base display</i>					
Ad5	RGD loop	<i>insertion</i>	Influenza A	9aa	Krause <i>et al.</i> , 2006
Ad3 dodecahedron	RGD loop or N- terlminus	substitution	Influenza A	18aa	Szurgot <i>et al.</i> , 2013

\* for detailed description see paragraph 3.3.6.

### 3.3.4 Penton base

Pentameric penton base is located in all 12 vertices of Ad capsid, resulting in total number of 60 copies in one viral particle. The monomeric penton base shows high similarity to hexon in its topology with a jelly-roll barrel domain located at the base, responsible for fiber binding and two loops formed at the distal end, containing conserved RGD motif implicated in interaction with  $\alpha_v$  integrins (Zubieta *et al.*, 2005).

Studies using penton base as a platform are much less frequent than for previously described capsomers. Penton base permissiveness for epitope insertion was first evaluated by Krause

*et al.*, in 2006. Epitope from influenza was placed in RGD loop, which resulted in good accessibility and Ab recognition *in vitro*. However, infectivity test showed that both CAR- and integrin-dependent pathway of infection was notably diminished. ELISA test failed to detect the induction of humoral response upon C57BL/6 or BALB/C mice vaccination. Interestingly, when using different mice strain, CBA, a weak signal was detected, suggesting small production of Abs. To our knowledge, after this first study there was no trial that successfully introduced heterologous epitopes into the penton base of the Ad viral particle for vaccination purposes. However, Ad3 dodecahedrons (Dds) were evaluated for their vaccination potential. Dds are VLP structures lacking viral genome, formed solely by penton base and recognized for their extraordinary capacity to enter the cells (Fender *et al.*, 1997). T cell epitope from Influenza M1 protein was selected and placed in variable loop or at the N-termini of penton base (Szurgot *et al.*, 2013). *In vitro* tests confirmed efficient uptake and cross-presentation of inserted antigen. After chicken immunization, Influenza-specific CD4<sup>+</sup> and CD8<sup>+</sup> cellular responses were induced. Addition of Freud's adjuvant did not result in reinforcement of induced response, emphasizing the dodecahedron adjuvant potential.

It would be interesting to compare vaccination performance of epitope display on dodecahedron and full Ad particle in one study in order to elucidate if efficient dodecahedron uptake could balance impoverished number of Ad-associated CD4<sup>+</sup> T cell epitope number.

### **3.3.5 Combining epitope display strategy with classical vaccination**

The encouraging results of epitope display strategy led to the idea of combining this new immunization approach with the classical vaccination design. One of the first experiments investigating this idea was study performed with Ad5 vector AdOprF.RGD.Epi8, displaying *P.aeruginosa* B cell epitope coming from OprF protein (Epi8) in hexon HVR5 and encoding the whole OprF protein in the form of transgene (Worgall *et al.*, 2007). Additionally, RGD motif was introduced into fiber knob to enhance DCs transduction in an integrin-dependent manner. Its vaccination potential was compared to Ad vector encoding OprF only. Two and four weeks after mice immunization no difference between IgG titers was observed between the groups. Analysis of Th2 cellular responses showed similar level of Il-4 production. On the contrary, detection of IFN- $\gamma$  demonstrated stronger CD4<sup>+</sup> Th1 and CD8<sup>+</sup> cellular response in mice immunized with vector combining epitope and transgene. This enhancement resulted also in better mice survival in *P.aeruginosa* pulmonary challenge test. Advantage of combining two vaccination strategies in one vector was also underpinned by results of boosting the immune response in a homologous injection. Antibody titers were increased only in the presence of OprF epitope in the capsid and this led to

better mice protection in pulmonary infection with *P. aeruginosa*. Nevertheless, it has to be underlined that differences in *in vivo* performance can also be related to better DCs infectivity by AdOprF.RGD.Epi8 due to the presence of RGD motif.

Another study evaluated immune responses induced upon vaccination by Ad5 vectors encoding Friend murine leukemia virus glycoprotein g70 and/or displaying whole Env protein in pIX (Bayer *et al.*, 2010). Boost injections for all groups of FV-susceptible CB6F1 mice were performed with the use of homologous vectors, having its Ad5 fiber pseudotyped with knob and shaft from serotype 35. After two injections vector displaying antigen in its capsid (without transgene) showed diminished viral load control, lower neutralizing Abs titers and lack of detectable CD4<sup>+</sup> T cell responses. On the contrary, vaccination with the vector combining antigen in the form of transgene and a pIX display led to more efficient humoral and cellular responses. It is worth noticing that titers of total binding IgG Abs were similar for both transgene/display and display-only vectors. Differences in the neutralizing Abs concentration between those groups could result from much weaker CD4<sup>+</sup> T cell response upon immunization with vector displaying the g70, causing the lack of Abs affinity maturation. This underlines the beneficial effect of the presence of CD4<sup>+</sup> T cell epitope in the antigen sequence. Analogous vectors using Ova protein model were used to evaluate CD8<sup>+</sup> T cell proliferations. Results of adoptive transfer with CFSE-stained, Ova-specific CD8<sup>+</sup> lymphocytes, analyzing kinetics of the response, again privileged vector combining two vaccination strategies (Ad5.pIXova) over classical vaccination vector (Ad5.ova). Production of IFN- $\gamma$ , IL-2 and TNF $\alpha$  by CD8<sup>+</sup> T cells, contrarily, was similar for both groups. Vector carrying antigen in pIX but not in the form of transgene was not tested in this part of study.

Matthews and colleagues proposed combining hexon HVR2 display of B cell epitope from HIV Env protein, MPER, with HIV Gag protein provided in the form of transgene (Matthews *et al.*, 2010). In this design the displayed epitope is not additionally present in the sequence of encoded protein and combining this two different antigens in one viral particle serves rather as a “vaccine cocktail”, stimulating simultaneously anti-Env humoral response and anti-Gag cellular immunity. Control vector were also constructed, encoding Env or Gag only, as well as vector carrying B cell epitope in HVR2 without transgene. After confirming *in vitro* transgene expression and epitope accessibility on the capsid surface mice were immunized twice in a homologous injection design. Vectors displaying MPER epitope elicited specific, strong humoral responses and were able to boost it upon second administration. Surprisingly, no anti-MPER Abs were detected in group vaccinated with Ad encoding whole Env protein. This was explained by the fact that Env, as a precursor protein, is cleaved into gp41, comprising MPER epitope, and gp120. Production of the latter could mask the Ab recognition of gp41. Additional presence of Gag transgene, providing CD4<sup>+</sup> T cell epitopes did not enhanced anti-MPER Ab titers. Evaluation of Gag-specific cellular response revealed no significant

difference between epitope display/transgene and transgene-only groups. On the contrary, upon boosting, vector encoding Gag and carrying MPER in HVR2 was able to reinforce anti-Gag CD8<sup>+</sup> T cell response. These results could be limited by weaker anti-Ad immune response triggered by vector with alteration in hexon.

Interesting study combining transgene expression with fiber and hexon display in one viral particle was performed by Shiratsuchi and his team (Shiratsuchi *et al.*, 2010). In a *Plasmodium yoelii* circumsporozoite (CS) protein model different recombinant Ad were constructed. All of them were encoding whole CS protein in their genome (WT/CS-GFP) and additionally some of the vectors were displaying CS B cell epitope in one of the following sites: hexon HVR1 (B-HVR1/CS-GFP), hexon HVR5 (B-HVR5/CS-GFP), fiber HI loop (B-Fib/CS-GFP) or simultaneously in hexon HVR1 and fiber HI loop (B-HVR1/B-Fib/CS-GFP). Interestingly, CS peptide placed in fiber was recognized *in vitro* to the similar extend as during its incorporation into hexon, although total copy number differs 20 fold. While after single vector administration no difference was observed among the groups, upon three injections of increasing virus doses into mice, Ab titers recognizing the displayed epitope were significantly higher for all capsid modified vectors while compared to WT/CS-GFP. Sera from mice injected with B-HVR1/CS-GFP and B-HVR1/B-Fib/CS-GFP were the most potent in *Plasmodium yoelii* CS neutralization test *in vitro*, proving their ability to recognize not only synthetic peptide but also native epitope. Adding additional epitope copy in fiber does not seem to increase the efficacy of HVR1-display immunization. Cellular responses, on the contrary, were weaker for all the groups having epitope placed in capsid proteins, resulting probably from altered infectivity of this vectors as shown *in vivo*. However, in mice with pre-existing anti Ad5 immunity only HVR1/CS-GFP and B-HVR1/B-Fib/CS-GFP could elicit CD8<sup>+</sup> T cell immunity, hinting about the presence of Ad5 neutralizing epitope in HVR1. Pathogen challenge assay showed that injections with HVR1/CS-GFP protected mice better than immunization with WT/CS-GFP.

Non-human Ad was also assessed for the potential of mounting potent adaptive immune responses in this vaccination strategy. Chimpanzee-derived AdC68 vectors were generated, displaying B cell epitope from influenza M2 protein ectodomain in hexon HVR1 or HVR4. Additional vectors were produced by introducing the sequence encoding NP-M2e fusion protein into the genome of wild-type or M2e-modified hexon (Zhou *et al.*, 2013). All vectors induced detectable anti-M2e responses in mice, however, both Ad vectors with epitope in HVR1 triggered markedly higher anti-M2e Abs titers. Double injection with these constructs resulted in stronger humoral responses than in a group undergoing heterologous injection of AdC68 and AdC6 vectors, both encoding NP-M2e. What is worthy of note, is that additional presence of antigen in a form of transgene did not enhance the strong vaccination performance of epitope displaying vectors as the titers were not increased. Nevertheless, Abs avidity was strengthened by concomitant NP-M2e

expression, suggesting that protracted display of transgene products over time drives maturation of B cells. Weaker efficiency of HVR4 display upon prime and boost could be explained by reduced Abs accessibility of this site observed *in vitro*. After priming NP-specific CD8<sup>+</sup> T cells were detected for both hexon modified vectors encoding NP-M2e but their frequencies were not increased by boost injection. Induction of potent humoral and cellular responses by hexon modified vectors expressing NP-M2e conferred higher protection in a pathogen challenge assay than in case of epitope display only vector.

The strategy to combine classical vaccination with new epitope display approach offers an interesting features. Transgene form ensures strong Abs production upon priming and peptide present in the capsid allows for efficient boosting of induced humoral response. By providing supplementary stimulation to the B cells, encoded antigen drives Abs affinity maturation and serves also as a target for cellular responses.

### 3.3.6 Epitope display strategy in alternative context of vaccination

Johrden with his group compared immunization potential of virus displaying Ova in pIX protein (Ad5.pIXova) with those of coated polystyrene particles loaded with recombinant Ova. UV-inactivated Ad5.pIXova (UV-Ad5<sup>pIXova</sup>) was also included in the study to evaluate the influence of *in vivo* viral genome expression (Johrden *et al.*, 2013). Upon two injections, Ad5.pIXova induced the most significant IgG1 and IgG2c anti-Ova Ab titers, surmounting humoral response triggered by UV-Ad5<sup>pIXova</sup> or polystyrene-Ova particles. Similar patterns could be observed in the analysis of cellular CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses, highlighting the importance of *in vivo* transgene expression. Ad vectors adjuvant properties were emphasized in *in vitro* DC activation assay, where Ad5.pIXova and UV-Ad5<sup>pIXova</sup> induced IFN- $\alpha$  and IFN- $\beta$  production comparable to LPS stimulation.

Recently performed study evaluated in monkeys the vaccination potential of the replicative Ad vector, bearing B cell epitope from the circumsporozoite protein (CSP) of *Plasmodium falciparum* in the hexon HVR1 (Karen *et al.*, 2014). Four *Aotus nancymaae* monkeys were used in this study. In order to confirm the virus ability to replicate, Ad vectors encoding GFP protein were first orally administered to 2 animals. Due to the problems with GFP detection following immunization schedule was based on direct delivery into the trachea by percutaneous puncture (IT), which resulted in efficient transgene expression. Interestingly, monkeys that first received orally GFP-encoding control virus responded differently to subsequent IT administration of epitope-display vector. These two animals developed high-level of anti-CSP responses as soon as after the first or the second intratracheal immunization. Monkeys which had not received oral immunization with control vector

displayed weak anti-CSP titers after the first exposure. The anti-CSP Ab titer gradually increased with the second and third virus administration, to finally reach the titer of orally pre-immunized mice. This highlights the very potent priming of oral virus administration and role of anti-Ad immunity in inducing the adaptive responses against displayed peptide. Produced anti-CSP Abs recognized full-length CSP expressed normally on the sporozoite surface. Result of sporozoite neutralization *in vitro* varied significantly between the four tested animals. This could be the result of slight genetic differences of this outbred monkeys. It would be interesting to compare in parallel the outcome of vaccination with the homologous, non-replicative vector. This could shed a light on the relevance of the presence of newly synthesized virus proteins and DNA on the vector immunogenicity.

The importance of Ad proteins expressions in the context of efficient vaccination is still a burning question. The interesting conclusions could be drawn from the study where empty capsid or UV-inactivated vector were compared to non-replicative and replicative vector in a homologous setting.

### 3.4 Conclusions and perspectives

Epitope display strategy is a promising approach to vaccinate against the infectious diseases and cancer. Genetic insertion of heterologous epitope of interest into the Ad capsid offers many advantages. One of them is contribution of invaluable adjuvant properties of Ad viral particles to the magnitude of anti-insert adaptive responses. Indeed, some studies discussed in this review compared that the same purified peptide, administered with a standard adjuvant such as Freund's complete adjuvant, gives weaker immune responses than the same epitope in the context of Ad capsid insertion. This is due to the efficient stimulation of innate immunity by Ad but also, supposedly, could result from better access to APC and good peptide exposure on the capsid surface. It has been also suggested that repetitive display of the epitope on the capsid surface can lead to cross-linking of BCRs upon the peptide recognition. This can in turn activate B cells and reinforce humoral response. Techniques to enhance stability of the B cell epitopes conformations are also being developed and include use of special scaffolds, flanking the epitope by small sequences having propensity for folding into  $\alpha$ -helix or adding the linkers to improve presentation. As for T cell epitopes, a few studies showed that flanking the selected peptide by 2-5aa long fragments coming from the natural protein sequence may enhance the epitope MHC presentation.

Little is known about the molecular basics of the epitope display immunogenicity. Results of various studies show efficient induction of immune responses even in presence of preexisting Ad Abs, hinting that gene transfer is not necessary in order to mount efficient immune responses against the displayed epitope. Dodecahedrons particles, deprived of viral DNA, were still able to trigger strong cellular responses against inserted influenza T cell epitope. This suggests that stimulation of TLR9 or cytosolic DDX41 /cGAS, the innate sensors for Ad DNA, is not a crucial step in the induction of adaptive responses against the heterologous peptide. Results obtained with UV inactivated Ad, however, suggest that DNA cross-linking diminishes the vector performance. It would be interesting to compare in parallel the immunogenicity of dodecahedron particles, Ad viral particles deprived of viral genome, so-called Ad empty capsids and fully formed Ad particles. One would assume that due to the lack of many additional Ad CD4<sup>+</sup> epitopes present in the hexon protein, dodecahedrons would display the weakest immunogenic potential. Also, role of another Ad associated PAMPs, such as TLR2 or TLR4 remains to be elucidated.

Another factor having an impact on the vaccination outcome is MHC molecules allele variability. Evaluation of humoral responses in different strains of mice disclosed that T cell epitope comprised in the sequence of displayed peptide plays a big role in the total IgG production and influences percentage of particular IgG subclasses contribution to the final titer. This genetic restriction of vaccination performance suggests that vectors displaying B cell epitope with

intrinsically embedded T cell epitope can induce different humoral responses in immunogenetically heterogeneous population, depending on the avidity of peptide anchoring to MHC molecule as well as on the strength of interaction with TCR.

The importance of T cell epitope presence was confirmed in studies combining B cell epitope display with the whole antigen protein encoded as a transgene. Even though the total Abs amount was not altered by the additional presence of transgene antigen, the neutralizing Abs titer and the avidity was significantly enhanced. Helper T lymphocytes induced against the supplementary epitopes provided by encoded heterologous protein along with protracted display of the transgene contributed to additional B cell activation and maturation of humoral response. On the level of cellular responses, there are strong evidences favoring antigen administration in the form of transgene as both CD4<sup>+</sup> and CD8<sup>+</sup> are stronger and confer better protection against pathogen. Nevertheless, as far as humoral response is concerned, transgene form of antigen on its own, although capable of inducing high antibody titers, was unable to further increase the Ig production after second administration. On the contrary, epitope display, especially in fiber protein, upon several administrations triggered as potent humoral immunity as transgenic form of antigen or even surpassed it. Hence, the interest of combining this two vaccination strategies in one viral particle would assume strong induction of both cellular and humoral responses by transgene antigen upon priming and contribution of capsid-displayed epitope to further increase in Abs titers upon boosting.

Discovery of various sites in the Ad capsid, permissive for epitope insertion, created an opportunity of placing more than one copy of epitope, or what's more, inserting two different peptides in one viral particle. Simultaneous display of identical peptide in hexon and fiber protein revealed that additional epitope copy in fiber did not further enhanced good immunization performance of hexon display. Studies evaluating the vaccination with two different epitopes in various hexon HVRs shown that concomitant display may result in unequal vaccination potency, presumably due to the sterical hindrance and uneven access to the epitopes. This results, however, vary depending on the size of inserted epitope or the distance between insertion sites in the final conformation of hexon protein. So far, no study has evaluated the immunization abilities of vector with alteration in two distinct sites of fiber. In regard to its remarkable boosting potential this could generate very promising tool in vaccination field. Exploiting multiple insertion sites in the Ad capsomers is a very appealing strategy, especially since it has been shown that placing multiple epitopes in one single site results in humoral responses targeting new epitopes generated by adjacent peptide sequences.

Another limit is the insert size that particular capsomer is able to tolerate. Hexon has been evaluated for their insertion capacity and depending on the HVR, peptide of up to 30-40aa seems to be the limiting size. Even though viruses with longer epitopes have been obtained, their infectivity

and stability was decreased. As for fiber loops, 22aa is the longest insert successfully introduced into its structure. No study so far investigated the ability of this capsomer to accommodate increasing peptide fragments. Protein pIX, on the other hand, possess a remarkable ability to display not only the selected peptides but also the entire heterologous proteins. This is of big interest not only in the vaccination field but also in gene therapy where the entire therapeutic protein needs to be provided to counteract the pathology.

Except T cell epitope from influenza A placed on Ad3 dodecahedron surface, no other study so far evaluated the outcome of vaccination with Ad displaying CD8<sup>+</sup> T cell epitope. Whether this strategy would repeat the success of B cell epitope display, remains to be elucidated. In case of obtaining sufficient cellular response stimulation, combining T cell and B cell epitope display on one viral particle could be an interesting perspective.

Finally, another viruses were also used as a vaccine platform in this approach. A study using Influenza virus, Vaccinia virus or Rabies virus vectors displaying *B.anthraxis* epitope showed that only heterologous injection consisting in priming with Influenza vector resulted in production of neutralizing Abs (Langley *et al.*, 2010). This is a drawback in comparison to Ad efficiency, where the same vector can be re-injected in order to reinforce potent adaptive responses. Rabies virus pseudotyped with SIV-1 glycoprotein, however, triggered production of neutralizing Abs and cellular responses controlling viremia in rhesus macaques (McKenna *et al.*, 2004). Adeno-associated virus (AAV) was also tested and B cell epitope form Ova placed in its surface protein induced humoral responses (Manzano-Szalai *et al.*, 2014). Still, for each vector administration an additional adjuvant was included, masking the natural adjuvantic properties of AAV.

All things considered, in spite of certain limitations Ad vectors remain one of the most promising platform in vaccination field. *Epitope display* vaccination strategy, reviewed in this paper, proved its efficacy in a various vaccination designs. Nevertheless, a few questions still remain to be answered and further investigation of molecular mechanisms of this relatively new immunization approach could result in a very potent vaccination tools.

## References

1. Abe, S., et al., Adenovirus type 5 with modified hexons induces robust transgene-specific immune responses in mice with pre-existing immunity against adenovirus type 5. *J Gene Med*, 2009. 11(7): p. 570-9.
2. Alba, R., et al., Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood*, 2010. 116(15): p. 2656-64.
3. Alexander, J., et al., Pre-clinical development of a recombinant, replication-competent adenovirus serotype 4 vector vaccine expressing HIV-1 envelope 1086 clade C. *PLoS One*, 2013. 8(12): p. e82380.
4. Amalfitano, A., et al., Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. *J Virol*, 1998. 72(2): p. 926-33.
5. Baden, L.R., et al., First-in-human evaluation of the safety and immunogenicity of a recombinant adenovirus serotype 26 HIV-1 Env vaccine (IPCAVD 001). *J Infect Dis*, 2013. 207(2): p. 240-7.
6. Balandya, E., et al., Adenovirus serotype 26 and 35 vectors induce simian immunodeficiency virus-specific T lymphocyte responses in foreskin in rhesus monkeys. *J Virol*, 2014. 88(7): p. 3756-65.
7. Bangari, D.S. and S.K. Mittal, Development of nonhuman adenoviruses as vaccine vectors. *Vaccine*, 2006. 24(7): p. 849-62.
8. Bayer, W., et al., Vaccination with an adenoviral vector that encodes and displays a retroviral antigen induces improved neutralizing antibody and CD4+ T-cell responses and confers enhanced protection. *J Virol*, 2010. 84(4): p. 1967-76.
9. Boyer, J.L., et al., Protective immunity against a lethal respiratory *Yersinia pestis* challenge induced by V antigen or the F1 capsular antigen incorporated into adenovirus capsid. *Hum Gene Ther*, 2010. 21(7): p. 891-901.
10. Bradley, R.R., et al., Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. *J Virol*, 2012. 86(1): p. 625-9.
11. Bradley, R.R., et al., Adenovirus serotype 5-specific neutralizing antibodies target multiple hexon hypervariable regions. *J Virol*, 2012. 86(2): p. 1267-72.
12. Chmielewska, A.M., et al., Combined adenovirus vector and hepatitis C virus envelope protein prime-boost regimen elicits T cell and neutralizing antibody immune responses. *J Virol*, 2014. 88(10): p. 5502-10.
13. Crawford-Miksza, L. and D.P. Schnurr, Analysis of 15 adenovirus hexon proteins reveals the location and structure of seven hypervariable regions containing serotype-specific residues. *J Virol*, 1996. 70(3): p. 1836-44.
14. Crompton, J., et al., Expression of a foreign epitope on the surface of the adenovirus hexon. *J Gen Virol*, 1994. 75 ( Pt 1): p. 133-9.
15. Danthinne, X. and M.J. Imperiale, Production of first generation adenovirus vectors: a review. *Gene Ther*, 2000. 7(20): p. 1707-14.
16. de Vrij, J., et al., Adenovirus targeting to HLA-A1/MAGE-A1-positive tumor cells by fusing a single-chain T-cell receptor with minor capsid protein IX. *Gene Ther*, 2008. 15(13): p. 978-89.
17. Dechecchi, M.C., et al., Heparan sulfate glycosaminoglycans are involved in adenovirus type 5 and 2-host cell interactions. *Virology*, 2000. 268(2): p. 382-90.

18. Dmitriev, I.P., E.A. Kashentseva, and D.T. Curiel, Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J Virol*, 2002. 76(14): p. 6893-9.
19. Farrow, A.L., et al., Immunization with Hexon modified adenoviral vectors integrated with gp83 epitope provides protection against *Trypanosoma cruzi* infection. *PLoS Negl Trop Dis*, 2014. 8(8): p. e3089.
20. Fender, P., et al., Adenovirus dodecahedron, a new vector for human gene transfer. *Nat Biotechnol*, 1997. 15(1): p. 52-6.
21. Fitzgerald, J.C., et al., A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J Immunol*, 2003. 170(3): p. 1416-22.
22. Freimuth, P., et al., Coxsackievirus and adenovirus receptor amino-terminal immunoglobulin V-related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. *J Virol*, 1999. 73(2): p. 1392-8.
23. Gahery-Segard, H., et al., Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. *J Virol*, 1998. 72(3): p. 2388-97.
24. Gall, J.G., R.G. Crystal, and E. Falck-Pedersen, Construction and characterization of hexon-chimeric adenoviruses: specification of adenovirus serotype. *J Virol*, 1998. 72(12): p. 10260-4.
25. Gu, L., et al., Using multivalent adenoviral vectors for HIV vaccination. *PLoS One*, 2013. 8(3): p. e60347.
26. Hansra, S., S. Pujhari, and A.N. Zakhartchouk, Exploration of New Sites in Adenovirus Hexon for Foreign Peptides Insertion. *Open Virol J*, 2015. 9: p. 1-6.
27. Johrden, L., et al., Comparison of polystyrene nanoparticles and UV-inactivated antigen-displaying adenovirus for vaccine delivery in mice. *Virol J*, 2013. 10: p. 108.
28. Karen, K.A., et al., A replicating adenovirus capsid display recombinant elicits antibodies against *Plasmodium falciparum* sporozoites in *Aotus nancymae* monkeys. *Infect Immun*, 2015. 83(1): p. 268-75.
29. Keefer, M.C., et al., A phase I double blind, placebo-controlled, randomized study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults. *PLoS One*, 2012. 7(8): p. e41936.
30. Khare, R., et al., Identification of adenovirus serotype 5 hexon regions that interact with scavenger receptors. *J Virol*, 2012. 86(4): p. 2293-301.
31. Krause, A., et al., Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity. *J Virol*, 2006. 80(11): p. 5523-30.
32. Langley, W.A., et al., Induction of neutralizing antibody responses to anthrax protective antigen by using influenza virus vectors: implications for disparate immune system priming pathways. *J Virol*, 2010. 84(16): p. 8300-7.
33. Lanzi, A., et al., Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid. *Vaccine*, 2011. 29(7): p. 1463-71.
34. Leen, A.M., et al., Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific CD8+ T cells. *Blood*, 2004. 104(8): p. 2432-40.
35. Lemckert, A.A., et al., Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol*, 2005. 79(15): p. 9694-701.

36. Liu, H., et al., Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science*, 2010. 329(5995): p. 1038-43.
37. Liu, H., L. Wu, and Z.H. Zhou, Model of the trimeric fiber and its interactions with the pentameric penton base of human adenovirus by cryo-electron microscopy. *J Mol Biol*, 2011. 406(5): p. 764-74.
38. Manzano-Szalai, K., et al., Adeno-associated virus-like particles as new carriers for B-cell vaccines: testing immunogenicity and safety in BALB/c mice. *Viral Immunol*, 2014. 27(9): p. 438-48.
39. Matthews, Q.L., et al., HIV antigen incorporation within adenovirus hexon hypervariable 2 for a novel HIV vaccine approach. *PLoS One*, 2010. 5(7): p. e11815.
40. Matthews, Q.L., et al., Genetic incorporation of a herpes simplex virus type 1 thymidine kinase and firefly luciferase fusion into the adenovirus protein IX for functional display on the virion. *Mol Imaging*, 2006. 5(4): p. 510-9.
41. Matthews, Q.L., et al., Optimization of capsid-incorporated antigens for a novel adenovirus vaccine approach. *Virol J*, 2008. 5: p. 98.
42. McConnell, M.J., X. Danthinne, and M.J. Imperiale, Characterization of a permissive epitope insertion site in adenovirus hexon. *J Virol*, 2006. 80(11): p. 5361-70.
43. McCoy, R.D., et al., Expression of human interleukin-1 receptor antagonist in mouse lungs using a recombinant adenovirus: effects on vector-induced inflammation. *Gene Ther*, 1995. 2(7): p. 437-42.
44. McKenna, P.M., et al., Immunogenicity study of glycoprotein-deficient rabies virus expressing simian/human immunodeficiency virus SHIV89.6P envelope in a rhesus macaque. *J Virol*, 2004. 78(24): p. 13455-9.
45. Olive, M., et al., The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. *Hum Gene Ther*, 2002. 13(10): p. 1167-78.
46. Onion, D., et al., The CD4+ T-cell response to adenovirus is focused against conserved residues within the hexon protein. *J Gen Virol*, 2007. 88(Pt 9): p. 2417-25.
47. Ouedraogo, A., et al., A phase 1b randomized, controlled, double-blinded dosage-escalation trial to evaluate the safety, reactogenicity and immunogenicity of an adenovirus type 35 based circumsporozoite malaria vaccine in Burkina Faso healthy adults 18 to 45 years of age. *PLoS One*, 2013. 8(11): p. e78679.
48. Palma, C., et al., Adenovirus particles that display the *Plasmodium falciparum* circumsporozoite protein NANP repeat induce sporozoite-neutralizing antibodies in mice. *Vaccine*, 2011. 29(8): p. 1683-9.
49. Pinto, A.R., et al., Induction of CD8+ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol*, 2003. 171(12): p. 6774-9.
50. Poulin, K.L., et al., Retargeting of adenovirus vectors through genetic fusion of a single-chain or single-domain antibody to capsid protein IX. *J Virol*, 2010. 84(19): p. 10074-86.
51. Reddy, V.S., et al., Crystal structure of human adenovirus at 3.5 Å resolution. *Science*, 2010. 329(5995): p. 1071-5.
52. Reyes-Sandoval, A., et al., Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. *J Virol*, 2004. 78(14): p. 7392-9.
53. Roberts, D.M., et al., Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature*, 2006. 441(7090): p. 239-43.

54. Rosa-Calatrava, M., et al., Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. *J Virol*, 2001. 75(15): p. 7131-41.
55. Rowe, W.P., Propagation of group A Coxsackie viruses in denervated adult mouse muscle. *Science*, 1953. 117(3052): p. 710.
56. Roy, S., et al., Circumvention of immunity to the adenovirus major coat protein hexon. *J Virol*, 1998. 72(8): p. 6875-9.
57. Rux, J.J. and R.M. Burnett, Adenovirus structure. *Hum Gene Ther*, 2004. 15(12): p. 1167-76.
58. Schirmbeck, R., et al., The immunogenicity of adenovirus vectors limits the multispecificity of CD8 T-cell responses to vector-encoded transgenic antigens. *Mol Ther*, 2008. 16(9): p. 1609-16.
59. Seregin, S.S., et al., Adenovirus capsid-display of the retro-oriented human complement inhibitor DAF reduces Ad vector-triggered immune responses in vitro and in vivo. *Blood*, 2010. 116(10): p. 1669-77.
60. Sharma, A., et al., Adenovirus-based vaccine with epitopes incorporated in novel fiber sites to induce protective immunity against *Pseudomonas aeruginosa*. *PLoS One*, 2013. 8(2): p. e56996.
61. Shiratsuchi, T., et al., Replacing adenoviral vector HVR1 with a malaria B cell epitope improves immunogenicity and circumvents preexisting immunity to adenovirus in mice. *J Clin Invest*, 2010. 120(10): p. 3688-701.
62. Shott, J.P., et al., Adenovirus 5 and 35 vectors expressing *Plasmodium falciparum* circumsporozoite surface protein elicit potent antigen-specific cellular IFN-gamma and antibody responses in mice. *Vaccine*, 2008. 26(23): p. 2818-23.
63. Sumida, S.M., et al., Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol*, 2005. 174(11): p. 7179-85.
64. Sung, R.S., L. Qin, and J.S. Bromberg, TNFalpha and IFNgamma induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression. *Mol Ther*, 2001. 3(5 Pt 1): p. 757-67.
65. Szurgot, I., et al., Self-adjuvanting influenza candidate vaccine presenting epitopes for cell-mediated immunity on a proteinaceous multivalent nanoplatfrom. *Vaccine*, 2013. 31(40): p. 4338-46.
66. Tang, J., et al., Adenovirus hexon T-cell epitope is recognized by most adults and is restricted by HLA DP4, the most common class II allele. *Gene Ther*, 2004. 11(18): p. 1408-15.
67. Tang, J., et al., Human CD8+ cytotoxic T cell responses to adenovirus capsid proteins. *Virology*, 2006. 350(2): p. 312-22.
68. Thorner, A.R., et al., Immunogenicity of heterologous recombinant adenovirus prime-boost vaccine regimens is enhanced by circumventing vector cross-reactivity. *J Virol*, 2006. 80(24): p. 12009-16.
69. Vellinga, J., et al., Efficient incorporation of a functional hyper-stable single-chain antibody fragment protein-IX fusion in the adenovirus capsid. *Gene Ther*, 2007. 14(8): p. 664-70.
70. Vigant, F., et al., Substitution of hexon hypervariable region 5 of adenovirus serotype 5 abrogates blood factor binding and limits gene transfer to liver. *Mol Ther*, 2008. 16(8): p. 1474-80.
71. Worgall, S., et al., Protective immunity to *pseudomonas aeruginosa* induced with a capsid-modified adenovirus expressing *P. aeruginosa* OprF. *J Virol*, 2007. 81(24): p. 13801-8.
72. Worgall, S., et al., Protection against *P. aeruginosa* with an adenovirus vector containing an OprF epitope in the capsid. *J Clin Invest*, 2005. 115(5): p. 1281-9.

73. Wu, H., et al., Identification of sites in adenovirus hexon for foreign peptide incorporation. *J Virol*, 2005. 79(6): p. 3382-90.
74. Xia, D., et al., Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. *Structure*, 1994. 2(12): p. 1259-70.
75. Xue, C., et al., Construction and characterization of a recombinant human adenovirus type 3 vector containing two foreign neutralizing epitopes in hexon. *Virus Res*, 2014. 183: p. 67-74.
76. Yang, T.C., et al., Detailed analysis of the CD8<sup>+</sup> T-cell response following adenovirus vaccination. *J Virol*, 2003. 77(24): p. 13407-11.
77. Youil, R., et al., Hexon gene switch strategy for the generation of chimeric recombinant adenovirus. *Hum Gene Ther*, 2002. 13(2): p. 311-20.
78. Zhong, T., et al., Characterization of malleability and immunological properties of human adenovirus type 3 hexon hypervariable region 1. *Arch Virol*, 2012. 157(9): p. 1709-18.
79. Zhou, D., et al., Hexon-modified recombinant E1-deleted adenovirus vectors as dual specificity vaccine carriers for influenza virus. *Mol Ther*, 2013. 21(3): p. 696-706.
80. Zubieta, C., et al., The structure of the human adenovirus 2 penton. *Mol Cell*, 2005. 17(1): p. 121-35.

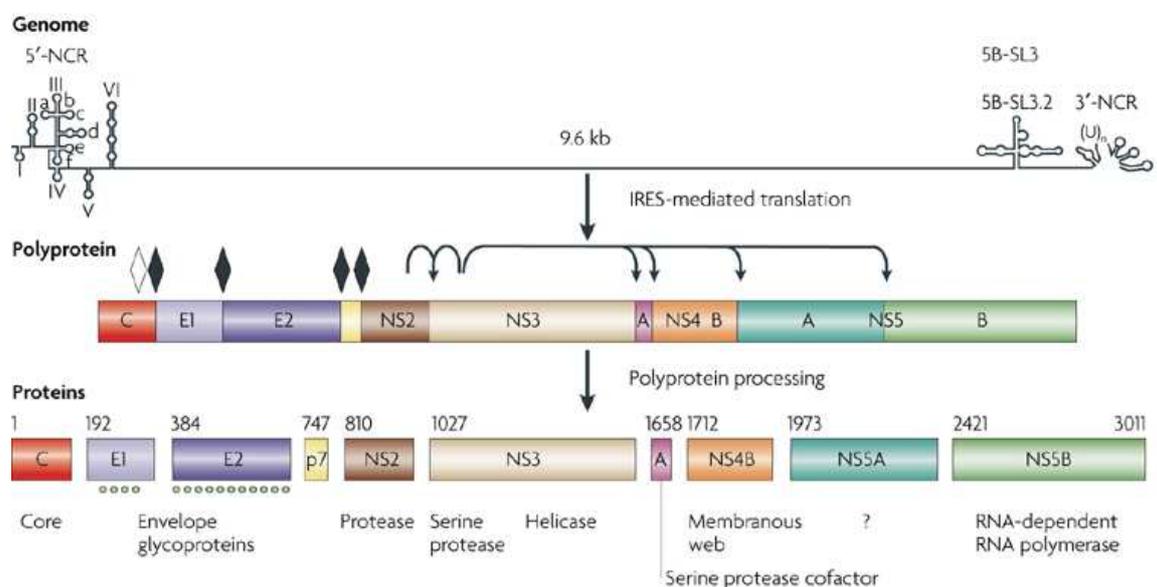


## 4 Hepatitis C virus

Hepatitis C virus (HCV), identified in 1989, is a major human pathogen causing chronic hepatitis and related liver pathologies. Infection often goes unnoticed for a long time as virus manifests itself very slowly, initially displaying only flu-like symptoms. Being able to persist and replicate undetected for many years, HCV slowly and gradually attacks the liver, where it can cause cirrhosis or cancer. Around 180–200 million people are now predicted to be infected worldwide and the virus continues to spread in developing countries. It is estimated that more than 350,000 people worldwide die from HCV-related liver disease every year. The current treatment is expensive, generates heavy side effects and its efficacy depends on the HCV genotype. All these constraints underline a need for a successful HCV vaccine, which so far is not available.

### 4.1 Molecular virology of HCV

HCV belongs to the *Flaviviridae* family of viruses, which share some specific molecular characteristics of capsid structure and genome organisation. Among members of this family we can find dengue fever virus, yellow fever virus, tick-borne encephalitis virus or Japanese encephalitis virus.



**Figure 4.1. Genetic organization and polyprotein processing of HCV.** Hepatitis C virus genome is flanked with 5' and 3' non-coding regions (NCR). Following IRES-mediated translation, one polyprotein is generated which upon cleavage by viral and cellular proteases give rise to 10 proteins. Structural proteins C, E1 and E2 form viral capsid, while non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B perform various functions during virus life cycle (Moradpour *et al.*, 2007).

#### 4.1.1 Genome

HCV genome in the form of positive single stranded RNA is divided in three regions: a 5'UTR or noncoding region, a 9000 nucleotides long open reading frame (ORF) and a short 3'UTR (**Figure 4.1**). The 341 base long 5'UTR contains the internal ribosomal entry site (IRES) which after binding to the 40S ribosomal subunit forms a translation pre-initiation complex (Thurner *et al.*, 2004). This region is the most conserved one in the whole HCV genome. The 3'UTR plays a role in minus-strand priming during HCV replication (Thurner *et al.*, 2004). In the ORF, the structural proteins are encoded by the N-terminal part while the remaining part codes for the non-structural proteins.

#### 4.1.2 Proteins and capsid structure

HCV genome encodes one polyprotein, which after cleavage by viral and cellular proteases generates 10 proteins, roughly divided into structural and non-structural proteins (**Figure 4.1**).

##### 4.1.2.1 Structural proteins

One of the three non-structural proteins is the nucleocapsid-forming core protein. This RNA-binding protein has been reported to interfere with numerous cell mechanisms by interacting with a variety of cellular proteins (Ray & Ray, 2001). It has anti- and pro-apoptotic functions (Meyer *et al.*, 2005) and is involved in carcinogenesis as well as in lipid metabolism (Moriya *et al.*, 1998; Moriya *et al.*, 1997).

Two remaining proteins forming the viral particle are glycoproteins E1 and E2, embedded in a host cell-derived lipid bilayer surrounding the capsid. These type-I transmembrane proteins assemble in a non-covalent heterodimer (Deleersnyder *et al.*, 1997). Both proteins are heavily glycosylated, and some glycans were described to participate in proper protein folding as well as in interaction with cell receptors (Goffard *et al.*, 2005). For that reason they are good targets for anti-HCV Abs. Indeed, in infected individuals the majority of circulating anti-HCV Abs recognizes epitopes present in E1 and E2 sequence (Keck *et al.*, 2008; Meunier *et al.*, 2008; Perotti *et al.*, 2008).

In the E2 protein sequence we distinguish fragments of very modest variability which are described as Hyper Conserved Regions (HCR). They are involved in binding to HCV putative receptor CD81. In these regions a few neutralizing epitopes were identified, such as 412–419aa epitope located in HCR1 (Zhang *et al.*, 2007). In the sequence of E1 and E2 we can also distinguish areas with significantly increased mutation rate, and their variability is most probably driven by the pressure to

escape the immune system. They are described as Hyper Variable Regions (HVRs), and so far three of them were discovered. HVR1 maps to the first 27aa in the E2 ectodomain (residues 384–410) (Kato *et al.*, 1992) and is recognized as the main HCV neutralizing epitope. Despite its big sequence variability, the overall conformation and positively charged amino acid residues at certain positions are conserved, in order to preserve the role of this region in virus attachment (Penin *et al.*, 2001). Downstream of HVR1, 9 residues are forming HVR2 (residues 474–482) (Kato *et al.*, 1992), a region involved in heterodimer formation, receptor binding and virus entry (McCaffrey *et al.*, 2011). Finally, HVR3 (residues 431–466) was more recently identified and also seems to be involved in the cell entry process (Troesch *et al.*, 2006).

#### 4.1.2.2 Non-structural proteins

Seven remaining proteins obtained after the polyprotein cleavage are performing various functions during virus genome replication and formation of new virions.

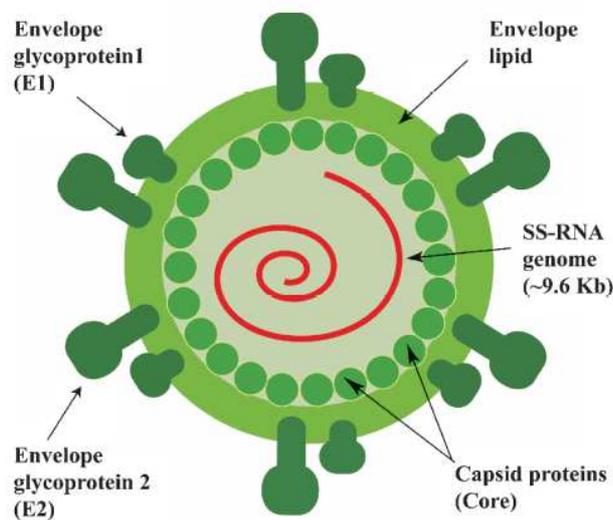
The p7 polypeptide is found on the junction between structural and non-structural proteins. This small membrane protein is involved in assembly and release of infectious virions (Steinmann *et al.*, 2007). NS2 protein exerts protease activity and after self-cleavage from NS3 is degraded in the proteasome (Franck *et al.*, 2005). NS3 is a multifunctional protein with serine-type protease domain in N-terminal end and a RNA helicase/NTPase domain in the C-terminus. Together with NS4A which is a cofactor for its protease activity, this protein is essential for the HCV life cycle. It catalyzes cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions, crucial for the generation of the viral RNA replication complex components. Consequently, NS3-NS4A protease is nowadays one of the most popular targets for directly acting antivirals (DAAs) in HCV treatment. NS4B is responsible for intracellular membrane alterations, serving as a mechanical support for the RNA replicating complex (Egger *et al.*, 2002). NS5A regulates HCV replication, although its exact mechanism of action is not clear (Elazar *et al.*, 2003). Finally, NS5B has activity of RNA-dependent RNA polymerase and is responsible for the synthesis of new copies of genomic RNAs (Ago *et al.*, 1999). Because of its nondispensable function in HCV life cycle, NS5B constitutes another major target for antiviral therapy (Scheel & Rice, 2013). NS5B lacks proof-reading function and it is one of the main reasons why HCV genotypes as well as quasispecies emerge so quickly.

Non-structural proteins, and most importantly NS3 and NS5B are the main targets for cellular responses raised against HCV. Numerous class I-restricted epitopes were found in the NS3 sequence (Cerny *et al.*, 1995; Kurokohchi *et al.*, 1996; He *et al.*, 1999). Moreover, it has been described that patients responding to IFN- $\alpha$  therapy develop cytotoxic T lymphocytes specific for one

of these epitopes (Vertuani *et al.*, 2002). These reports makes NS3 a promising vaccine candidate in the perspective of inducing a protective T cell-mediated immunity.

#### 4.1.2.3 Capsid structure

First analysis of the virus morphology revealed a 30-60nm particles (He *et al.*, 1987; Yuasa *et al.*, 1991). Subsequent tests involving detergent treatment of infectious sera disclosed 30-40 nm icosahedron-shaped particles containing both core protein and RNA (Takahashi *et al.*, 1992). Similarly to the other members of *Flaviviridae* family, this nucleocapsid built-up from core protein ensapsidating the viral genome is enveloped by a host-cell derived lipid bilayer. E1 and E2 viral glycoproteins are embedded as heterodimers into the viral envelope (Figure 4.2).



**Figure 4.2. HCV viral particle structure.** Viral genome is enclosed in a capsid formed by a core protein. Viral capsid is enveloped by a bilipid layer originating from host cell membranes into which glycoproteins E1 an E2 are embedded (Sharma, 2010).

The circulating HCV particles can be present in various forms. In the sera of infected individuals two distinct virus populations were found. One of them comprised infectious virus particles in interaction with low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) (Andre *et al.*, 2005). In parallel, immune complexes of HCV particles associated with immunoglobulins are also present, and considered to be less infectious (Andre *et al.*, 2002; Hijikata *et al.*, 1993; Thomssen *et al.*, 1992). Interestingly, proportions between these two subpopulations reflect the stage of liver disease and fluctuate over the course of infection (Kanto *et al.*, 1994; Petit *et al.*, 2003). Non-enveloped nucleocapsids were also found in the sera of infected patients. Recent findings suggest that they can enter immune cells, such as B- or T cells. Once inside CD4<sup>+</sup> T cells, they

are capable of activating production of IL-2, IL-10, and IFN- $\gamma$ , which in turn can lead to partial T cell exhaustion and T<sub>reg</sub> expansion (Doumba *et al.*, 2013).

#### 4.1.3 HCV live cycle

HCV has a very restricted host range as it infects only humans and chimpanzees. This pathogen displays strong liver tropism, albeit infection of DCs, B cells and other cell types has also been reported.

##### 4.1.3.1 Cell attachment and entrance

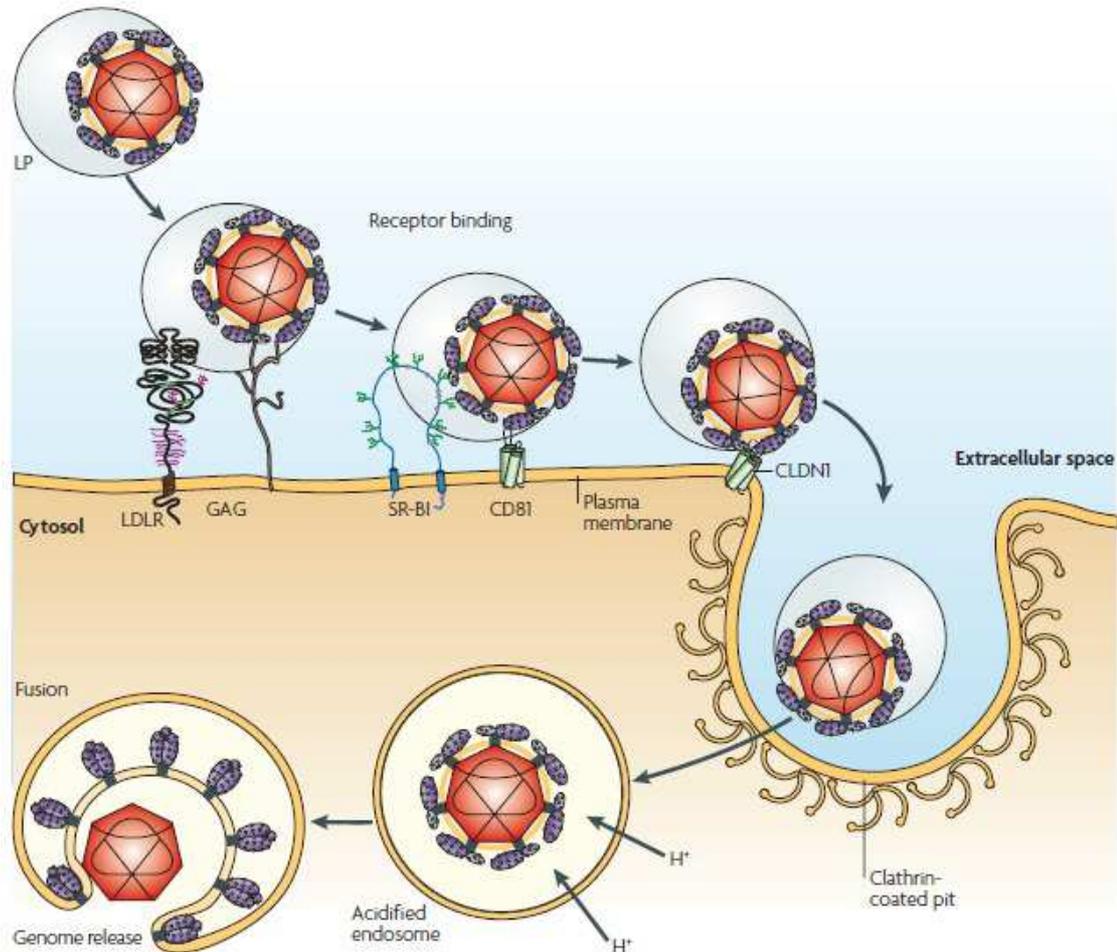
Circulating HCV gains access to the liver through liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KC). These cells, respectively, express on their surface liver/lymph-node-specific intercellular adhesion molecule 3-grabbing integrin (L-SIGN) and dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Both molecules are able to bind E2 glycoprotein with high affinity, and presumably to transfer virus to hepatocytes (**Figure 4.3**).

The first stage of HCV life cycle is the attachment to its receptors on the hepatocytes. The most important role in HCV cell entry is played by CD81, class B type I scavenger receptor (SR-BI), LDL receptor (LDLR) and two proteins found in tight junctions, occludine-1 (Ploss *et al.*, 2009) and claudin-1 (CLDN1).

CD81 and SR-BI are considered as main HCV entry receptors (Pileri *et al.*, 1998; Scarselli *et al.*, 2002). CD81 is a tetraspanin found on many cell types. SR-BI is a fatty acylated protein found in lipid raft domains, highly expressed on hepatocytes (Babitt *et al.*, 1997). Both CD81 and SR-BI interact with E2 glycoprotein, however, amino acid residues in E2 sequence involved in this binding remain to be identified. E2 region 502-520aa is a possible candidate, as mutations in this region influence HCV usage of SR-BI and CD81 receptors (Harman *et al.*, 2014). CD81 and SR-BI are both crucial, but not sufficient factors for HCV cell entry, as non-hepatocyte cells expressing these two molecules remain not permissive for the infection (Bartosch *et al.*, 2003).

LDLR is an endocytic receptor, involved in lipoprotein transport into the cells through receptor-mediated endocytosis (Chung and Wasan, 2004). It is implicated in the HCV infection process due to virus interactions with LDL and VLDL, and most probably serves as an initial anchoring molecule (Wunschmann *et al.*, 2000). Other molecules involved in HCV cell entry are glycosaminoglycans (GAG), ubiquitously expressed on various cell types. HVR-1 of E2 has been shown to bind HSPG with a strong affinity, and this interaction, along with binding to LDLR, is docking HCV

particle to the host cell surface. While attached to the cell, HCV particle can subsequently interact with main entry receptors, CD81 and SR-BI. Claudin-1, a tight junction component, was more recently identified as another entry receptor (Evans *et al.*, 2007).



**Figure 4.3. HCV entry.** HCV initial attachment to cell surface involves low density lipoprotein receptor (LDLR) and glycosaminoglycans (GAG) and allows for subsequent interaction with class B type-I scavenger receptor (SR-BI), CD81 tetraspanin and claudin-1 (CLDN1). Upon clathrin-dependent internalization, in acidified endosome, viral envelope bilayer fuses with endosome membrane releasing viral capsid into the cytoplasm (Moradpour *et al.*, 2007).

HCV enters the cell in a clathrin-dependent manner and entering particles are associated with CD81 and CLDN1 (Blanchard *et al.*, 2006; Coller *et al.*, 2009). Once internalized, the virion is transported to early endosomes where, in a presumably pH-dependent manner, fusion between viral and host membranes takes place. Then, the nucleocapsid gains access to the cytosol, where viral RNA is translated to viral polyprotein and initiates viral replication.

#### 4.1.3.2 Translation

The internal ribosome entry site (IRES) element, present in the 5'-UTR of the viral RNA is able to directly bind ribosomal subunit 40S. This interaction allows for the recruitment of the translation complex to the viral genome and helps to overcome the need for the capping process. In order to initiate the translation of its proteins, HCV needs host cellular factors, such as eukaryotic initiation factors (eIF)-2 and -3 (Ji *et al.*, 2004; Otto and Puglisi, 2004). Upon translation of one ORF, the long polyprotein is generated and subsequently cleaved by host and viral proteases, as indicated in section 4.1.2.2.

#### 4.1.3.3 Replication

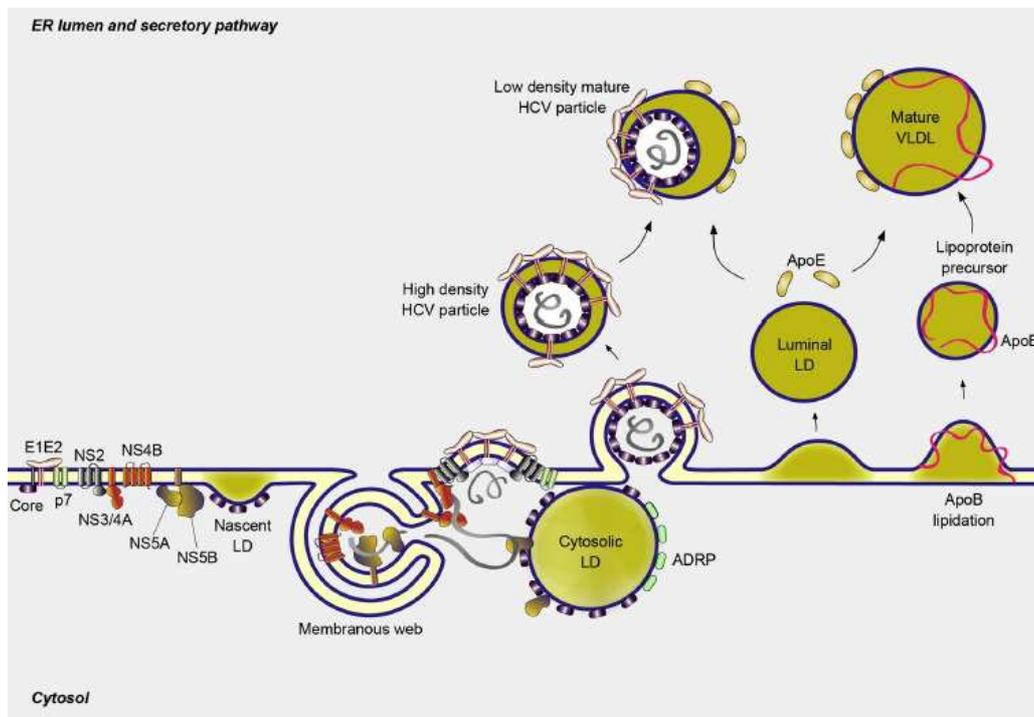
HCV genome replication is a semi-conservative and asymmetric process. In the first turn negative-strand intermediate is synthesized using positive-strand genome RNA as a template. This RNA of negative polarity serves as a template to produce numerous copies of positive strand genome, which will subsequently serve in the process of polyprotein translation, synthesis of new replication intermediates or will be encapsidated in new virus particles (Bartenschlager *et al.*, 2004).

The replication of HCV genome takes place in special alterations of ER membranes induced by NS4B, called *membranous web*. This solution offers a few important advantages, such as local concentration of viral products, physical support and arrangement of replication complex and stabilisation of viral RNA during unwinding. Additionally, this organization provides protection from the host defence mechanisms or RNA interference and facilitates access to lipid factors, crucial for the replication (Penin *et al.*, 2004). Pivotal role in replication process is played by NS3, NS4B, NS5A and NS5B viral proteins as well as cellular proteins, such as phosphatidylinositol-4-kinase-III (PI4KIII) (Reiss *et al.*, 2011), ARFGAP1 (Li *et al.*, 2014) or vesicle-associated membrane protein-associated protein A (VAP-A) and VAP-B (Paul *et al.*, 2013). Lipid droplets are also found in the *membranous web* and most probably are playing an important part in viral RNA synthesis and new virion morphogenesis (Miheyani *et al.*, 2007).

#### 4.1.3.4 New virions formation and release

The process of HCV assembly and release appears to be closely linked to lipid metabolism. Newly produced homodimerized core proteins are transferred to cytosolic lipid droplets (Barba *et al.*, 1997), which seems to be an essential step for the recruitment of remaining viral proteins implicated

in virion assembly. Indeed, mutations blocking interaction between core and lipid droplets dramatically alter assembly of new virus particles (Boulang *et al.*, 2007). The newly produced viral genome copies are directed to the assembly sites thanks to the NS3/4A or NS5A protein. In parallel, glycoproteins E1 and E2 mature in ER, undergoing glycosylation and forming a heterodimer (Dubuisson *et al.*, 1994). Then, thanks to the interactions between E1 and NS2 as well as E2 and p7, E1E2 complex approaches lipid droplets where virion assembly takes place (Ma *et al.*, 2011). Subsequent steps of virions assembly are tightly related to VLDL secretion pathway, as virion is associated with lipids resembling in their composition VLDL and LDL (Huang *et al.*, 2007). Thus, apolipoproteins such as apoE or apoB associate with new virion and are also considered essential for the final assembly. Finally, after budding from the ER, HCV particles are transported through the secretory pathway to the cell surface where they are released (**Figure 4.4**).



**Figure 4.4. HCV replication and new virions assembly.** Upon polyprotein cleavage, the non-structural proteins associate with cellular factors to form double-membrane vesicles known as membranous web where replication takes place. Core protein is then loaded onto lipid droplets (LDs) which interact with the ER-embedded replication-complex to create the virion assembly site. Replicated viral genomes, through the interaction with NS3/4A or NS5A, are transferred to the assembling virions. Glycoproteins are brought to the assembly site via NS2 and p7. Subsequent events of HCV virion morphogenesis are tightly related to the VLDL assembly metabolism, albeit this process is poorly understood (Dubuisson & Cosset, 2014).

Interestingly, direct cell-to-cell transmission is observed in cell culture (Brimacombe *et al.*, 2011) and in the patient's liver (Wieland *et al.*, 2014). This process could explain ability of this virus to remain ignored by the immune system and thus persist in the organism. The detailed mechanism of this process remains unknown, however CD81, occludin and CLDN-I were shown to be crucial in this event (Brimacombe *et al.*, 2011).

## 4.2 HCV infection, pathogenicity and treatment

High mutation rate of HCV sequence, due to the high error rate of viral RNA-dependent RNA polymerase and the host immune system pressure, has led to the evolution of 11 different virus genotypes, differing in their nucleotide sequence by as much as 30%-50% (Wang *et al.*, 2013). This sequence divergence causes significant discrepancies in virus responsiveness to the treatment. Moreover, after infection, virus gradually mutates inside the host, leading to the generation of so-called quasispecies, corresponding to closely related viral variants. This ability to quickly mutate allows the pathogen not only to evade the immune system and persist in the organism for decades, but also to acquire resistance to the applied treatment. Understanding both of these mechanisms is crucial in order to more efficiently counteract HCV epidemic. For a long time possibilities to analyze this pathogen were very limited due to the lack of suitable infection model. Development of the HCV replicon strategy as well as establishment of adequate animal models allowed a better understanding of the virus life cycle (Uprichard *et al.*, 2006).

### 4.2.1 Interplay with the immune system

Due to its vast arsenal of escape strategies allowing to hide from immune system HCV is a special challenge as a vaccine target. The virus is known to interfere and cause perturbation in the amount and functionality of various cells playing role in both innate and adaptive immune responses. Defects in the populations of Natural Killer cells, DCs, macrophages or T cells have been reported in chronically infected patients.

#### 4.2.1.1 Innate immunity

Natural Killer Cell (NK) are one of the cells affected by HCV infection. This cell subset is involved in direct killing of endangered and pathological cells without MHC restriction. In acute phase of HCV infection NK display “active state” with enhanced cytotoxicity. In chronic infection, however, they become crippled in their cytotoxic performance and have a tendency to produce IL-10 and TGF- $\beta$ . This impairs their capacity to activate DCs and favors the skewing of T cells into a T<sub>h</sub>2 T cells profile, more permissive for HCV (De Maria *et al.*, 2007; Jinushi *et al.*, 2004).

DCs are both decreased in their number and impaired in functionality during chronic HCV infection. They do not display fully mature profile (Auffermann-Gretzinger *et al.*, 2001) and secrete IL-10, thus generating immunosuppressive environment (Averill *et al.*, 2007) and favoring proliferation of T<sub>reg</sub> subpopulation (Dolganiuc *et al.*, 2008). Additionally, increased expression of

programmed death molecule ligand (PD-L1), having an inhibitory effect on T cells, was observed (Dolganiuc *et al.*, 2008).

Kupffer cells, liver-resident macrophages are also aberrant in HCV chronic infection. Similarly to DCs, they display PD-L1, along with another inhibitory molecule, galectine-9, also important in suppressing T cell responses (Mengshol *et al.*, 2010). In parallel, HCV can activate secretion of IL-10 and TGF- $\beta$  through the stimulation of TLR2/MyD88 pathway of signalization (Dolganiuc *et al.*, 2004).

All these alterations conjointly lead to insufficient and defective adaptive responses, that are described in the following paragraphs.

#### **4.2.1.2 Humoral response**

In viral infections, neutralizing Abs (nAbs) usually play a critical role. They help to control viremia and protect from re-infection. The majority on Abs generated during HCV infection is directed against glycoproteins E1 and E2. Their implication in controlling HCV pathogenesis remains a controversial issue. Studies in chimpanzees showed that administration of hyperimmune sera, targeting mostly HVR1 sequence, conferred protection against homologous HCV challenge (Farci *et al.*, 1996). This was confirmed by the observation that induction of nAbs during the early stage of infection allows for HCV control (Pestka *et al.*, 2007). On the contrary, the reports that the virus can be controlled in patients suffering from hypogammaglobulinemia suggests that Abs are not required for HCV eradication (Semmo *et al.*, 2006). It is now believed that in the context of chronic infection delayed antibody response allows for development of viral mutants with escape mutations at Ab epitopes (Zeisel *et al.*, 2008). In parallel, an interesting mechanism allowing HCV to escape the humoral response was described. It involves the induction of interfering Abs, non-neutralizing immunoglobulins directed against HCV. Upon binding to the virus they are masking the neutralizing epitopes and thus allow HCV to avoid neutralization (Zhang *et al.*, 2007; Zhang *et al.*, 2009). Additionally, lipoproteins associated with the virus could shield the viral surface glycoproteins and influence the induction of humoral responses (Tao *et al.*, 2009). Similarly, glycans presented on E1 and E2 by covering the neutralizing epitopes assure protection from neutralization (Helle *et al.*, 2011). Finally, ability to move between cells without entering the extracellular environment, also known as cell-to-cell spread, limits HCV arecognition by the humoral arm of the immune system.

#### 4.2.1.3 Cellular response

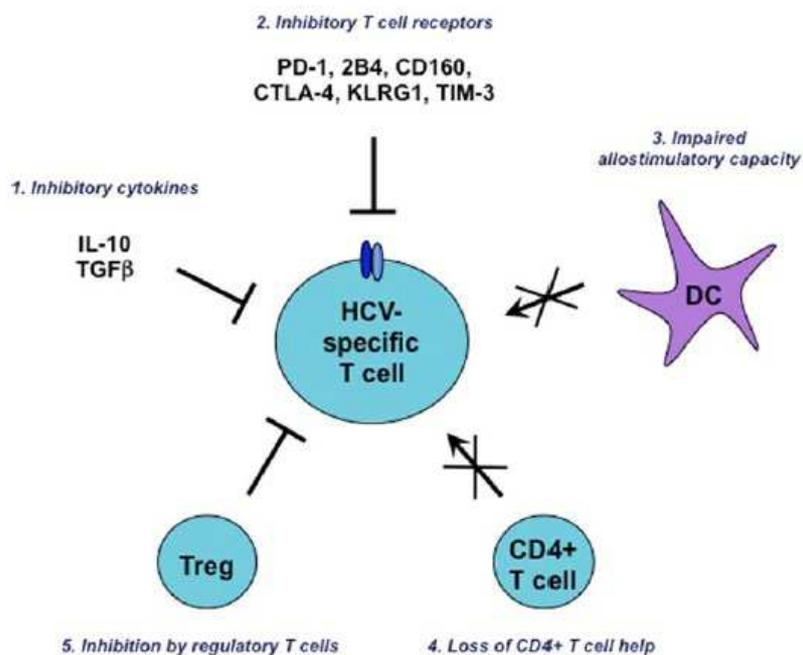
HCV-specific T cells can be detected in the organism around 4-8 weeks after the initial infection (Cox *et al.*, 2005). During acute infection, induction of sustained, robust, and multi-specific CD4<sup>+</sup> T cell responses correlates with the rate of spontaneous HCV clearance in both experimental models and infected patients (Lechner *et al.*, 2000; Thimme *et al.*, 2002). More precisely, T<sub>h</sub>1 CD4<sup>+</sup> cells provide protection and ensures HCV control, while T<sub>h</sub>2 CD4<sup>+</sup> cells lead to the development of chronic HCV infection (Tsai *et al.*, 1997). HCV-specific CD4<sup>+</sup> lymphocytes play a key role in viral clearance by producing antiviral cytokines such as IFN- $\gamma$  and by boosting the performance of antiviral B cells and CD8<sup>+</sup> T cells. This contribution is underlined by a reduction of this population in the stage of chronic infection (Urbani *et al.*, 2006). Moreover, a strong correlation is found between the recurrent viremia and loss of CD4<sup>+</sup> T cell responses after initial viral clearance (Gerlach *et al.*, 1999), highlighting additionally the importance of these cells.

Virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells found in chronically infected patients showed reduced proliferative response and impaired effector functions. Their specific cytotoxicity is hampered and IFN- $\gamma$  production decreased (Gruener *et al.*, 2001; Wedemeyer *et al.*, 2002). Even though they are found in the liver with 30-fold greater frequency in comparison to peripheral blood (He *et al.* 1999), most of the time the phenotype of these T cells shows incomplete differentiation (Appay *et al.*, 2002). This disabled adaptive response is a result of many impairments in immune system functionality induced by HCV (**Figure 4.5**).

Failure of T-lymphocyte responses in HCV chronic infection is primarily caused by exhaustion of these cells. Exhausted T cells are characterized by their inability to proliferate or secrete antiviral cytokines. One of the hallmark of this state is an upregulation of inhibitory receptors, such as PD-1, CTLA-4 or TIM-3 and low expression of CD127 (Radziewicz *et al.*, 2007; Nakamoto *et al.*, 2009; McMahan *et al.*, 2010). Additionally, some studies reported that intrahepatic HCV-specific CD8<sup>+</sup> T cells expressing high levels of PD-1 are likely to undergo apoptosis (Radziewicz *et al.*, 2007). The presence of inhibitory cytokines is also influencing T cell exhaustion and in parallel favors development of T<sub>reg</sub> subpopulation. Production of IL-10 and TGF- $\beta$  at high levels by innate immunity cells, such as DCs, macrophages or NK and by CD4<sup>+</sup> T cells plays a pivotal role in this process. Additionally, insufficient DC maturation and reduced number of CD4<sup>+</sup> T cells result in lack of adequate stimulation. HCV infection alters also liver chemokine profile and an increased CCL17 and CCL22 expression in this organ favors recruitment of T<sub>h</sub>2 and T<sub>reg</sub>, resulting in HCV persistence (Riezu-Boj *et al.*, 2011).

Another very important mechanism allowing to avoid cellular responses is the selection of escaping quasispecies dictated by the pressure of the immune system. High proliferation rate and

polymerase lacking proofreading function allow to rapidly adapt to the immune pressure through selection of escape, beneficial mutations. In the context of cellular responses, mutations were shown to influence epitope processing (Seifert *et al.*, 2004), MHC binding (Chang *et al.*, 1997) and TCR stimulation (Chang *et al.*, 1997). In order to induce effective T cell responses against HCV, a conserved epitope needs to be selected that will not tolerate the mutations due to the high fitness cost (Uebelhoer *et al.*, 2008). Among the suitable candidates, NS3 and NS5B proteins seem to be the most promising ones due to their critical role in HCV life cycle.



**Figure 4.5. Factors resulting in T cell exhaustion.** The main factors contributing to the development of HCV-specific T cell exhaustion are i) inhibitory cytokines (IL-10 and TGF- $\beta$ ), ii) inhibitory T cell receptors (PD-1, 2B4, CD160, CTLA-4, KLRG1 and TIM-3), iii) Impaired allostimulatory capacity of DCs, iv) loss of CD4<sup>+</sup> T cell help and v) inhibition by T<sub>reg</sub> cells (Brenndorfer & Sallberg; 2012).

#### 4.2.2 HCV pathogenicity

HCV spreads mainly through blood contact while sexual transmission accounts only for a minority of cases. Depending on the transmitted viral load, virus RNA can be detected in the blood between 1-3 weeks after infection (Farci *et al.*, 1991). Along with the increase of viral genome yield, the concentration of serum alanine aminotransferase (ALT) also rises, indicating hepatic injury. Still, first stages of HCV acute infection usually pass unnoticed, as clinical symptoms are unspecific: fatigue, nausea, abdominal pain, loss of appetite or mild fever. Liver-related jaundice appears in

50-84% of individuals (Loomba *et al.*, 2011). In some patients HCV infection can be self-limiting, and level of HCV RNA decreases below the threshold of detection in 3-4 months after the onset of acute infection (Santantonio *et al.*, 2008). However, in 54-86% of cases, patients fail to clear the virus and chronic infection is established, defined by viral RNA persistence 6 months after the initial infection (Seeff & Hoofnagle; 2002). The factors influencing HCV eradication are not fully explored, but age (Vogt *et al.*, 1999), sex (Bakr *et al.*, 2006), or single nucleotide polymorphism in IL-28B locus (Ge *et al.*, 2009) seem to be implicated.

During the chronic infection, various extrahepatic manifestations (EHM) of the virus can be detected. Among the most relevant is mixed cryoglobulinemia, defined by the presence of Abs precipitating at a temperature lower than 37 °C. HCV infection influences insulin metabolism, and the prevalence of insulin resistance and type-2 diabetes is increased in chronically infected patients (White *et al.*, 2008). Many others associated pathologies can be observed in e.g. neural system, skin, or eyes. Prolonged infection is linked to the development of liver cirrhosis. Viral proteins can interfere with various cell signaling pathways and mechanisms. These interactions lead to the deregulation of cytokine production and subsequently to the excess in synthesis and deposition of extracellular matrix (ECM). The matrix metalloproteinases (MMPs) are unable to remove all the ECM excess, and thus the balance between fibrogenesis and fibrolysis is disturbed. TGF- $\beta$  is the most potent pro-fibrogenic cytokine released during inflammation. It is produced at high levels by deregulated cells of the immune system, described in previous sections, thus favoring cirrhosis development.

Ultimately, chronic HCV infection can lead to the hepatocellular carcinoma (HCC). It is estimated that HCV infection is responsible for approximately 25% of HCC cases (Tanaka *et al.*, 2006). As in fibrosis process, the main reason for HCC development is the interaction of viral proteins with host cell proteome. Various proteins, such as core protein, NS3, NS5A and NS5B display an oncogenic potential, mostly by deregulating the cell cycle control or apoptosis (**Table 4.1**). It has been described that core protein is a sufficient factor to induce HCC in transgenic mice (Moriya *et al.*, 1998). This protein is implicated in the apoptosis, cell proliferation and formation of reactive oxygen species (Kang *et al.*, 2009). NS3 was shown to interact with p53 and inhibit activity of p21 (He *et al.*, 2003). Besides its interaction with p53 (Lan *et al.*, 2002), NS5A induces also chromosome instability (Wu *et al.*, 2008). Finally, NS5B is promoting cellular proliferation by downregulation of Rb protein (Munakata *et al.*, 2007).

Additional stimulation in the process of HCC development is provided by the chronic inflammation established in the liver, most probably resulting from the cell cycle deregulation. This state is presumably involved in the increase of mutation rate, and thus HCC development and progression (Fabregat *et al.*, 2009). Nevertheless, the exact molecular mechanism of this process are

not fully understood. It is estimated that establishment of this pathology can take around 10-30 years after the initial HCV encounter. Until today, HCC treatment is very limited. There is no effective chemotherapy available and patients with small and slow-growing tumors usually undergo liver transplantation. The benefit of this operation is hampered by the frequent recurrence of HCC in the graft. It is thus crucial to limit the viremia at earlier stages of HCV pathology to prevent HCC development. The arsenal of available compounds counteracting chronic HCV infection is described in the next section.

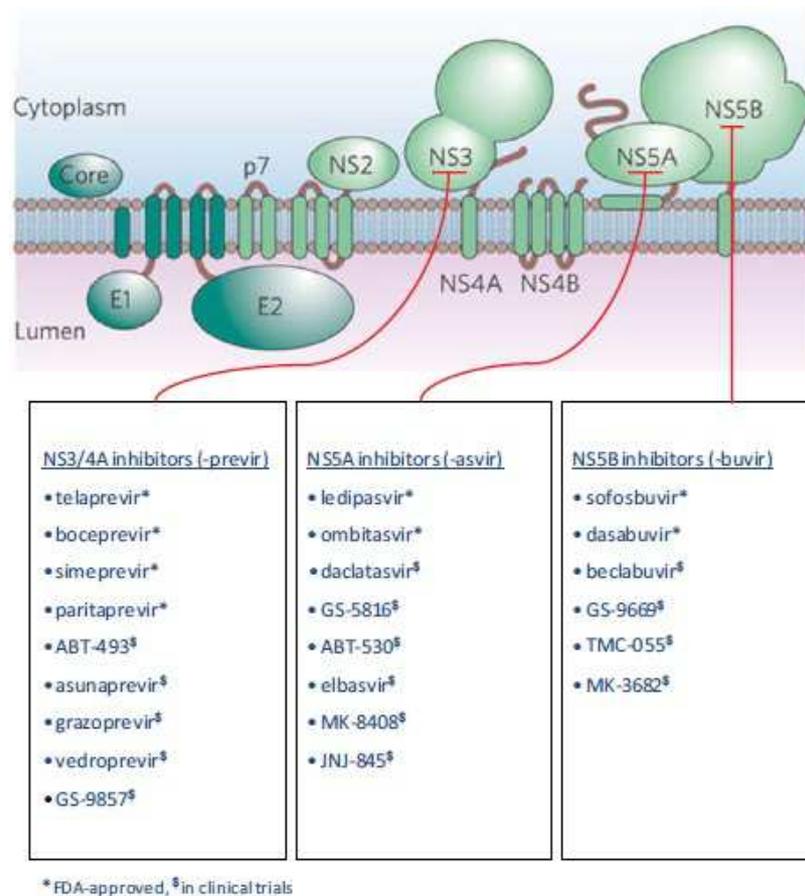
**Table 4.1. Interactions of HCV proteins with cellular proteins favoring oncogenesis (Ming *et al.*, 2015).**

Protein	Functions/effects
Core	Inhibits apoptosis Inactivates tumor-suppressor genes Promotes cellular proliferation Suppresses immune response and increases cell survival Induces reactive oxidative stress Promotes fibrogenesis Causes direct mitochondrial and chromosomal DNA damage
E1-E2	Induces endoplasmic reticulum stress
E2	Inhibits apoptosis Promotes cellular proliferation Induces cytokine production and cytotoxic granule release
NS3	Inhibits apoptosis Promotes cellular proliferation Inhibits tumor-suppressor genes
NS5A	Inhibits apoptosis Promotes cellular proliferation and survival Induces mitotic dysregulation and chromosomal instability

#### 4.2.3 Treatment of HCV chronic infection

For a long time the “golden standard” of anti-HCV therapy was a combination of IFN- $\alpha$  and ribavirin. Both compounds were limiting HCV infection in an unspecific manner. IFN- $\alpha$  is boosting the immune system and inducing anti-viral state in uninfected cells. Its efficacy in infected individuals was proven even before the identification of HCV as a causative agent. Nevertheless, monotherapy was long and had limited success (Di Bisceglie *et al.*, 2002). Addition of ribavirin significantly improved the therapy outcome, raising the sustained virologic response (SVR) rate to 35-40% (McHutchison & Poynard, 1999). Ribavirin is a guanosine analogue and can prematurely block virus RNA replication (Maag *et al.*, 2001). In another proposed mode of action, the molecule is causing an “error catastrophe” by increasing the mutagenesis rate in HCV genome, especially in NS5A region (Contreras *et al.*, 2002).

Further improvement in the regimen performance was achieved by covalently attaching the polyethylene glycol (PEG) molecule to recombinant IFN- $\alpha$ . It resulted in longer drug half-life and thus better pharmacokinetic performance. This ameliorated combination of (PEG)-IFN- $\alpha$  and ribavirin raised the treatment efficacy to 54-56% (Manns *et al.*, 2001). Depending on the genotype, therapy lasted 24 or 48 weeks and was associated with various severe side effects, such as anemia, flu-like symptoms, irritability, depression, anxiety or fatigue. Interestingly, the therapy outcome was strictly linked to HCV genotype, with the worst predictions for genotype 1, being also the most prevalent in US and Europe (Hézode *et al.*, 2014).



**Figure 4.6. Currently evaluated DAAs inhibiting HCV viral replication.** In order to counteract virus replication, different proteins crucial for this process are targeted by directly acting antivirals (DAAs). Among them the most promising targets are NS3/4A, NS5A and NS5B and their inhibitors are already FDA approved or still evaluated in clinical trials (Lam *et al.*, 2015).

A new era in HCV treatment began with the discovery of the first generation of directly acting antivirals (DAAs). Initially, the attention was drawn to NS3 protease inhibitors, and the first molecules of this type, boceprevir and telaprevir, became available in 2011. They were included in the standard (PEG)-IFN- $\alpha$ /ribavirin treatment of patients infected by HCV genotype 1, rising SVR to 70%. Notwithstanding, even though new drugs proved themselves efficacious in fighting the virus,

they added additional layer of side effects. Another burden was a necessity of high dose administration, resulting in the intake of as much as three pills per day (Hézode *et al.*, 2014). Subsequently, treatment focus expanded to other non-structural proteins involved in virus replication: NS5A and NS5B. Many molecules are already approved by US Food and Drug Administration (FDA) or are currently being evaluated in clinical trials (**Figure 4.6**). Among the most intensively discussed molecules is Sofosbuvir. It is a uridine nucleotide pro-drug, whose active metabolite is incorporated into virus RNA by NS5B, resulting in chain termination. It was first approved by FDA in December 2013 and released by Gilead under the market name Sovaldi®. This drug is used in clinics in combination with ribavirine or (PEG)-IFN- $\alpha$ / ribavirin, in patients infected with virus genotype. In late 2014, the same company obtained FDA approval to introduce to the market single tablet regimen (Harvoni®) for the treatment of genotype 1 chronic HCV infection. It is a combination of Sofosbuvir and Ledipasvir, an NS5A replication complex inhibitor. In 12 week treatment this (PEG)-IFN- $\alpha$ /ribavirin-free regimen leads to the SVR in around 97-99% of individuals. Another combination of drugs involving Sofosbuvir was accepted in late 2014 for the treatment of genotype 1 patients. Janssen Therapeutics supplemented the Gilead's Sofosbuvir with their drug named Olysio®, containing NS3/4A inhibitor, Simeprevir. Combinations of Sofosbuvir with different molecules e.g. Daclatasvir, are also being tested (Hézode and Bronowicki, 2016).

Various cocktails of DDAs are currently being used in patients. Ribavirin, due to its low price and good tolerability in IFN-free regimen still remains a useful tool. It can improve the results of anti-HCV regimens and allows to reduce the treatment duration. The final combination of DDAs +/- ribavirin is tailored to each patient, depending on the HCV genotype, previous HCV treatment history and outcome as well as the eventual presence of liver cirrhosis (Hézode and Bronowicki, 2016). Unfortunately, the majority of current DAAs does not have a pan-genotypic efficacy. Also, new DAAs-based therapies are very expensive and associated with secondary effects. Altogether, this still makes a discovery of an effective cross-reactive anti-HCV vaccine a high priority, not only to prevent the initial infection but also to counteract re-infection after successful cure with costly DAAs (Walker and Grakoui, 2015).

### **4.3 Vaccination against HCV**

Vigorous humoral and cellular responses are associated with better resolution rate of HCV infection. Furthermore, individuals who managed to spontaneously clear the virus have significantly increased chances to prevent the virus persistence upon the second encounter. This advocates the demand for the development of a successful vaccine. Since the discovery of the virus as a causative agent of hepatitis C, many vaccination strategies were evaluated. Until today, a few vaccine candidates based on recombinant proteins, synthetic peptides, DNA plasmids or recombinant viruses have been tested in non-human primates and humans (Ghasemi, 2015).

#### **4.3.1 Peptide vaccines**

Peptide vaccine IC41 developed by Intercell (Austria) consisted of five peptides derived from core, NS3, and NS4 proteins of HCV (genotypes 1 and 2) and was adjuvanted with poly-L-arginine. The results of a phase-I study in 128 HLA-A2<sup>+</sup> healthy volunteers showed that IC41 is safe, well-tolerated and capable of inducing dose-dependent T cell responses (Firbas *et al.*, 2006). The same formulation was tested in 60 HLA-A2<sup>+</sup> chronic HCV patients. Up to 67% of enrolled patients displayed T cell proliferation while IFN- $\gamma$  responses were detected in almost 42% of individuals. Another team evaluated the vaccination potential of peptide vaccine with synthetic peptides from E1, E2, NS3 and NS5A proteins immersed in incomplete Freund's adjuvant. Results from 12 individuals showed induction of HCV-specific cellular responses in all patients and 3 of them showed reduction in HCV RNA levels (Yutani *et al.*, 2007).

#### **4.3.2 Recombinant protein vaccines**

The first HCV vaccine based on recombinant protein tested in humans was a truncated E1 protein immersed in aluminum hydroxide adjuvant (T2S-918/InnoVac-C). The formulation showed induction of anti-E1 Abs in healthy individuals (Leroux-Roels *et al.*, 2004). Another recombinant protein vaccine, based on E1E2 heterodimer with MF59C adjuvant, proposed by Chiron, was evaluated in 60 healthy individuals. It not only induced Ab production, but also provided stimulation for CD4<sup>+</sup> lymphocytes, important in the control of acute hepatitis. This combination allows to neutralize virus infectivity but to also prevent persistence. The vaccine development was allegedly hampered by the problems with E1E2 manufacturing (Frey *et al.*, 2010; Law *et al.*, 2013). As another strategy Innogenetics NV (Belgium) proposed InnoVAC-C, a therapeutic vaccine candidate containing E1 protein in aluminum hydroxide adjuvant. Study in 122 chronically infected HCV subjects showed

an increase in anti-E1 Ab titers and presence of HCV-specific cellular responses but these responses did not stop the progression of liver disease and thus no further studies were performed (Wedemeyer *et al.*, 2008).

Core protein with ISCOMATRIX™ adjuvant was another vaccine candidate evaluated in a phase-I study enrolling 30 healthy individuals. Anti-core Abs were induced in 29 subjects, but anti-HCV T cell responses were detected in only two subjects (Drane *et al.*, 2009). Another therapeutic vaccine candidate formulated with core and NS3 proteins was GI-5005. Results of phase-I clinical trial confirmed its immunogenicity in chronically infected patients. Subsequent evaluation of GI-5005 in combination with the (PEG)-IFN- $\alpha$ /ribavirin in phase-II study involving more than 250 chronic HCV-1 individuals resulted in increase in their SVR (Habersetzer *et al.*, 2009).

### **4.3.3 DNA vaccines**

As for the DNA vaccination, CIGB-230 was the first vaccine candidate to reach clinical trial. It contained a mix of plasmids expressing Core/E1/E2 proteins and the core protein itself. The results of phase-I showed a production of neutralizing Abs and induction of core-specific T cell responses in the majority of individuals with half of them presenting an improvement in liver histology and fibrosis reduction (Castellanos *et al.*, 2010). ChronVac-C proposed by the Tripep Company (Sweden) was the second DNA HCV vaccine to reach a human trial. This formulation, based on the conserved HCV regions (NS3/NS4A) was administrated to 12 chronic HCV individuals i.m. through electroporation (Sallberg *et al.*, 2009). The results were promising, indicating the reduction of HCV RNA in 2/3 of the patients, and thus ChronVac-C is currently further investigated in a combination with standard IFN-based therapy in phase-II clinical trial (ClinicalTrials.gov Identifier: NCT01335711).

### **4.3.4 Viral vector-based vaccines**

Viral vectors are another potent formulation in HCV vaccination and Ad vectors appears to be the most potent vaccine design for T cell priming in both non-human primates and humans (Casimiro *et al.*, 2003). One vaccine candidate based on Ad was designed by Okairos (Italy). Initially, the vaccination schedule was based on a heterologous prime-boost regimen. Ad6 and AdC63 vectors encoding the entire genotype 1b NS3-NS5B region were constructed and induced both CD8<sup>+</sup> and CD4<sup>+</sup> cellular responses in chimpanzees (Folgori *et al.*, 2006). In later stages, these constructs were evaluated in 36 human volunteers having low risk of HCV exposure. The results were encouraging, as the induced T cell responses were multifunctional and cross-reactive (Barnes *et al.*, 2012). Then,

a regimen modification was introduced, consisting in priming with chAd63 and boosting with modified vaccinia Ankara virus (MVA) (Swadling *et al.*, 2014). This provided an increase in CD8<sup>+</sup> and CD4<sup>+</sup> T cell frequencies and also ensured higher percentage of cells displaying central or effector memory cell phenotype. The currently recruiting phase-I/II study in high-risk individuals will provide us with important information on the efficacy of this vaccine (ClinicalTrials.gov Identifier: NCT01296451).

Another study evaluated MVA vector encoding HCV core, E1, E2 and NS3 genes in a heterologous prime-boost regimen with DNA vaccination in chimpanzees (Transgene). Upon immunization, strong anti-HCV CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were observed. However, three out of four animals developed chronic infection upon pathogen challenge (Rollier *et al.*, 2007).

A MVA vector encoding NS3, NS4, and NS5B proteins has shown to be effective in HLA-A2.1 and HLA-B7.2 transgenic mice, as it induced strong and long-lasting, cross-reactive, anti-HCV CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Moreover, 6 months later a boosting injection was able to recall these responses, indicating the induction of memory T cells by this construct (Fournillier *et al.*, 2007). The same formulation (TG4040) was then assessed for its efficacy in 15 chronically infected HCV individuals. The induction of vigorous CD8<sup>+</sup> T cell responses allowed for HCV viral load reduction in six of them (Habersetzer *et al.*, 2009). After acceptable safety profile demonstrated in phase-I, TG4040 entered the phase-II of clinical trials in combination with (PEG)-IFN- $\alpha$ /ribavirin therapy (ClinicalTrials.gov Identifier: NCT01055821). A total of 153 individuals received the standard-of-care treatment either alone or in combination with 6 or 13 injections of TG4040. The results indicate that addition of TG4040 vaccine to (PEG)-IFN/Ribavirin treatment increases SVR rate.

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# ***Objectives***

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Several advantages such as their ability to transduce different cell types, the high level of transgene expression as well as the ease of their manipulation and production makes Ad5 a promising platform for vaccine development. Nevertheless, the efficacy of immunization with these transgene-encoding vectors is hampered by anti-Ad Abs, highly prevalent in the human population. A few strategies were proposed to overcome Ad immunity, among them epitope display vaccination approach, consisting in genetic insertion of selected epitopes into the viral capsid. Few teams, including ours, evaluated the immunization efficacy of Ad5 vectors carrying B-cell epitopes. We previously showed that B-cell epitopes (Ova<sub>323-339</sub>) derived from ovalbumin (Ova) model antigen, when inserted into Ad5 capsid, were capable to trigger anti-Ova humoral responses in both naïve and Ad5-immune mice. Moreover, a remarkable boosting potential was observed with an Ad displaying Ova epitope inserted into capsid fiber protein (Lanzi *et al.*, 2011).

The present studies seek to go further in our understanding of epitope display strategy of vaccination. More precisely, the objectives of my thesis aims at **i)** understanding the parameters controlling the efficacy of Ad displaying B cell epitopes in fiber protein, **ii)** characterizing the capacity of Ad displaying T-cell epitopes to trigger cellular responses and **iii)** evaluating the potential of Ad displaying epitopes to elicit humoral and cellular responses against HCV.

**i) First objective:** this part of my work was conducted to determine molecular bases controlling the induction of humoral responses by Ad displaying B-cell epitopes. For this, we took advantage of previous results of our laboratory showing the key role of Ad interactions with cellular receptors and factors in shaping Ad tropism and inflammatory profile. More precisely, mutations in hexon (HVR5 deletion) and in penton base (RGD deletion) ablated Ad5 interaction with FX and integrins, respectively (Vigant *et al.*, 2008; Vigne *et al.*, 2003). Additionally, the modification of Ad5 fiber with Ad3 shaft was shown to reduce blood toxicity, cytokine production (Raddi *et al.*, 2016) and liver uptake (Raddi, in revision). Based on these previous data, we introduced different capsid mutations/modifications in an Ad displaying Ova<sub>323-339</sub> B cell epitope in the fiber protein in order to evaluate the influence of cell entry on vector vaccination potential. In parallel, we also pursued studies in mice deficient in innate immune pathways in order to understand their contribution to the establishment of humoral responses elicited by Ad displaying Ova<sub>323-339</sub> in the fiber protein.

**ii) Second objective:** previous unpublished data of our laboratory investigated the capacity of Ad displaying T cell epitopes inserted into hexon or fiber protein to induce anti-Ova cellular responses. Ad displaying Ova<sub>257-264</sub> immunodominant T cell epitope were constructed and produced and preliminary results have confirmed their ability to trigger anti-Ova cellular responses (thesis

Anastasia Lanzi). To go further in the characterization of these vectors, we monitored the phenotypes and kinetics of anti-Ova cellular responses. In particular, these immune responses were assessed in both Ad-naive and Ad-immune mice in order to analyze the influence of pre-existing Ad immunity on the efficacy of Ad displaying T cell epitopes.

**iii) Third objective:** In this last part of my thesis, I applied the epitope display strategy of vaccination to a therapeutic target, HCV. To this end, HLA-A2-restricted, class-I epitopes from non-structural protein NS3 were inserted into Ad hexon or fiber protein. In parallel, Ad5 vectors displaying B cell epitopes from structural E2 protein were inserted into Ad hexon or fiber protein. Ad displaying NS3- or E2-derived epitopes were produced, characterized and we assessed their ability to trigger cellular or humoral responses, respectively.

For each of these objectives, the data obtained are presented as manuscripts in preparation in separate chapters of the results section.

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# ***Results***

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# **PART 1**

## **Molecular bases controlling the efficacy of epitope display strategy**



## ARTICLE I

**TLR/MyD88 pathway but not the process of Ad infection shapes the humoral responses elicited by adenovirus displaying epitopes on their capsid.**

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## **Abstract**

Performance of adenoviral (Ad) vectors in vaccination studies is heavily limited due to the strong prevalence of anti-Ad antibodies, hampering vector transgene expression. As an alternative approach, epitope display strategy was developed, bypassing the need for a gene transfer. To better understand the molecular mechanisms of this new vaccination approach, various virus- and host-related factors were evaluated. Vectors ablated in the interaction with their receptors were still able to induce potent anti-epitope humoral responses. Vaccination studies in MyD88<sup>-/-</sup> MAVS<sup>-/-</sup> mice unraveled that TLR and RLR stimulation is not a crucial element in this immunization strategy. Altogether, our data shed a new light on the molecular mechanisms involved in the induction of humoral responses against the heterologous epitopes presented on Ad capsid.

## 1. Introduction

Adenoviruses (Ad) belong to a family of non-enveloped DNA viruses containing a linear double-strand DNA genome. The accumulation of knowledge regarding their biology led more than 20 years ago to the development of Ad-derived vectors [1]. The ease of Ad manipulation, their production at high titers as well as the strong level of gene expression achieved by these vectors make them attractive tools for gene therapy but also for vaccination. Indeed, Ad-mediated gene transfer of DNA fragments encoding heterologous proteins was shown to elicit strong humoral and cellular responses towards transgene-encoded proteins [2]. The efficacy of this approach of vaccination (hereafter referred to as classical approach) stems in Ad's ability to transduce *in vivo* a large set of cells and in the intrinsic immunogenic properties of this vector [3].

Several studies investigated Ad capsid proteins and cell receptors controlling Ad infection. Thus, in the case of the well-characterized serotype 5 Ad (Ad5), interaction of fiber protein, and more precisely its knob, with Coxsackie and Ad receptor (CAR) was shown to be responsible for initial virus attachment. Then, subsequent binding of a penton base-located RGD motif to cellular integrins allows virus endocytosis through a clathrin-dependent pathway [3]. The role of integrins and CAR in controlling Ad distribution *in vivo* was for a long time a matter of debate. CAR was shown to play only a minor role in the transduction of different tissues, including liver and spleen [4][5]. Integrin-ablated Ad led to a reduced transgene expression in spleen and lung [6]. Of note, ablation of both CAR and integrin binding was unable to reduce liver gene transfer [5][7](and for a review see [3]). Besides CAR and integrins, different studies demonstrated a role for Ad shaft in controlling liver but also spleen transduction [8, 9][4]. More recently, different Ad serotypes were shown to bind to plasma proteins such as vitamin K-dependent coagulation factors, leading to liver transduction [10]. Among numerous coagulation factors, factor X (FX) plays a key role in liver transduction by bridging Ad capsid to liver heparan sulfate proteoglycans (HSPG). More precisely, mutations of Ad capsid helped to identify Ad hexon protein as the capsomer directly involved in FX binding [11-13].

Besides their role in cell transduction followed by transgene expression, Ad receptors contribute to the intrinsic immunogenic properties of this vector. For example, interaction with CAR and integrins were at the origin of pro-inflammatory cytokines and chemokines production in epithelial cells and macrophages (for a review see [3]). Innate immune responses to Ad are also triggered through the stimulation of pathogen recognition receptors (PRR). Several studies reported a role of membrane-anchored sensors such as TLR9 and more surprisingly TLR2 in controlling cytokine production [14][15]. In addition, mice deficient in MyD88, an adaptor protein common to different TLR signalling pathways, displayed reduced levels of serum pro-inflammatory cytokines and chemokines [14]. After endosome escape, one can anticipate that Ad stimulate cytosolic sensors. Indeed, following Ad infection, synthesis of viral associated (VA) RNA elicits type I interferon (IFN) through RIG-I mediated pathway [16]. Finally, comparison of the transcriptome in the spleen after administration of wild-type and FX-ablated Ad unmasked an unanticipated key role of FX in activating NFκB pathway leading to pro-inflammatory cytokine production [17].

Despite their efficacy in transducing cells *in vivo* and their strong adjuvant properties, the use of Ad in the classical vaccination approach is hampered by the highly prevalent anti-Ad5 immunity. Moreover, Ad vector immunogenicity impairs the efficiency of homologous prime-boost administrations. Several strategies were developed to overcome these limitations (for a review see [2]), among them epitope display relying on genetic insertion of relevant epitopes into Ad capsid. This approach was successful at inducing antibody responses against *P. Aeruginosa* [18], *B. anthracis* [19] or Plasmodium [20]. Using a B cell epitope derived from Ova model antigen, we previously uncovered that Ad pre-existing antibodies (Abs) strongly increased antibody response elicited by Ad displaying the epitope into the fiber protein [21]. The present results seek to go further in our understanding of this strategy of vaccination by defining the role of Ad interaction with its receptors, as well as the influence of innate immune pathways.

## 2. Material and methods

### 2.1. Mice

Seven-week-old mice C57BL/6 female mice were purchased from Harlan (Gannat, France). MyD88- (MyD88<sup>-/-</sup>) and MAVS- (MAVS<sup>-/-</sup>)-deficient mice were bred in animal facilities of TAAM-UPS44 (Orléans) or UMR0892 (VIM, Jouy-en-Josas), respectively. All mice were conditioned for at least one week in the animal facilities before beginning of the experiments. All animal experiments were conducted in accordance with the institutional guidelines of Gustave Roussy Institutional Animal Care and Use Committee.

### 2.2. Virus construction and production

AdWT (described as AE18 in ref. [22]) is based on Ad5 and possess deletions in E1 and E3 regions. The expression cassette cloned instead of the E1 region contains the promoter/enhancer from the immediate early gene of human cytomegalovirus (CMV), the Escherichia coli lacZ gene with a nuclear localization signal, and the SV40 late polyA signal (SV40pA). AdH-3OVA2 and AdF-3OVA2 derived from AdWT and displaying OVA<sub>320-341</sub> (3OVA2) respectively in hexon or fiber protein were described previously [21]. AdP\*F-3OVA2, AdH\*F-3OVA2, AdH\*P\*F-3OVA2 and AdS\*F3-OVA2, disabled in Ad interactions with its natural receptors, were derived from AdF-3OVA2 (**Table 1**). More precisely, AdP\*F-3OVA2 was derived from AE74 [9] bearing a deletion of penton base RGD motif, impairing interaction with integrins. AdH\*F-3OVA2 was derived from AdH[GA]24 [12] bearing a deletion of hypervariable region 5 of hexon protein, impairing FX binding. AdH\*P\*F-3OVA2 contains both deletions. Finally, AdS\*F-3OVA2 was obtained from AdWT by replacing the fiber shaft with the shaft from Ad serotype 3 [23]. All capsid-modified viruses (Table 1) were constructed by recombinational cloning in *E. coli* [24].

Ad-OVA (provided by Dr D. Descamps, INRA, Jouy-en-Josas) has a wild-type capsid and encodes the full ovalbumin protein. Ad control encoding no transgene was described previously [25].

All viruses were obtained using previously described procedures [5], stored at -80°C in PBS-7% glycerol, and titrated by spectrophotometry ( $1 \text{ OD}_{260} = 1.1 \times 10^{12}$  viral particle [vp]/ml).

### 2.3. Cell lines

The 293A human embryonic kidney cell line (R705-07, Invitrogen) was maintained in modified Eagle medium (MEM) supplemented with 10% FBS and 1% of non-essential amino acids (NEAA). CHO-k1-hCAR and CHO-k1-pCDNA were kindly provided by J.M. Bergelson (School of Medicine, University of Pennsylvania). L929 murine fibrosarcoma cells were kindly provided by Dr U. Greber (Institute of Molecular Biology, Zurich, Switzerland) and maintained in DMEM supplemented with 10% FBS.

### 2.4. SDS-PAGE

Purified viruses ( $10^{10}$  vp) were resuspended in Laemmli lysis buffer, boiled for 10 min, and loaded onto a 10% NuPage gel (Novex, Invitrogen, CA). After electrophoresis, the gel was stained with a silver staining kit (Invitrogen, Carlsbad, CA).

### 2.5. In vitro cell transduction

In order to evaluate virus infectivity, cells were plated out in 12-well plates at  $1 \times 10^5$  cells /well 48 h prior to infection. On the day of infection, cells were counted and infected with different multiplicity of infection (MOI) of different Ad in 400µl of serum-free medium. After 24 h, βgalactosidase (βgal) activity was measured using a chemiluminescent assay (BD Biosciences, Clontech, CA) and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Marnes-la-Coquette, France). Results were presented as relative light units (RLU) per microgram of proteins.

In order to evaluate FX-dependent cell transduction, viruses were incubated with or without human FX (1 U/ml, Cryopep) for 1h in order to ensure efficient Ad coating. Then, virus-FX solution was added to CHO-k1-pCDNA cells and cells were incubated for 24h at 37°C.  $\beta$ gal activity was measured as described above.

### *2.6. Epitope detection on virions*

To confirm the presence and accessibility of the epitopes on the capsid surface, denatured or native viral particles were coated on 96-well plates (Nunc, Roskilde, Denmark). Viruses were inactivated by incubation at 56 °C for 30 min, followed by addition of 0.1% SDS. Spectrophotometric measurements at 215 and 225nm allowed to determine viral protein concentrations, and subsequently 100ng was coated on 96-well plates. To analyse epitope presence and accessibility on native virions, non-denatured viruses (100ng) were coated on the plates. After overnight incubation at 4°C, non-specific sites were blocked with 5% milk PBS-Tween for 2 h, then plates were washed and incubated for 1 h with an anti-ovalbumin rabbit polyclonal antibody (AB1225, Millipore, MA). Upon washing, an anti-rabbit IgG peroxidase-linked Ab (NA934, Amersham Biosciences, Saclay, France) was added for 1 h and peroxidase activity was revealed by incubation with the substrate O phenylenediamine dihydrochloride (Sigma–Aldrich, Lyon, France) for 30 min. The reaction was stopped by addition of 3N HCl and spectrophotometric readings were performed at 490 nm.

### *2.7. In vivo experiments*

Capsid-modified viruses ( $10^{10}$  vp) in PBS (200 $\mu$ L) were injected intraperitoneally. Repeated injections were performed at 2 weeks intervals with a total number of injections ranging between 2 and 3. Blood samples were collected from the submandibular vein, before virus injection and at different time points thereafter. Mice sera were prepared and analyzed for the presence of anti-ovalbumin, anti- $\beta$ gal and anti-Ad Abs by ELISA as described below.

In some experiments, mice were depleted of coagulation factors by subcutaneous injection of 133µg of warfarin in 100µl of PBS at day 3 and 1 prior to virus administration. Ad naïve or Ad-immune mice were obtained after injection of PBS or AdWT ( $10^{10}$  vp).

### *2.8. Measurement of specific antibodies*

Sera were analysed for the presence of specific Abs by ELISA. After coating of 96-well plates (Nunc) with 1µg of ovalbumin (Sigma), 100ng of βgal (Sigma) or 100ng of denatured AdWT viral particles, serial dilutions of the sera in 5% milk PBS-Tween were added. Bound Abs were detected with peroxidase-conjugated anti-mouse IgG, IgG1, IgG2a, or IgG2b isotype goat Abs (Southern Biotechnology Associates, Birmingham, AL). The peroxidase activity was revealed by incubation with the substrate O-phenylenediamine dihydrochloride (Sigma–Aldrich) for 30 min. The reaction was stopped by addition of 3N HCl and spectrophotometric readings were performed at 490 nm. Titers were defined as the reciprocal of the highest dilution giving an OD<sub>490</sub> 2-fold above background values.

### *2.9. Statistical analysis*

A Mann–Whitney test, recommended for groups fewer than 30 mice, was conducted. Differences were considered significant when  $P < 0.05$ . All graphs and statistical tests were obtained with the use of GraphPad Prism software.

### 3. Results

#### **Antibody responses elicited by Ad displaying epitopes does not require gene transfer**

Our previous results have shown that pre-existence of anti-Ad Abs strongly enhances anti-ovalbumin (Ova) antibody responses elicited by AdF-3OVA2 bearing OVA<sub>320-341</sub> B cell epitope [21], suggesting that cell transduction is dispensable for the efficacy of this vector. To address specifically this question, we compared the ability of capsid-modified AdF-3OVA2 and AdH-3OVA2 (**Table 1**) to elicit anti-Ova antibody responses relative to AdOVA, encoding the whole Ova protein as a transgene, in both naïve and immune mice. C57BL/6 mice were injected with PBS or with an Ad bearing a wild-type capsid (AdWT, 10<sup>10</sup> viral particle) and two weeks later received one intraperitoneal injection of AdH-3OVA2, AdF-3OVA2 or AdOVA (10<sup>10</sup> vp). In Ad-naïve mice, AdOVA induced high levels of anti-Ova antibodies from day 14 p.i. up to 210 days p.i. In accordance with our previous study [21], AdH-3OVA2 led to a significantly higher anti-ovalbumin Ab responses compared to AdF-3OVA2 (**Figure 1, upper panel**). However, this response remains lower than the one observed in AdOVA-injected mice. In Ad-immune mice, AdF-3OVA2 triggered strong anti-ovalbumin antibody responses compared to AdH-3OVA2 as described previously [21] while AdOVA was unable to trigger any antibody response (**figure 1, lower panel**). Interestingly, the immune response elicited by AdF-3OVA2 was long lasting with strong Ab titers detectable up to 7 months after the immunization. While strong anti-βgalactosidase (βgal) Ab responses were elicited by AdF-3OVA2 and AdH-3OVA2 in Ad-naïve mice, none of them triggered significant anti-βgal Ab responses in Ad-immune mice (**Supplementary Figure 1**).

Altogether, our data indicate that, in contrast to AdOVA, AdF-3OVA2 was able to trigger Ab responses in both naïve and immune mice, suggesting that virus transduction is not mandatory for the efficacy of this vector.

## **Production and characterization of Ad displaying ovalbumin epitope in the fiber and ablated in their native receptor interaction**

To examine more precisely whether virus transduction plays any role in controlling the efficacy of AdF-3OVA2, different vectors were produced displaying both 3OVA2 epitope inserted into the fiber protein and capsid modifications impairing the binding to specific receptors. AdP\*F-3OVA2 presents a deletion of the RGD motif in penton base protein in order to ablate interaction with integrins [9]. AdH\*F3-OVA2 contains a mutation in HVR5 hexon impairing binding to FX [12]. AdH\*P\*F-3OVA2 possesses both hexon and penton base mutations. Finally, AdS\*F-3OVA2 contains an Ad3 fiber shaft instead of Ad5 shaft [9] impairing binding to CAR receptor. All vectors were produced at titers comparable to AdF-3OVA2 (**Table 1**). SDS-PAGE analyses showed no difference in virus composition between the different Ad. As expected, all viruses but AdS\*F-3OVA2 displayed a similar migration pattern for the modified fiber. For AdS\*F-3OVA2, the migration pattern of the fiber protein is consistent with its reduced size (**Figure 2A**). Additionally, 3OVA2 epitope was detected on native virions by ELISA (**Figure 2B**) using a polyclonal Ova antibody. With the exception of AdS\*F-3OVA2, all vectors possess the same capacity to transduce CHO-k1-hCAR cells, a cell line overexpressing the Ad primary receptor. AdS\*F-3OVA2 led to a reduced cell transduction (**Figure 3A**) consistent with previous results of our laboratory (Raddi et al. in revision). Next, we examined transduction efficiency using specific cell lines allowing to monitor the detargeting from native Ad receptors. AdP\*F-3OVA2 and AdH\*P\*F-3OVA2 were tested on CAR-negative integrin-positive L929 cells. Both vectors led to a reduced  $\beta$ gal activity compared to AdF-3OVA2, confirming their reduced ability to bind integrins (**Figure 3B**). AdP\*H\*F-3OVA2 was used to transduce CAR-negative CHO-pcDNA cells in the presence or absence of FX (**Figure 3C**). The results showed vector displayed reduced ability to use FX in accordance with the role of hexon protein in FX binding (**Figure 3C and 3D, right part**).

## **Humoral responses elicited by Ad displaying ovalbumin epitope in the fiber and ablated in their native receptor interactions**

After the characterization of the different vectors, we examined their capacity to mount anti-Ova humoral responses. C57BL/6 mice were twice intraperitoneally injected with  $10^{10}$  vp. Sera were collected two weeks after each administration and anti-OVA, anti- $\beta$ gal and anti-Ad Abs were quantified by ELISA. Remarkably, no difference in their ability to trigger anti-ovalbumin Abs was observed between detargeted vectors (AdH\*F-3OVA2, AdP\*F-3OVA2, AdP\*H\*F-3OVA2 and AdS\*F-3OVA2) and AdF-3OVA2 neither after priming nor after boosting (**Figure 4A**). In contrast, anti- $\beta$ gal antibody titers were significantly reduced after priming in AdH\*P\*F-3OVA2- and AdS\*F 3OVA2 injected mice (**Figure 4B**,  $P < 0.05$ ) compared to AdF-3OVA2-injected mice. After boosting, all detargeted vectors were significantly impaired in their ability to trigger anti- $\beta$ gal Ab (**Figure 4B**). Of note, all vectors induced comparable anti-Ad Ab responses at all time points (data not shown). Altogether, these results underline that while detargeting Ad for its natural receptors reduce its ability to trigger humoral responses against the transgene, it has no impact on its ability to trigger humoral responses against an epitope displayed on the capsid.

Besides FX-detargeting through hexon modification, we also used a pharmacological approach based on administration of warfarin, a drug able to deplete all vitamin K-dependent blood factors. To do so, mice were pre-treated with warfarin or PBS before each AdF-3OVA2 administration. Blood factor depletion was confirmed by the measurement of FX activity in mice sera harvested prior to virus delivery (data not shown). Measurement of anti-Ova titers showed no significant difference between warfarin- and PBS-pre-treated mice neither after priming nor after boosting (**Supplementary Figure 2**). Additionally, levels of anti- $\beta$ gal and anti-AdAbs also remained unmodified in warfarin-pretreated mice (**Supplementary figure 2**). Thus, both genetic and pharmacological approaches used to detarget Ad from FX were unable to decrease humoral responses towards 3OVA2 epitope.

Collectively, these results underline that in contrast to humoral responses against  $\beta$ gal transgene, the humoral responses against 3OVA2 epitope displayed on fiber protein do not rely on Ad interaction with their cellular receptors.

### **Role of innate immune pathways in humoral responses elicited by Ad displaying ovalbumin epitope inserted into the fiber protein**

To get further insights into molecular bases controlling the efficacy of vaccination with Ad displaying epitopes, we investigated the role innate immune pathways. First, since TLRs and MyD88 were shown to participate in Ad innate immunity, we examined their role in shaping humoral responses by using MyD88-deficient mice. No significant difference was found in anti-Ova humoral responses in wild-type and MyD88-deficient mice, neither after priming nor after boosting (**Figure 5A**). In addition, both strains elicited comparable levels of anti- $\beta$ gal (**Figure 5B**) and anti-Ad Abs (**Figure 5C**). Interestingly, compared to AdF3-OVA2-injected wild-type mice, AdF3-OVA2-injected MyD88-deficient mice displayed a strong increase in IgG1 anti-Ova Abs and a trend to a reduced level of IgG2b anti-Ova Abs without any difference in IgG2a (**Figure 5D,  $p < 0.05$** ). In contrast, MyD88<sup>-/-</sup> mice displayed very low titers of anti- $\beta$ gal IgG1 and IgG2b and a strong reduction in IgG2a (**Figure 5D,  $p < 0.01$** ). No difference in anti-Ad isotypes were found between MyD88<sup>-/-</sup> and wild-type mice (**Figure 5E**). Then, we examined the role of RIG-I-induced innate immune pathway using MAVS-deficient mice. The levels of total anti-Ova IgG Abs (**Figure 6A**) as well as IgG1, IgG2a and IgG2b Abs (**Figure 6B**) were comparable in wild-type and in MAVS-deficient mice, ruling out a major role of RIG-I/MAVS pathway in induction of anti-epitope humoral responses.

Altogether, these results underline that TLR/MyD88 and RIG-I/MAVS pathways are both dispensable in mounting antibody responses against 3OVA2 epitope. However, they also reveal that TLR/MyD88 pathway influences the isotype nature of antibody responses against the displayed epitope.

## 4. Discussion

Vectors derived from Ad were used in different preclinical studies as well as in clinical trials for purpose of vaccination. The high seroprevalence of neutralizing Abs as well as the induction of strong anti-vector immunity after first administration led to the development of different strategies to overcome these limits [2]. Among them are the use of other Ad serotypes or even xenotypes but also epitope display on Ad capsid. This latter approach relies on insertion of peptides within a capsid protein (most of the time hexon protein). Generally, those peptides were B cell epitopes and they were found to successfully elicit antibody responses against model antigens [21] but also against several pathogens such as influenza virus [18], *B. anthracis* [19] or *Plasmodium yoelii* [20]. While several studies investigated the role of the site of epitope insertion and thus the number of motifs per capsid [21][27] or the size of the peptides [19], no study investigated molecular mechanisms controlling the efficacy of Ad displaying epitopes.

In a previous study, we discovered a key role of anti-Ad Abs in increasing antibody responses induced by Ad displaying Ova-derived epitopes in the fiber protein. At the same time, anti-Ad Ab were impeding the efficacy of Ad displaying epitopes into the hexon protein [21]. These previous results suggested that when anti-Ad Abs were able to neutralize the particle, Ad displaying epitopes were still able to trigger antibody responses. Two observations of the present paper confirmed that Ad infectious process is not mandatory to allow AdF-3OVA2 to elicit antibody responses. First, lacZ recombinant AdH-3OVA2 failed to trigger significant anti- $\beta$ gal antibody responses in Ad-immune mice while being more efficient in inducing anti-Ova Ab responses than in Ad-naïve mice (**Figure 1**). Second, detargeting AdF-3OVA2 from its native receptors reduced its ability to elicit antibody responses against  $\beta$ gal transgene without significant impairment of Ab production against 3OVA2 epitope (**Figure 4A**). Altogether, these results indicate that epitope display strategy of vaccination does not rely on gene delivery in contrast to the classical Ad vaccine approach that requires transgene expression.

Detargeting Ad from integrin receptor or from FX did not impact Ad's capacity to induce humoral responses toward  $\beta$ gal transgene. However, ablation of both integrin and FX binding or modification of Ad5 shaft translated into a significant reduction of anti- $\beta$ gal antibody titers (**Figure 4B**). These results may stem in the reduced ability of Ad impaired in hexon:FX and penton:integrin binding to transduce the spleen and to induce cytokine production [28]. AdS\*F-3OVA2 also led to the decrease in anti  $\beta$ gal responses. This behaviour could be related to the reduced ability of shaft-mutated Ad to transduce different cells and tissues *in vivo* (Raddi et al. *in revision*) but may also be linked to their reduced ability to trigger pro-inflammatory cytokine and chemokine production [23].

Innate immune responses are key factors for the establishment of adaptive immune responses. Among the different innate immune pathways, Ad were shown to activate TLR/Myd88 signalling. At all analysed time points, no significant modification of total anti-IgG Abs against Ad,  $\beta$ gal or 3OVA2 epitope was found in MyD88<sup>-/-</sup> mice compared to their wild-type counterparts (**Figure 5A**). These data suggest that TLR/MyD88 pathway is dispensable to mount efficient humoral responses or, alternatively, that other innate immune pathways could compensate the lack of MyD88. However, it should be noticed that Hartman *et al.* reported previously a reduction in anti-Ad IgG Ab in MyD88<sup>-/-</sup> mice compared to heterozygous MyD88<sup>+/-</sup> mice [29]. The discrepancies between their study and our study could be linked to differences in mouse strains, virus dose and mode of administration.

Increase of anti-epitope IgG1 Abs (**Figure 5B**) unravelled a role of MyD88 in shaping Ig isotype switching. Interestingly, MyD88 was not mandatory for the production of different anti-Ad IgG isotypes (**Figure 5F**) but was required for the production of anti- $\beta$ gal IgG2a Abs (**Figure 5D**). The difference in requirement of MyD88 for the production of Abs against the inserted epitope, the vector or the transgene product could be linked to their intrinsic nature (soluble protein or particle, monomeric or multimeric protein). The precise TLR involved in MyD88 activation was not investigated in this study. However, previous studies have unravelled Ad's capacity to trigger different TLRs such as TLR2 [30], TLR4 [17] and TLR9 [14] [30].

Modification of IgG isotype balance in MyD88<sup>-/-</sup> mice compared to their wild-type counterparts was also reported for other non-enveloped DNA viruses ([31][32][33]) but also for enveloped RNA viruses ([34][35]). The role of MyD88 may be linked to its ability to trigger type-I IFN production by dendritic cells. This cytokine was shown to promote IgG2b and IgG2c production while reducing IgG1 level [36][37]. MyD88 expressed by B cell could also directly promote isotype switching and affinity maturation as described by [32][38].

Several studies have previously shown that Ad triggers RIG-I pathway [16]. Our study revealed that mice deficient in MAVS, a protein downstream of RIG-I, did not show modification in total IgG production nor specific IgG isotype production against the epitope displayed into the capsid (**Figure 6**). This suggests that MAVS/RIG-I pathway did not play any significant role in modifying humoral responses against the epitope nor against the transgene product. Alternatively, other innate immune sensor pathways such as TLR/MyD88 may compensate the absence of functional RIG-I/MAVS pathway.

To summarize, our results shows that the efficacy of epitope display does not depend on Ad infection process nor on Ad interaction with its natural receptors. Interestingly, whereas mice deficient in TLR/MyD88 or RIG-I/MAVS pathways mount IgG antibody responses comparable to control mice, we unmasked a key role of MyD88/MAVS pathway in shaping antibody isotype production not only against the epitope but also against the transgene product. Thus, the present study improved our understanding of molecular bases controlling the efficacy of Ad displaying epitopes on their capsid. The results pave the way for the development of vaccines based on epitope display on Ad capsid.

## **Acknowledgments**

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The authors have no competing financial interests to declare.

## References

1. Benihoud, K., P. Yeh, and M. Perricaudet, *Adenovirus vectors for gene delivery*. Curr Opin Biotechnol, 1999. **10**(5): p. 440-7.
2. Lasaro, M.O. and H.C. Ertl, *New insights on adenovirus as vaccine vectors*. Mol Ther, 2009. **17**(8): p. 1333-9.
3. Descamps, D. and K. Benihoud, *Two key challenges for effective adenovirus-mediated liver gene therapy: innate immune responses and hepatocyte-specific transduction*. Curr Gene Ther, 2009. **9**(2): p. 115-27.
4. Smith, T.A., et al., *Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice*. Hum Gene Ther, 2003. **14**(8): p. 777-87.
5. Martin, K., et al., *Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism*. Mol Ther, 2003. **8**(3): p. 485-94.
6. Smith, T.A., et al., *Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates*. Hum Gene Ther, 2003. **14**(17): p. 1595-604.
7. Mizuguchi, H., et al., *CAR- or alphav integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice*. Gene Ther, 2002. **9**(12): p. 769-76.
8. Nakamura, T., K. Sato, and H. Hamada, *Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber*. J Virol, 2003. **77**(4): p. 2512-21.
9. Vigne, E., et al., *Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation*. Gene Ther, 2003. **10**(2): p. 153-62.
10. Parker, A.L., et al., *Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes*. Blood, 2006. **108**(8): p. 2554-61.
11. Kalyuzhniy, O., et al., *Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo*. Proc Natl Acad Sci U S A, 2008. **105**(14): p. 5483-8.
12. Vigant, F., et al., *Substitution of hexon hypervariable region 5 of adenovirus serotype 5 abrogates blood factor binding and limits gene transfer to liver*. Mol Ther, 2008. **16**(8): p. 1474-80.
13. Waddington, S.N., et al., *Adenovirus serotype 5 hexon mediates liver gene transfer*. Cell, 2008. **132**(3): p. 397-409.
14. Yamaguchi, T., et al., *Role of MyD88 and TLR9 in the innate immune response elicited by serotype 5 adenoviral vectors*. Hum Gene Ther, 2007. **18**(8): p. 753-62.
15. Appledorn, D.M., et al., *Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors*. Gene Ther, 2008. **15**(24): p. 1606-17.
16. Minamitani, T., D. Iwakiri, and K. Takada, *Adenovirus virus-associated RNAs induce type I interferon expression through a RIG-I-mediated pathway*. J Virol, 2011. **85**(8): p. 4035-40.
17. Doronin, K., et al., *Coagulation factor X activates innate immunity to human species C adenovirus*. Science, 2012. **338**(6108): p. 795-8.
18. Worgall, S., et al., *Protection against P. aeruginosa with an adenovirus vector containing an OprF epitope in the capsid*. J Clin Invest, 2005. **115**(5): p. 1281-9.
19. McConnell, M.J., X. Danthinne, and M.J. Imperiale, *Characterization of a permissive epitope insertion site in adenovirus hexon*. J Virol, 2006. **80**(11): p. 5361-70.

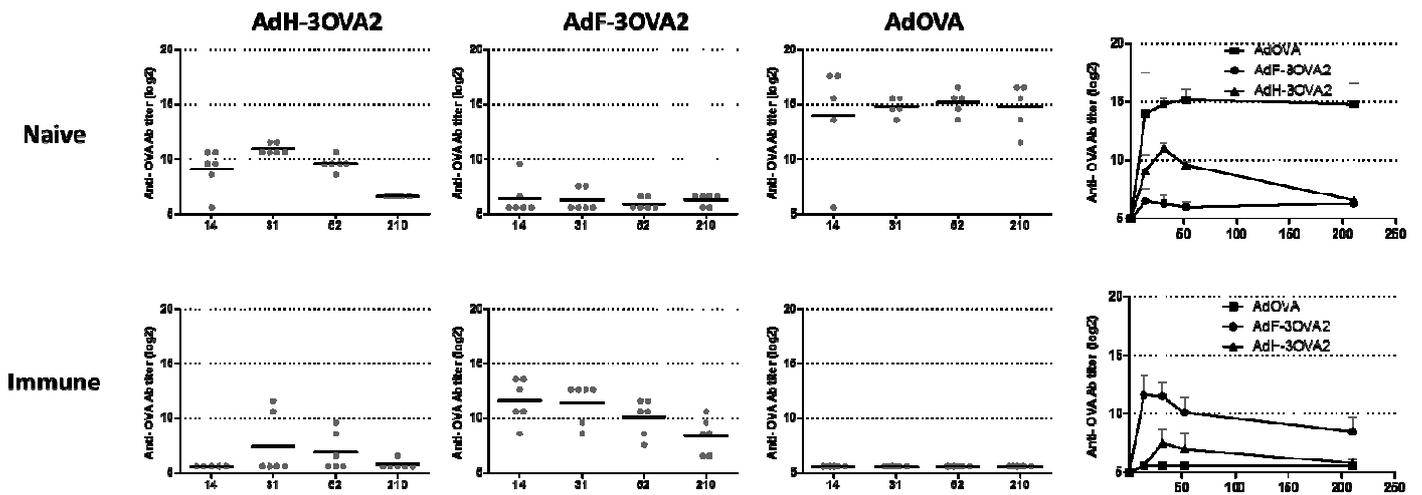
20. Shiratsuchi, T., et al., *Replacing adenoviral vector HVR1 with a malaria B cell epitope improves immunogenicity and circumvents preexisting immunity to adenovirus in mice.* J Clin Invest, 2010. **120**(10): p. 3688-701.
21. Lanzi, A., et al., *Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid.* Vaccine, 2011. **29**(7): p. 1463-71.
22. Vigne, E., et al., *RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection.* J Virol, 1999. **73**(6): p. 5156-61.
23. Raddi, N., et al., *Pseudotyping Serotype 5 Adenovirus with the Fiber from Other Serotypes Uncovers a Key Role of the Fiber Protein in Adenovirus 5-Induced Thrombocytopenia.* Hum Gene Ther, 2016. **27**(2): p. 193-201.
24. Crouzet, J., et al., *Recombinational construction in Escherichia coli of infectious adenoviral genomes.* Proc Natl Acad Sci U S A, 1997. **94**(4): p. 1414-9.
25. Galaup, A., et al., *Full kringles of plasminogen (aa 1-566) mediate complete regression of human MDA-MB-231 breast tumor xenografted in nude mice.* Gene Ther, 2005. **12**(10): p. 831-42.
26. Liszewski, M.K., I. Tedja, and J.P. Atkinson, *Membrane cofactor protein (CD46) of complement. Processing differences related to alternatively spliced cytoplasmic domains.* J Biol Chem, 1994. **269**(14): p. 10776-9.
27. Krause, A., et al., *Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity.* J Virol, 2006. **80**(11): p. 5523-30.
28. Bradshaw, A.C., et al., *Biodistribution and inflammatory profiles of novel penton and hexon double-mutant serotype 5 adenoviruses.* J Control Release, 2012. **164**(3): p. 394-402.
29. Hartman, Z.C., et al., *Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo.* J Virol, 2007. **81**(4): p. 1796-812.
30. Appledorn, D.M., et al., *Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 in vivo.* J Immunol, 2008. **181**(3): p. 2134-44.
31. Sudres, M., et al., *MyD88 signaling in B cells regulates the production of Th1-dependent antibodies to AAV.* Mol Ther, 2012. **20**(8): p. 1571-81.
32. Rogers, G.L., et al., *Unique Roles of TLR9- and MyD88-Dependent and -Independent Pathways in Adaptive Immune Responses to AAV-Mediated Gene Transfer.* J Innate Immun, 2015. **7**(3): p. 302-14.
33. Yang, R., et al., *B lymphocyte activation by human papillomavirus-like particles directly induces Ig class switch recombination via TLR4-MyD88.* J Immunol, 2005. **174**(12): p. 7912-9.
34. Kang, S.M., et al., *MyD88 plays an essential role in inducing B cells capable of differentiating into antibody-secreting cells after vaccination.* J Virol, 2011. **85**(21): p. 11391-400.
35. Uchiyama, R., et al., *MyD88-mediated TLR signaling protects against acute rotavirus infection while inflammasome cytokines direct Ab response.* Innate Immun, 2015. **21**(4): p. 416-28.
36. Heer, A.K., et al., *TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses.* J Immunol, 2007. **178**(4): p. 2182-91.
37. Zhu, J., X. Huang, and Y. Yang, *The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice.* J Clin Invest, 2009. **119**(8): p. 2388-98.
38. Hou, B., et al., *Selective utilization of Toll-like receptor and MyD88 signaling in B cells for enhancement of the antiviral germinal center response.* Immunity, 2011. **34**(3): p. 375-84.

## Tables and figures

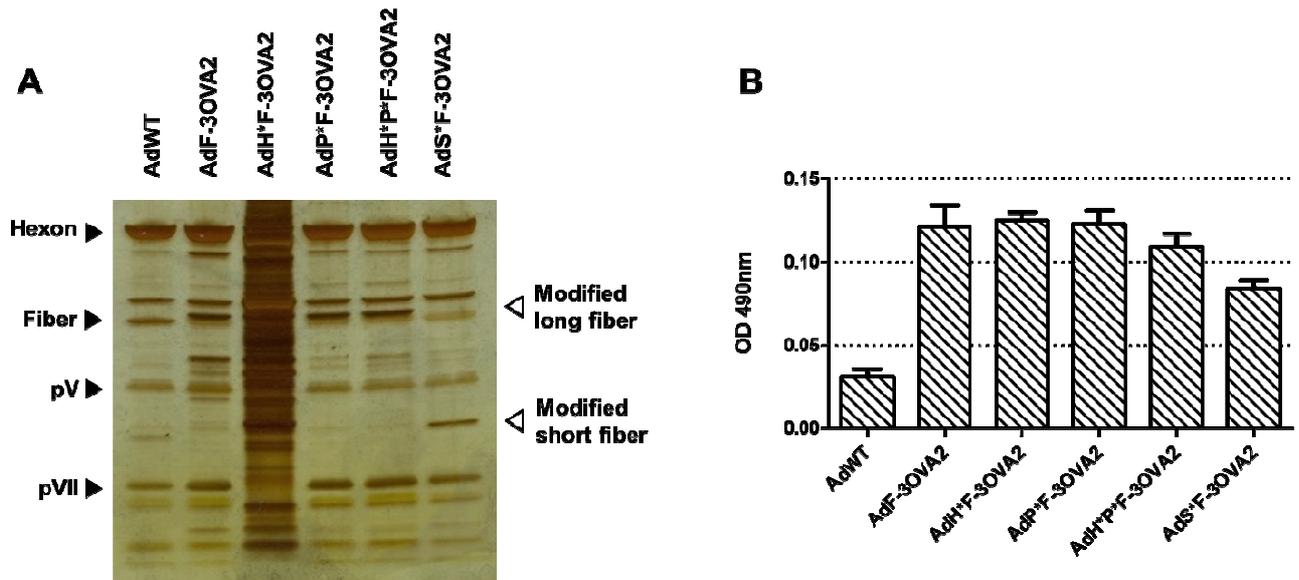
**Table 1. Characteristics of Ad displaying ovabumin epitopes.**

Virus	Transgene	Ovalbumin <sub>323-339</sub> epitope (3OVA2)		Additional alterations		Titer <sup>a</sup> ( $\times 10^{12}$ vp/ml)
		Modified protein	Inserted sequence	Modified protein	Alteration	
<b>AdWT</b>	$\beta$ gal	-	-	-	-	4.4 $\pm$ 0.8
<b>AdOVA</b>	Ovalbumin	-	-	-	-	7.3 $\pm$ 2.1
<b>AdH-3OVA2</b>	$\beta$ gal	Hexon	+	-	-	4.5 $\pm$ 2.9
<b>AdF-3OVA2</b>	$\beta$ gal	Fiber	+	-	-	4.6 $\pm$ 1.9
<b>AdP*F-3OVA2</b>	$\beta$ gal	Fiber	+	Penton base	RGD deletion	2.3 $\pm$ 1.0
<b>AdH*F-3OVA2</b>	$\beta$ gal	Fiber	+	Hexon	HVR5 deletion	2.1 $\pm$ 0.6
<b>AdP*H*F-3OVA2</b>	$\beta$ gal	Fiber	+	Penton base Hexon	RGD deletion HVR5 deletion	2.5 $\pm$ 1.2
<b>AdS*F-3OVA2</b>	$\beta$ gal	Fiber	+	Fiber	Ad3 shaft	1.7 $\pm$ 0.6

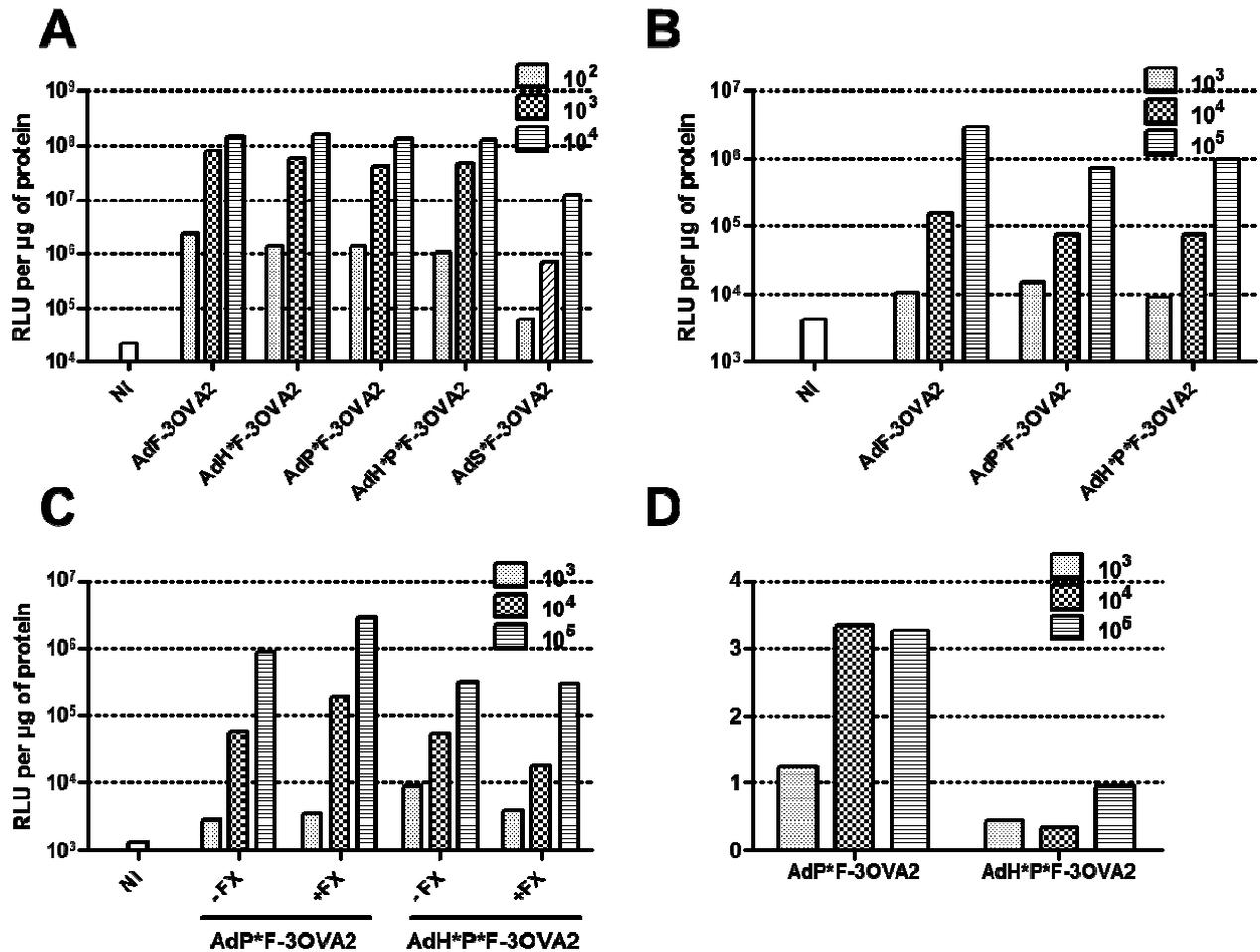
<sup>a</sup> Mean  $\pm$  SD



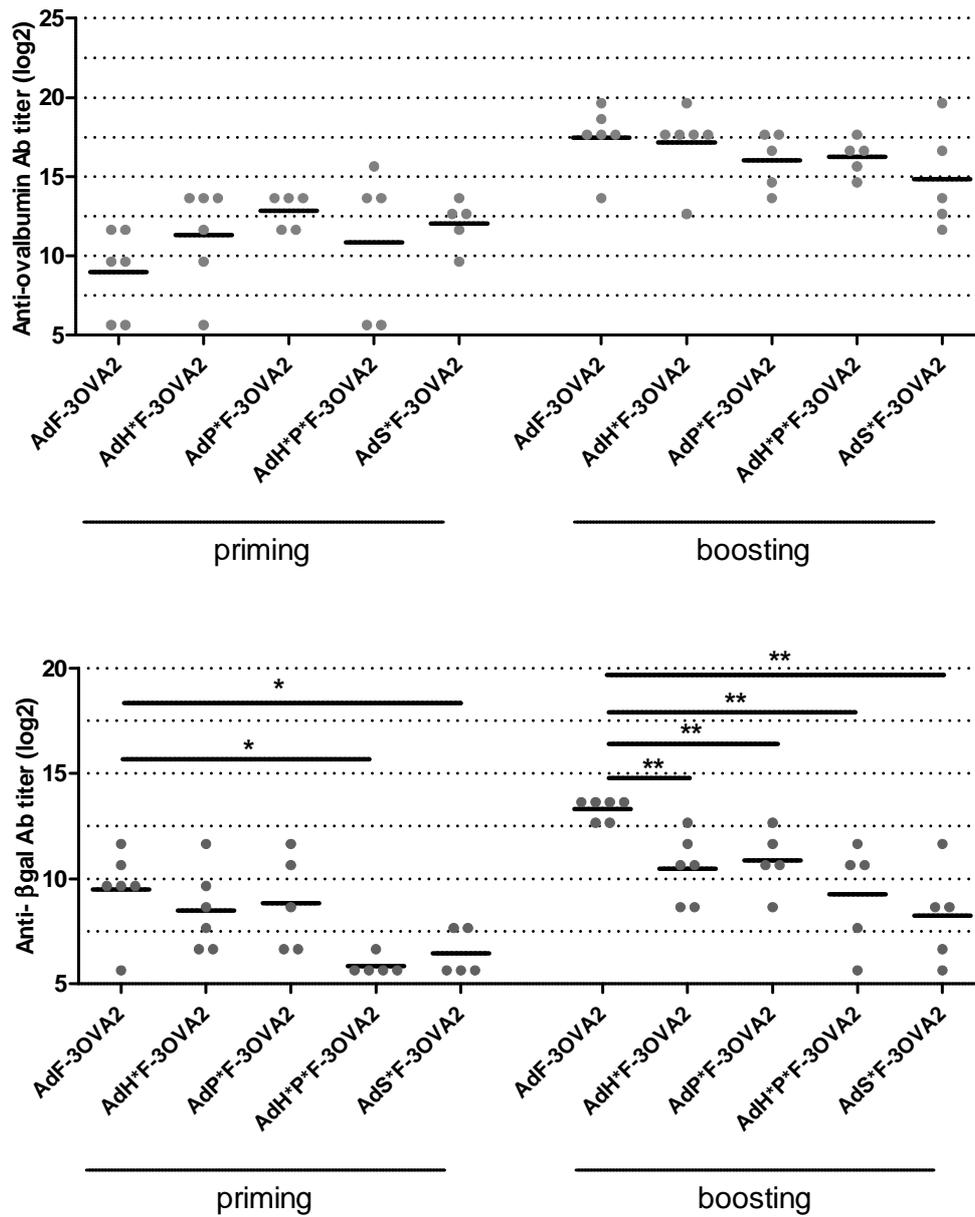
**Figure 1** Kinetics of anti-OVA humoral response. Naive (upper panel) or Ad immune (lower panel) C57Bl/6 mice were immunized intraperitoneally with  $10^{10}$  vp of capsid-modified Ad (AdF-3OVA2 or AdH-3OVA2) or with an Ad vector encoding Ova as a transgene (AdOVA). Anti-Ova IgG titers were determined by ELISA at different time points after injection. Left part, circles and bars represent results of individual mice ( $n = 5-6$ ) and means, respectively. Titers below 100 were plotted as 50. Right part, means + SEM for each group.



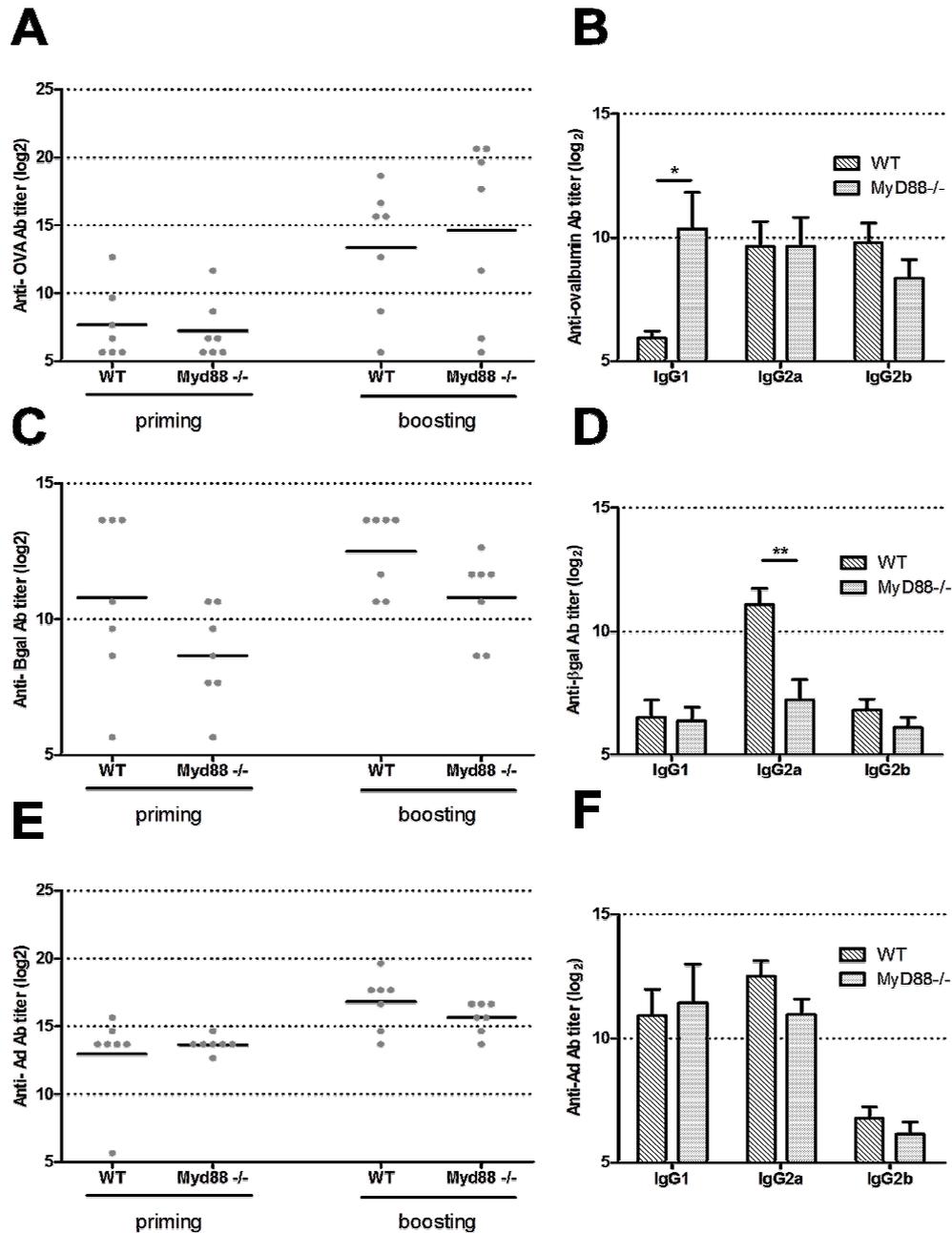
**Figure 2** Epitope detection on capsid-modified vectors. **(A)** Silver staining of capsid modified Ad.  $10^{10}$  vp of either a control Ad (AdWT) or a capsid-modified Ad (AdF-3OVA2, AdH\*F-3OVA2, AdP\*F-3OVA2, AdH\*P\*F-3OVA2 and AdS\*F-3OVA2) were separated on a 10% polyacrylamide gel. Modified fibers are indicated with white arrows while hexon, penton base, polypeptide IIIa (pIIIa) and native fiber proteins are labelled with black ones. **(B)** Detection of OVA epitopes on virions. ELISA plates were coated with 100 ng of native viruses and incubated with a rabbit polyclonal antibody against the Ova protein. The binding was detected with HRP-conjugated secondary antibody. One of two experiments is shown, means  $\pm$  SD of four replicates.



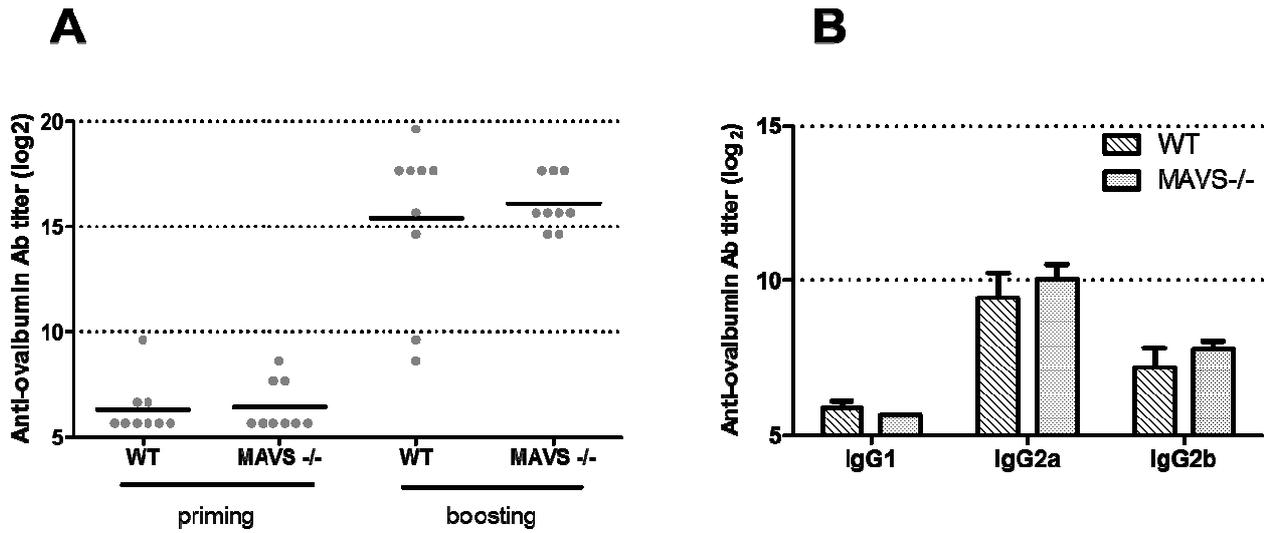
**Figure 3** *In vitro* infectivity of capsid modified vectors. (A) Transduction of CHO-k1-hCAR. Cells were mock-infected (NI) or infected with different MOI of capsid-modified Ad (AdF-3OVA2, AdH\*F-3OVA2, AdP\*F-3OVA2, AdH\*P\*F-3OVA2 and AdS\*F-3OVA2). (B) Transduction of L929 cells. Cells were mock-infected (NI) or infected with different MOI of capsid-modified Ad (AdP\*F-3OVA2 and AdH\*P\*F-3OVA2). (C and D) Transduction of CHO-k1-pCDNA. Cells were mock infected (NI) or infected with different MOI of capsid modified Ad (AdH\*F-3OVA2 and AdH\*P\*F-3OVA2) with or without FX coagulation factor. (A-C) For each panel,  $\beta$ gal activity was measured in cells harvested 24 h post-infection and expressed as relative light unit (RLU) per  $\mu$ g of protein. (D) Fold-inductions of  $\beta$ gal activity 24 hours after CHO-k1-pCDNA infection in the presence of physiological levels (1 U/ml) of FX in comparison with  $\beta$ gal activity in the absence of FX. The representative results of at least 2 experiments are shown.



**Figure 4** Humoral responses elicited by Ad displaying Ova epitopes and ablated in their native receptor interaction. C57BL/6 mice were immunized intraperitoneally with  $10^{10}$  vp of capsid-modified AdF-3OVA2, AdH\*F-3OVA2, AdP\*F-3OVA2, AdH\*P\*F-3OVA2 and AdS\*F-3OVA2. Anti-ovalbumin (A) and anti-βgal (B) IgG titers were determined by ELISA at day 14 after first (priming) and second (boosting) administration. Titers below 100 were plotted as 50. One of two experiments is shown, dots and bars represent results from individual mice (n = 5-6 mice) and means, respectively. \*,  $p < 0.05$  versus AdF-3OVA2; ns, non significant.

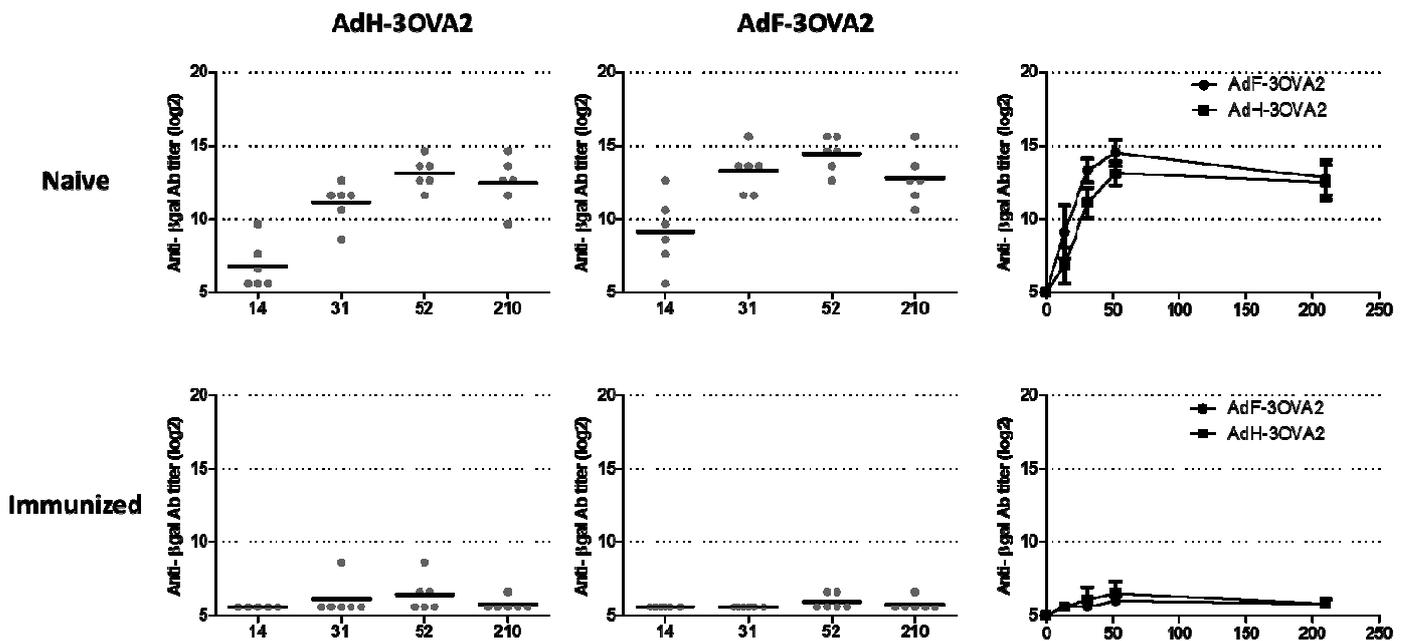


**Figure 5** Influence of MyD88 on humoral responses elicited by Ad displaying Ova epitope. Mice were immunized intraperitoneally with  $10^{10}$  vp of AdF-3OVA2. Total anti-Ova (A), anti-βgal (C) and anti-Ad (E) IgG were measured by ELISA at day 14 after first (priming) and second (boosting) administration. Dots and bars represent results from individual mice ( $n = 6-7$  mice) and means, respectively. Anti-Ova (B), anti-βgal (D) and anti-Ad (F) antibody isotypes IgG1, IgG2a and IgG2b were measured by ELISA at day 42 after the first administration. Titers below 100 were plotted as 50. \*,  $P < 0.05$  and \*\*,  $p < 0.01$  versus wild-type mice.

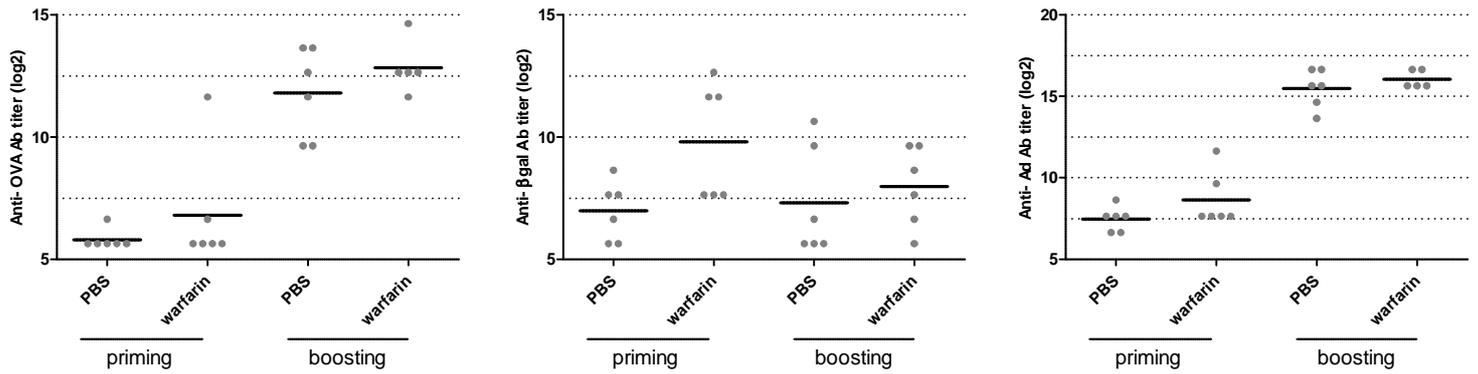


**Figure 6** Influence of RIG-I/MAVS pathway on humoral responses elicited by Ad displaying Ova epitope. Mice were immunized intraperitoneally with  $10^{10}$  vp of AdF-3OVA2. Total IgG (**A**) and IgG isotypes (**B**) specific for Ova were determined by ELISA (**A**) at day 14 after first (priming) and second (boosting) administration or (**B**) at day 42 post the first administration. Titers below 100 were plotted as 50. Circles represent individual mice and bars reflect means.

Supplementary figures



**Supplementary Figure 1** Kinetics of anti-βgal humoral response. Naïve (upper panel) or Ad immune (lower panel) C57Bl/6 mice were immunized intraperitoneally with  $10^{10}$  vp of capsid-modified Ad (AdF-3OVA2 or AdH-3OVA2) or with an Ad vector encoding Ova as a transgene (AdOVA). Anti-βgal IgG titers were determined by ELISA at different time points after injection. One experiment of two is shown. Left part, dots and bars represent results of individual mice ( $n = 5-6$ ) and means, respectively. Titers below 100 were plotted as 50. Right part, means + SEM for each group.



**Supplementary Figure 2** Influence of coagulation factor FX on humoral responses elicited by Ad displaying Ova epitopes. Mice were immunized intraperitoneally with  $10^{10}$  vp of capsid-modified AdF-3OVA2. Before each injection, mice were pre-treated with PBS or warfarin. Anti-Ova (**A**), anti-βgal (**B**) and anti-Ad (**C**) IgG titers were determined by ELISA at day 14 after first (priming) and second (boosting) administration. Titers below 100 were plotted as 50. One of two experiments is shown, dots and bars represent results from individual mice (n = 5-6 mice) and means, respectively.



## PART 2

# **CD8<sup>+</sup> cellular responses elicited by Ad displaying T cell epitopes**



## ARTICLE 2

### **Efficient induction of CD8<sup>+</sup> T cell responses by adenovirus displaying T cell epitopes inserted into hexon capsid protein**

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Key words: adenovirus, hexon, fiber

## Abstract

Although the capacity of Ad vectors displaying heterologous epitopes to induce humoral responses was previously demonstrated for various epitopes, their potential to induce CD8<sup>+</sup> cellular responses upon class-I T cell epitope display remains an open question. We thus constructed Ad vectors with ovalbumin epitope SIINFEKL inserted into hexon or fiber proteins. The displayed epitopes were efficiently uptaken and processed by dendritic cells *in vitro*. Vaccination studies in naïve and immune mice confirmed the capacity of vector displaying epitope in hexon to induce robust cellular responses. Analysis of kinetics and phenotype of ovalbumin-specific CD8<sup>+</sup> T cells suggests that this vector induces potent effector memory differentiation.

## 1. Introduction

Vaccine development has been one of the most impactful contributions to the mortality reduction in the medicine history. During decades, conventional vaccines were based on attenuated or inactivated whole pathogens, and have contributed to the eradication or regression of diseases like smallpox or poliomyelitis [10]. However, this traditional approach does not answer the new needs of vaccinology, and especially its safety concerns. Increased knowledge in the field of immunology and molecular biology has allowed for the emergence of new subunit vaccines, based on selected fragments of the pathogen, injected as recombinant protein, DNA vaccine or expressed by recombinant vector [1]. Thus, the current goal of vaccinology is to present to the immune system only antigens of interest. The most precise and minimal targets recognized by the immune system are epitopes and thus a new class of vaccine, consisting in epitope presentation, has emerged.

Epitope-based vaccines allow to direct immune responses toward selected epitopes of the pathogen [14]. Thus, by controlling the type of pathogen fragment which will be presented, the epitope-based vaccine safety is surpassing traditional vaccines. In consequence, potentially harmful regions of the pathogen which could lead to auto-immune or oncogenic reactions are no longer included during vaccine preparation. Other advantage of this new vaccine type is the possibility to combine different peptides in one vaccine formulation and to select epitopes which are presented by highly prevalent alleles of HLA molecules.

Therefore, the main challenges of this vaccination strategy consist in identifying suitable epitopes and presenting them correctly to the immune system. A safe, practical and efficient delivery system is needed to expose the epitope and to activate immune responses. Numerous studies have focused on this subject and evaluated carriers like lipopeptide [9] MAP [8] or viral pseudo-particles [13]. In this work, we proposed the adenovirus (Ad)-derived vectors as a promising epitope delivery system. Indeed, Ad from serotype 5 are safe, easy to manipulate and produce, and they activate efficiently both innate and adaptive responses [16].

Recombinant Ad vectors have been extensively used in a classical vaccination strategy [11]. However, the vector gene transfer is heavily limited by the presence of anti-Ad neutralizing antibodies (Abs) in 50 to 90% of human population [15]. To circumvent this problem, another use of Ad has emerged, based on epitope insertion into adenoviral capsid protein. Different regions of capsid proteins have been identified for their capacity to accommodate modifications without affecting capsid self-assembly, enabling genetic insertion of heterologous epitopes in these regions [3].

The efficacy of epitope display strategy with the use of Ad capsid as a platform has already been demonstrated by other groups [4, 9, 19]. Worgall *et al.* have inserted a neutralizing epitope from *P.aeruginosa* into hexon protein, which has led to protection of immunized mice against a lethal infection by the pathogen [18]. In the same manner, we have previously demonstrated that insertion of a model B epitope from the ovalbumin (Ova) protein into hexon or fiber protein results in efficient humoral anti-epitope responses that are boosted after re-administrations of the vector. Interestingly, we highlighted the fact that anti-Ad Abs control the efficacy of the anti-epitope responses, depending on the insertion site. Nonetheless, the studies evaluating this strategy have concentrated exclusively on B cell epitope insertion. Therefore, we have decided to pursue the study of this vaccine approach by inserting a class I-restricted T cell epitope from Ova, OVA<sub>257-264</sub>, into hexon and fiber proteins. The aim of this work is to compare the vaccination efficiency depending on the insertion site and the epitope number. We have investigated the role of flanking sequences, by inserting the epitope either alone or with additional residues from the Ova sequence, since these flanking residues could potentially modulate the processing efficiency of the epitope [12]. The immunization potential of these Ad vectors was assessed in both presence and absence of anti-Ad immune responses and the kinetics and phenotype of the induced cellular responses were analyzed.

## 2. Materials and methods

### 2.1. Cells

The RF33.70 T cell hybridoma was generously provided by Dr. K.L. Rock (Dana Farber Cancer Institute, Boston, MA, USA), and was grown in RPMI supplemented with 10% SVF, 1% non-essentials amino acids, 1% glutamine and 1% hepes.

### 2.2. Mice

7-week-old C57BL/6 female mice were purchased from Janvier (Le Gesnest Saint Isle, France) or Harlan (Gannat, France). They were conditioned for at least one week in the animal facilities before beginning the experiments. All animal experiments were approved by Gustave Roussy Institutional Animal Care and Use Committee.

### 2.3. FLDC preparation

FLDC (Flt3 ligand-dependent dendritic cells) were generated from bone marrow precursors. Bone marrow cells were harvested from tibia and femurs of C57Bl/6 mice by flushing with complete RPMI medium (RPMI supplemented with 5% SVF, 1% non-essentials amino acids, 1% glutamine, 1% pyruvate and  $5 \cdot 10^{-5}$  M  $\beta$ -mercaptoethanol), and filtered through a 100  $\mu$ m cell strainer. After removal of blood cells by ACK Lysing Buffer (Invitrogen, Cergy-Pontoise, France), the cells were resuspended and the concentration was adjusted to  $2 \times 10^6$  cells/mL. Cells were plated in MW6 plates, and recombinant human Flt3 ligand was added at 250 ng/mL. Cultures were fed with fresh Flt3 complemented RPMI medium every 2-3 days. At day 10, FLDCs were harvested from the cultures by vigorously pipetting. Cells were labelled with Pacific blue anti CD11b (eBioscience, CA, USA), FITC anti I-A<sup>b</sup>, PE anti CD24, PE-Cy<sup>TM</sup>7 anti CD11b and PerCP anti CD45R/B220 (BD Bioscience, CA, USA) Abs. The diverse phenotypic characteristics of FLDC were confirmed using a FACScan (BD Biosciences).

#### 2.4. Adenoviral vectors

LacZ-recombinant control AdWT (AE18 in ref. [17]) was derived from Ad5 with E1 and E3 regions deleted. The expression cassette cloned instead of the E1 region contains the promoter/enhancer from the immediate early gene of human cytomegalovirus (CMV), the *E. coli*  $\beta$ -galactosidase gene with a nuclear localization signal, and the SV40 late polyA signal. AdH-SL, AdH-3SL2 and AdH-5SL2, derived from AdWT, contain respectively a short (OVA<sub>257-264</sub>: SIINFEKL, referred in the text as SL peptide), medium (OVA<sub>254-266</sub>: QLESIINFEKLTE, referred in the text as 3SL2 peptide), or a long (OVA<sub>252-266</sub>: LEQLESIINFEKLTE, referred in the text as 5SL2 peptide) peptide derived from Ova (Table 1). These peptides were inserted into the hexon protein in place of <sub>269</sub>TTEAAAGNGDNL<sub>281</sub> of hypervariable region 5. AdF-SL, AdF-3SL2 and AdF-5SL2 contain respectively SL, 3SL2 or 5SL2 peptide inserted in place of T<sub>539</sub>QETGDTTPS<sub>548</sub> into the HI loop of the fiber protein. All modified viruses (Table 1) were constructed using recombinational cloning in *E. coli* [14].

All viruses were obtained using standard procedures as described [6], stored at  $-80^{\circ}\text{C}$  in PBS-7% glycerol and titrated by spectrophotometry ( $1 \text{ OD}_{260} = 1.1 \times 10^{12}$  viral particle (vp)/ml).

#### 2.5. SDS-PAGE and silver stain analysis

Purified viruses ( $10^{10}$  vp) were resuspended in Laemmli lysis buffer, boiled for 10 min and loaded onto a 10% NuPage gel (Novex, Invitrogen, CA). After electrophoresis, the gel was stained with a silver staining kit (Invitrogen, Carlsbad, CA).

#### 2.6. Detection of SL, 3SL2 and 5SL2-epitopes on virions

Viruses were inactivated at  $56^{\circ}\text{C}$  during 30 minutes and 0.1% SDS was added. Spectrophotometric reading was performed at 215 and 225 nm to determine virus concentrations and 100 ng were coated on 96-well plates (Nunc, Roskilde, Denmark). After overnight incubation at  $4^{\circ}\text{C}$ , non-specific sites were blocked with 5% milk PBS-Tween, then plates were washed and incubated with a rabbit polyclonal antibody against the Ova protein (AB1225, Millipore, MA) for 1

hour. After washing, an anti-rabbit IgG peroxidase-linked Ab (NA934, Amersham Biosciences, Saclay, France) was added for 1 hour and peroxidase activity was revealed by incubation with the substrate o-Phenylenediamine dihydrochloride (Sigma-Aldrich, Lyon, France) for 30 min. The reaction was stopped by addition of 3N HCl and spectrophotometric reading was performed at 490 nm.

### *2.7. Antigen presentation assay*

FLDC were seeded on a 96-well culture plate at a density of  $10^5$  cells/well. Cells were incubated with control or capsid-modified Ads for 2 hours at  $10^4$  or  $10^5$  vp/cell in a final volume of 100  $\mu$ L of complete RPMI medium. Controls included untreated FLDC, FLDC pulsed for 2 hours with Ova protein (2mg/mL), or SIINFEKL peptide (10 $\mu$ g/mL). Then, RF33.70 cells ( $10^5$  cells/well in 100  $\mu$ L) were added and cells were co-cultured during 2 days at 37°C. The response of stimulated RF33.70 cells was assessed by determining the amount of IL-2 released into supernatants using a murine IL-2 ELISA Kit (BD Bioscience).

### *2.8. In vivo experiments*

Control or capsid-modified viruses ( $10^{10}$  vp) in PBS (200  $\mu$ L) were injected intraperitoneally in C57Bl/6 mice. Repeated injections were performed at intervals of 2 weeks. Spleens were collected at different time points after the second injection.

### *2.9. Epitope-specific cellular responses*

Spleen from naïve or immunized mice were crushed in complete RPMI medium, and filtered through a 100  $\mu$ m cell strainer. After removal of blood cells by ACK Lysing Buffer (Invitrogen, Cergy-Pontoise, France), the cells were resuspended and the concentration was adjusted at  $2.5 \times 10^6$  cells/mL. Splenocytes were then directly used for tetramer staining assay, IFN- $\gamma$  ELISPOT assay or were restimulated *in vitro*.

To assess specific cellular responses splenocytes were seeded on a 24-well plate ( $5 \times 10^6$  cells in 2mL) and restimulated in different conditions in triplicates: RPMI medium, Ova SIINFEKL peptide (5 $\mu$ g/mL), Ad peptide FALSNAEDL (5 $\mu$ g/mL) or  $\beta$ gal peptide ICPMYARV (5 $\mu$ g/mL). As a positive control a mix of ionomycine (1 $\mu$ M) and PMA (0.1 $\mu$ M) was used. Cells were incubated for 72 hours at 37°C. Then, supernatant was collected and assayed for the presence of IFN- $\gamma$  using a murine IFN- $\gamma$  kit (eBioscience).

For ELISPOT assay restimulants were prepared and added in triplicates to the ELISPOT plates (Eurobio) in 100 $\mu$ l/well: RPMI medium as a negative control, Ova SIINFEKL peptide (5  $\mu$ g/mL), Ad peptide FALSNAEDL (5 $\mu$ g/mL),  $\beta$ gal peptide ICPMYARV (5 $\mu$ g/mL), Ova protein (236,5 $\mu$ g/ml) and mix of ionomycine (1 $\mu$ M) and PMA (0.1 $\mu$ M) as a positive control. Cells were then added to the wells (100 $\mu$ l) and incubated 22 hours at 37°C. Plates were revealed with supplied reagents and spots were counted with the ImmunoSpot<sup>®</sup> S6 FluoroSpot Line Plate Reader (CTL). For tetramer staining assay, splenocyte suspension was stained with a phycoerythrin (PE)-coupled major histocompatibility complex (MHC) class I pentamer, containing H-2K<sup>b</sup> restricted SIINFEKL epitope (CliniScience) and with monoclonal antibodies anti : CD90.2 (clone:53-2.1) eFluor<sup>®</sup> 450-coupled; CD45R (B220) (clone:RA3-6B2) APC-eFluor<sup>®</sup> 780 coupled; CD8a (clone:53-6.7) FITC-coupled; CD44 (clone:IM7) PE-Cyanine5 or PerCP-Cyanine5.5 coupled; CD197 (CCR7) (clone:4B12) APC-coupled; CD127 (clone:A7R34) Alexa Flour<sup>®</sup> 700-coupled; CD62L (clone:MEL-14) PE-Cyanine7-coupled and KLRG1 (clone:2F1) PE-eFluor<sup>®</sup> 610-coupled (eBionscience). Cells were analyzed by MoFlo<sup>®</sup> Astrios™ Flow cytometer and cell sorter (Beckman Coulter) and software Summit 6.2 (Beckman Coulter).

### *2.10. Statistical analyses*

A Mann-Whitney test, recommended for groups fewer than 30 mice, was conducted. Differences were considered significant when  $P < 0.05$ .

### 3. Results

#### Production and characterization of Ad displaying ovalbumin-derived epitopes

In the first turn we sought to determine the role of the epitope insertion site and the presence of flanking sequences on the vaccination efficacy of Ad displaying epitopes on their capsid. To that end, the immunodominant class I epitope SIINFEKL (SL) from Ova protein was genetically inserted, with or without flanking sequences (SL, 3SL2 and 5SL2), into either hexon (AdH-SL, AdH-3SL2 and AdH-5SL2) or fiber (AdF-SL, AdF-3SL2 and AdF-5SL2) proteins of a lacZ-recombinant Ad. All capsid-modified Ads were produced and purified at titers comparable to AdWT presenting a wild-type capsid (**Table 1**). SDS-PAGE analyses confirmed no differences in virus composition and integrity between capsid-modified Ad and AdWT (**Fig. 1**).

The presence of the epitope coding sequence in the Ad genome was confirmed by PCR performed on purified virions (data not shown). Using a polyclonal anti-Ova antibody, 3SL2 and 5SL2 epitopes were detected on denatured, purified virions by ELISA. SL epitope was detected on AdH-SL but not on AdF-SL (**Fig. 2**). In addition, there was no difference in the ability of capsid-modified Ad to transduce CHO-CAR cells, cell line overexpressing Ad primary receptor (supplementary Fig. 1).

#### Epitope insertion site strongly influences epitope processing efficiency

The capacity of the epitope to be efficiently processed from the capsid protein into which it is inserted constitutes a mandatory step to allow the peptide to be presented by MHC-I molecules on antigen-presenting cells. To address specifically this question, Ad displaying Ova T cell epitope were incubated with FLDC as a source of APC, then RF33 T cell specific hybridoma, recognizing SL epitope presented by H-2K<sup>b</sup> MHC-I molecules, was added. Finally, measurement of IL-2 production by ELISA in the co-culture supernatants was used to monitor hybridoma activation consecutive to both antigen processing and presentation. IL-2 production was detected after FLDC incubation with

hexon-modified Ad, demonstrating that both in the presence and absence of flanking sequences epitope inserted into hexon protein was efficiently processed. The highest release of IL-2 was observed for non-flanked SL epitope, and 3SL2 epitope was associated with the lowest IL-2 production (**Fig. 3a**). In sharp contrast, after incubation with fiber-modified Ads, IL-2 production was much more modest, suggesting that the epitope presentation was less efficient than with hexon-modified Ad (**Fig. 3b**). This result suggested that the insertion site plays an important role in the efficiency of epitope processing by dendritic cells. However, the obtained hierarchy could be explained by the 20-fold difference in the total number of epitopes displayed on the surface, depending on the insertion site (240 x 3 and 12 x 3 for hexon and fiber, respectively). To assess the role of the number of epitopes incorporated into the capsid, an antigen presentation assay was performed by incubating FLDC with the same epitope copy number ( $10^4$  vp of hexon-modified Ads and  $2 \times 10^5$  vp of fiber-modified Ads). Again, FLDC epitope presentation was significantly better after infection by hexon-modified Ads compared to fiber-modified vectors, thus excluding a preponderant influence of epitope number on its presentation efficiency (**Fig. 3c**).

### **Anti-epitope cellular responses in naive and immune mice**

Although results we obtained *in vitro* indicated significantly weaker processing /presentation of the epitope displayed in fiber protein, we decided to further assess the vaccination potential of both hexon-modified and fiber-modified Ads. In particular, we examined their capacity to mount anti-epitope cellular responses in mice immune or non-immune to Ad. To that end, C57Bl/6 mice were first injected intraperitoneally with PBS or AdWT ( $10^{10}$  vp) and two weeks after animals received one injection of epitope-displaying Ad. Ten days after this second injection, spleens were harvested and splenocytes were restimulated *in vitro* using antigen-specific peptides. Upon restimulation with Ad peptide, no significant difference in IFN- $\gamma$  production was observed between splenocytes of mice injected with AdH-SL and AdF-SL, neither in naive nor in immune mice (**Fig. 4**).

However, as expected, a dramatic increase in IFN- $\gamma$  production was detected for splenocytes isolated from both Ad-immune groups. Analysis of anti- $\beta$ gal cellular responses unravelled a strong reduction of anti- $\beta$ gal responses in Ad-immune mice, consistent with the ability of neutralizing Abs to inhibit gene delivery. In case of SL epitope restimulation, a significant increase in IFN- $\gamma$  production was found for AdH-SL compared to AdF-SL, indicating that AdH-SL is more potent to elicit memory CD8<sup>+</sup> T cell responses than AdF-SL. Additionally, comparison of splenocyte response in naive versus immune mice showed a tendency to reduced cellular responses in Ad-immune mice (**Figure 4**). Altogether, these results demonstrate the induction of epitope-specific CD8<sup>+</sup> T cell responses upon immunization with Ad vectors displaying heterologous epitopes into their capsid proteins.

#### **Kinetics of cellular responses elicited by Ad displaying ovalbumin-derived SL epitope.**

Having confirmed the ability of epitope-displaying Ad vectors to trigger epitope-specific T cell responses, we sought to evaluate their kinetics. As previously, mice were immunised with PBS or AdWT and two weeks later with either AdH-SL or AdF-SL. Mice injected twice with PBS were used as a control group (NI). First, we assessed the cellular responses by measuring SL-specific CD8<sup>+</sup> T cells using tetramers and cytometry. At day 11-12 p.i., about 0.8% of tetramer-positive cells (TetOva<sup>+</sup>) were found among CD8<sup>+</sup> splenocytes in AdH-SL-injected Ad-naïve mice, in comparison to 0.2-0.3% detected in other groups (**Fig. 5A-B**). Interestingly, this hierarchy between the different Ads was preserved at later time points, with AdH-SL triggering about 0.6% of TetOva<sup>+</sup>CD8<sup>+</sup> T cells. Quantification of the number of TetOva<sup>+</sup>CD8<sup>+</sup> T cells in total splenocytes confirmed that AdH-SL was more potent than AdF-SL to induce epitope-specific cellular responses (**Fig. 5C**). Then, with ELISPOT assay, we analysed the ability of isolated splenocytes to produce IFN- $\gamma$  upon restimulation with specific peptides. At an early time point (day 11-12), the highest number of SL-specific IFN- $\gamma$  producing cells was detected in the splenocytes isolated from Ad-naïve mice immunized with AdH-SL (**Fig.6, upper panel**). Similarly to the results of splenocyte restimulation *in vitro* described above,

AdH-SL was able to trigger more robust cell responses than AdF-SL in both naive and immune groups of animals. In contrast, AdF-SL was less potent to elicit SL-specific T cell responses, regardless of the host status towards Ad. Analysis of the presence of Ad-specific IFN- $\gamma$  producing cells confirmed that anti-Ad immunity was more vigorous in pre-immunized group. Interestingly, strong anti- $\beta$ gal cellular responses were triggered in both Ad-naive and Ad-immune mice. Analysis of cellular responses at a later time point (day 25-26) disclosed the hierarchy of vector vaccination efficacy similar to the one obtained at the early time point, for all the experimental groups (**Fig. 6, lower panel**). Despite a decrease in the number of SL-specific IFN- $\gamma$  producing cells for all the analyzed vectors, AdH-SL elicited the strongest T cell responses against SL epitope.

Altogether, these results underline the capacity of AdH-SL to elicit *in vivo* the amplification of functional SL-specific T cells, able to produce IFN- $\gamma$  upon antigen encountering.

#### **Characterisation of the phenotype of SL-specific T cells elicited after administration of Ad displaying SL epitope.**

In the next step, we investigated the levels of CD44, CCR7, CD127, CD62L and KLRG1 molecules on the surface of CD8<sup>+</sup> CD90<sup>+</sup> T cells at both time points. For all groups of immunised mice, at the early time point a reduction in CCR7, CD127, CD62L as well as an increase in CD44 and KLRG1 was observed for TetOva<sup>+</sup> CD8<sup>+</sup> T cells when compared to total CD8<sup>+</sup> T cells (**Fig. 7A**). At the later time point all groups continued to exhibit increased expression of CD44 and KLRG1 molecules on TetOva<sup>+</sup> CD8<sup>+</sup> T cells compared to total CD8<sup>+</sup> cells. Interestingly, TetOva<sup>+</sup>CD8<sup>+</sup> T cells isolated from Ad-naïve mice immunized with AdH-SL displayed reduced amounts of CD127 and CD62 molecules (**Fig. 7B**).

Based on differential expression of CD62L, CD127<sup>+</sup> T cells can be divided in two main lineages of memory T cells, named central (T<sub>CM</sub>) and memory (T<sub>EM</sub>) T cells. Thus, in order to measure the phenotype of TetOva<sup>+</sup>CD8<sup>+</sup> T cells in immunised mice, we concomitantly analysed the data of CD62L and CD127 molecule expression on their surface (**Fig. 7C and 7D**). At the early time point the biggest percentage of effector cells (T<sub>E</sub>, CD62L<sup>-</sup>CD127<sup>-</sup>) among the TetOva<sup>+</sup>CD8<sup>+</sup> T cells was detected

in naïve mice infected with AdH-SL (**Fig. 7C and 7D**). This experimental group presented also the most elevated amount of effector memory T cells ( $T_{EM}$ ,  $CD62L^-CD127^+$ ).  $T_{EM}$  were induced more vigorously by AdH-SL than AdF-SL vector, regardless of host status towards Ad. On the other hand,  $T_{CM}$  cells ( $CD62L^+CD127^+$ ) were reduced in AdH-SL-injected mice in comparison to AdF-SL, whatever their Ad immune status. At a later time point, a trend to a reduction in  $T_{EM}$  and to an increase in  $T_{CM}$  was found for all groups. Yet, AdH-SL-injected mice displayed a reduced percentage of  $T_{CM}$  compared to AdF-SL.

#### 4. Conclusion

Epitope display strategy was successfully used by us [5] and others [7, 8, 18] to induce humoral responses. Some of these studies tried to analyze the cellular responses against class-II T cell epitopes, embedded in the original sequence of B cell epitope [7]. Nevertheless, the capacity of Ad vectors displaying class-I T cell epitopes to trigger effective anti-epitope cellular responses was never evaluated. Thus, in order to address this question we constructed a panel of vectors displaying T cell epitope SIINFEKL coming from Ova model antigen. Epitope sequences were genetically inserted (with or without additional flanking sequences coming from Ova) into the hexon or fiber protein of Ad5 capsid. Vectors were produced at titers comparable to AdWT and showed correct capsid integrity, disclosed by SDS-PAGE. Inserted epitopes were detected by anti-Ova polyclonal antibody, with better epitope accessibility for hexon insertion.

Using an *in vitro* antigen presentation assay, we demonstrated that Ova epitope was efficiently processed from capsid protein and presented to T cell specific to H-2K<sup>b</sup> + SL peptide, leading to their activation as documented by production of IL-2. Moreover, we showed that epitope insertion site strongly influences the efficacy of T cell activation, with stronger activation elicited by AdH-SL compared to AdF-SL. The difference in activation is not linked to the number of epitopes per capsid (hexon 240 x3 and fiber 12 x 3), since upon equalizing the total number of displayed SIINFEKL epitopes, AdH-SL still led to a significantly stronger T cell hybridoma activation than AdF-SL. Additionally, the presence of flanking sequences in the vectors has weak influence, if any, on epitope processing.

Then, we analyzed the cellular responses induced by both AdH-SL and AdF-SL in Ad-naïve and Ad-immune C57BL/6 mice. After ten days splenocytes were harvested and analyzed by various techniques in order to assess induction of cellular responses. First, we demonstrated that splenocytes from AdH-SL injected Ad-naïve mice were able to elicit, upon restimulation with SL peptide, significantly higher IFN- $\gamma$  production than AdF-SL. Interestingly, the splenocyte responses

were reduced in AdH-SL-injected Ad-immune mice compared to AdH-SL-injected Ad-naïve mice (**Figure 4**). Then, with the use of tetramers and by cytometry analyses we measured the number of SL-specific CD8<sup>+</sup> T cells in the spleen at two time point after virus delivery (day 11-12 and day 25-26). In both Ad-naïve and Ad-immune background, AdH-SL was able to elicit stronger increase in SL-specific T cells than AdF-SL (**Figure 5**). When the functionality of SL-specific T cells was analysed by ELISPOT, we observed that SL-specific T cells induced by AdH-SL were able to produce IFN- $\gamma$  upon *in vitro* restimulation (**Figure 6**).

During our studies we also documented the expression of different phenotypic markers on SL-specific T cells to better characterize the nature of these cells (effectors vs. memory). We quantified the percentage of effector, effector memory and central memory phenotype within SL-specific T cells. Effector cells were found to be most numerous in AdH-SL-injected mice compared to AdF-SL-injected mice, regardless of the mice status at the time of virus administration. Interestingly, the percentage of cells displaying an effector memory phenotype was reduced at day 25-26 p.i. while an increase in SL-specific central memory T cells was observed (**Figure 9**).

Altogether, our results underline that Ad displaying T cell epitopes in hexon protein are able to elicit functional CD8<sup>+</sup> T cells with memory phenotype.

## References

1. Arnon, R. and T. Ben-Yedidia, Old and new vaccine approaches. *Int Immunopharmacol*, 2003. 3(8): p. 1195-204.
2. Crouzet, J., et al., Recombinational construction in *Escherichia coli* of infectious adenoviral genomes. *Proc Natl Acad Sci U S A*, 1997. 94(4): p. 1414-9.
3. Krasnykh, V.N., J.T. Douglas, and V.W. van Beusechem, Genetic targeting of adenoviral vectors. *Mol Ther*, 2000. 1(5 Pt 1): p. 391-405.
4. Krause, A., et al., Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity. *J Virol*, 2006. 80(11): p. 5523-30.
5. Lanzi, A., et al., Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid. *Vaccine*, 2011. 29(7): p. 1463-71.
6. Martin, K., et al., Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism. *Mol Ther*, 2003. 8(3): p. 485-94.
7. McConnell, M.J., X. Danthinne, and M.J. Imperiale, Characterization of a permissive epitope insertion site in adenovirus hexon. *J Virol*, 2006. 80(11): p. 5361-70.
8. Nardin, E.H., et al., Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes. *J Infect Dis*, 2000. 182(5): p. 1486-96.
9. Pialoux, G., et al., Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. *AIDS*, 2001. 15(10): p. 1239-49.
10. Plotkin, S.A., Vaccines: past, present and future. *Nat Med*, 2005. 11(4 Suppl): p. S5-11.
11. Randrianarison-Jewtoukoff, V. and M. Perricaudet, Recombinant adenoviruses as vaccines. *Biologicals*, 1995. 23(2): p. 145-57.
12. Rueda, P., et al., Influence of flanking sequences on presentation efficiency of a CD8+ cytotoxic T-cell epitope delivered by parvovirus-like particles. *J Gen Virol*, 2004. 85(Pt 3): p. 563-72.
13. Sedlik, C., et al., Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. *Proc Natl Acad Sci U S A*, 1997. 94(14): p. 7503-8.
14. Sette, A. and J. Fikes, Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Curr Opin Immunol*, 2003. 15(4): p. 461-70.
15. Sumida, S.M., et al., Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol*, 2005. 174(11): p. 7179-85.
16. Tatsis, N. and H.C. Ertl, Adenoviruses as vaccine vectors. *Mol Ther*, 2004. 10(4): p. 616-29.
17. Vigne, E., et al., RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol*, 1999. 73(6): p. 5156-61.
18. Worgall, S., et al., Protective immunity to *Pseudomonas aeruginosa* induced with a capsid-modified adenovirus expressing *P. aeruginosa* OprF. *J Virol*, 2007. 81(24): p. 13801-8.
19. Worgall, S., et al., Protection against *P. aeruginosa* with an adenovirus vector containing an OprF epitope

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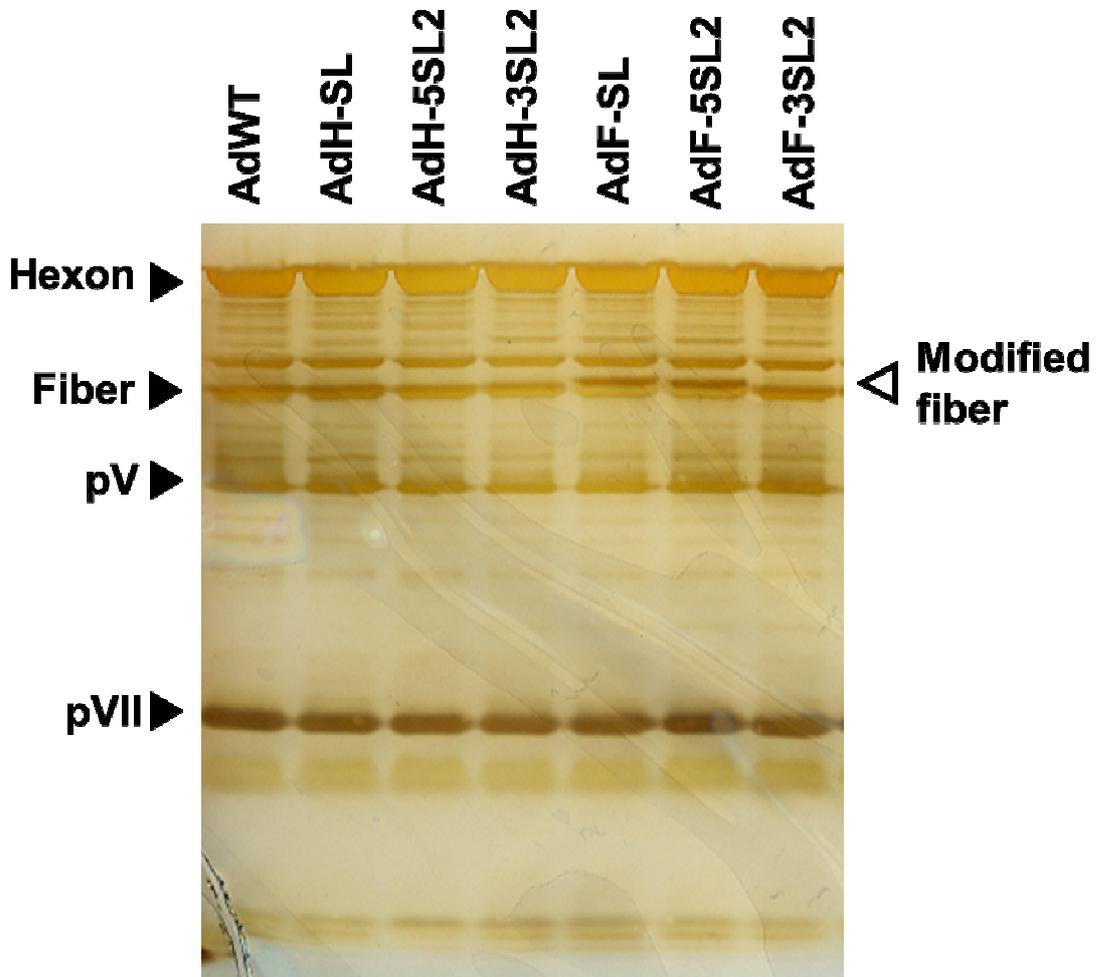
The authors have no competing financial interests to declare.



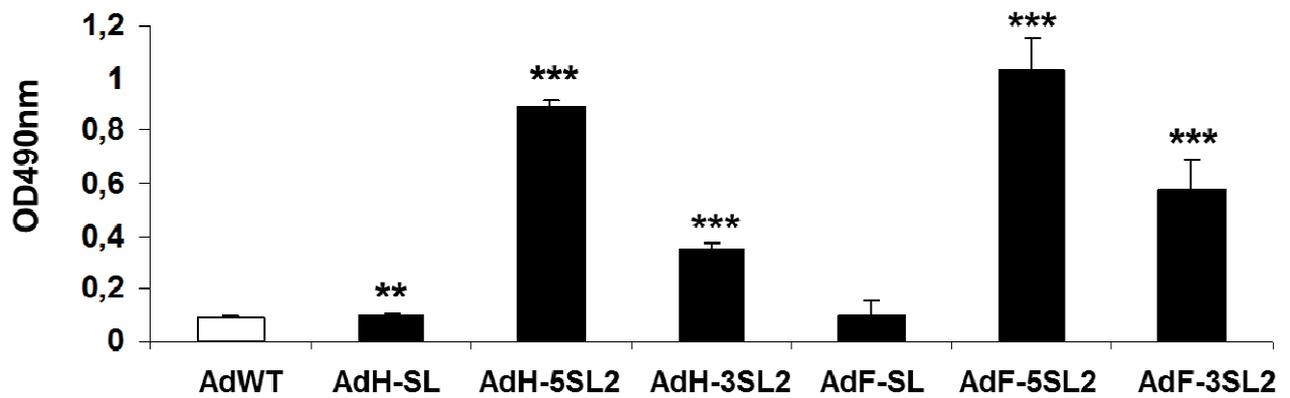
## Tables and figures

**Table 1: Features of wild-type Ad and Ad displaying ovalbumin-derived epitopes.**

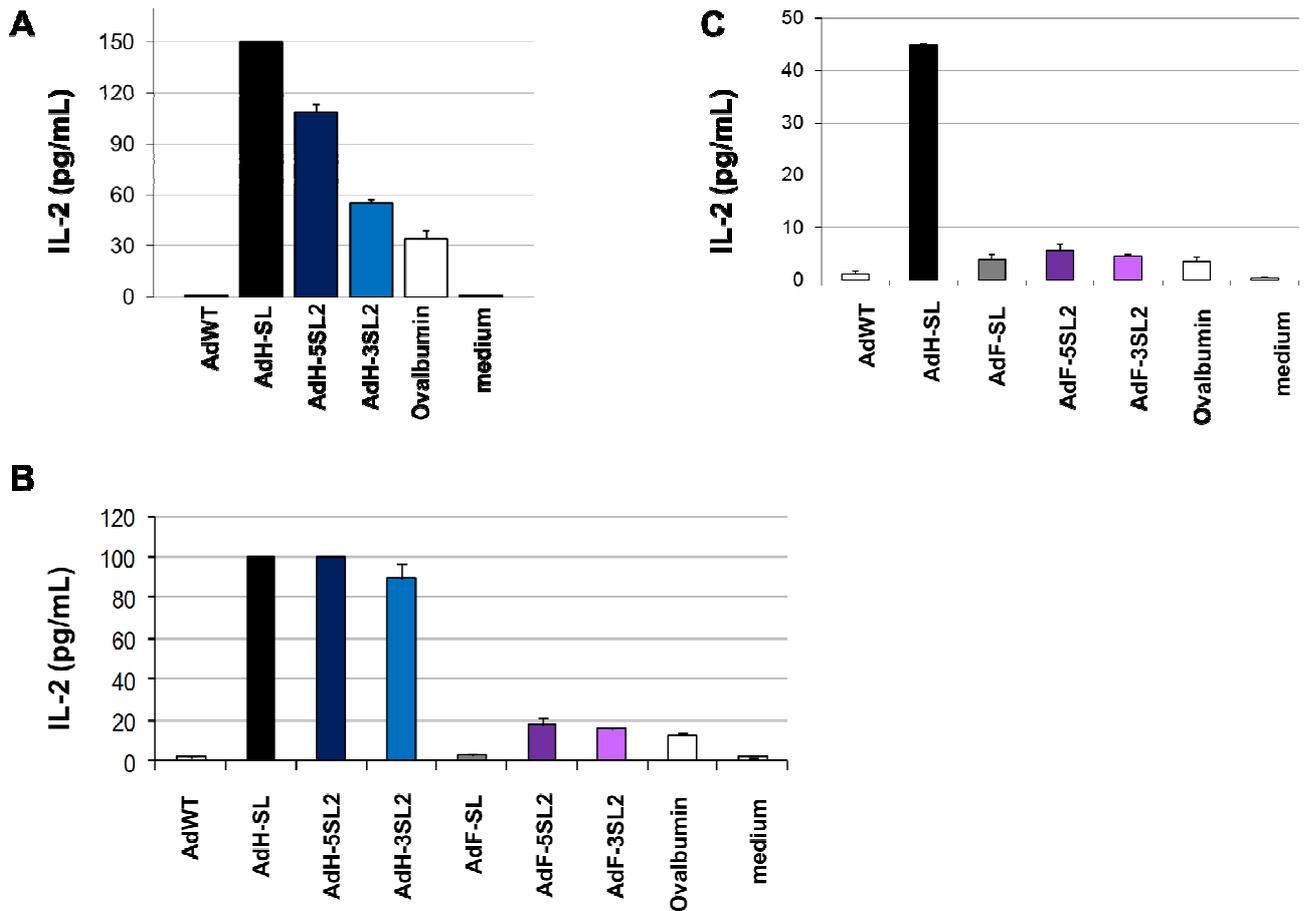
Virus	Modified protein	Adaptor	Inserted peptide	Adaptor	Titer ( $\times 10^{12}$ vp/ml)
AdWT	-	-	-	-	$7.7 \pm 0.8$
AdH-SL	hexon	G	SIINFEKL	LGG	$7.2 \pm 0.3$
AdH-5SL2	hexon	G	LEQLESIINFEKLTE	LGG	$2.9 \pm 0.7$
AdH-3SL2	hexon	G	QLESIINFEKLTE	LGG	$4.2 \pm 0.5$
AdF-SL	fiber	SS	SIINFEKL	GSS	$3.7 \pm 0.8$
AdF-5SL2	fiber	SS	LEQLESIINFEKLTE	GSS	4.2
AdF-3SL2	fiber	SS	QLESIINFEKLTE	GSS	5.8



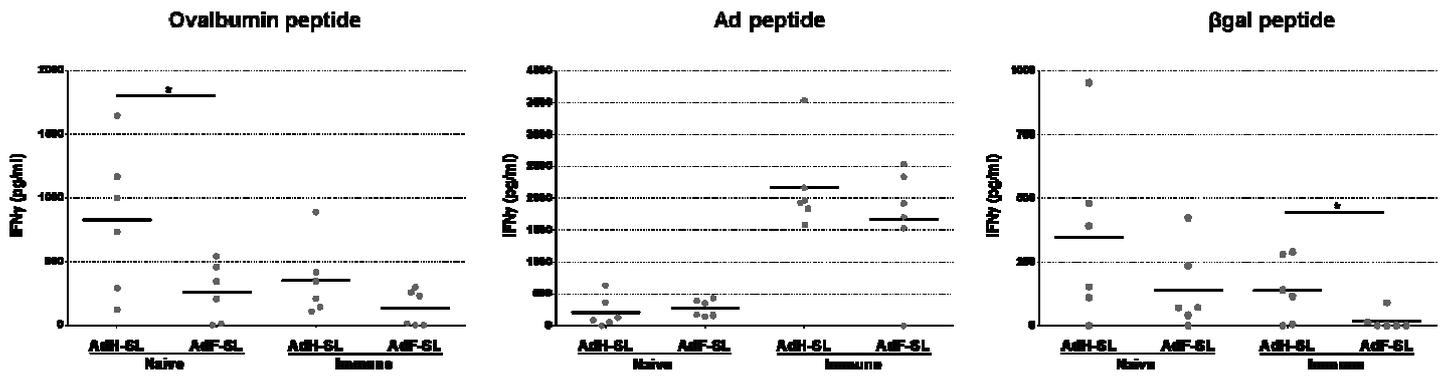
**Figure 1** Silver staining of capsid-modified Ad.  $10^{10}$  vp of either a control Ad (AdWT) or a capsid-modified Ad (AdH-SL, AdH-5SL2, AdH-3SL2, AdF-SL, AdF-5SL2, AdF-3SL2) were separated on a 10% polyacrylamide gel. Major capsid components (black arrow) and modified fiber (white arrow) are identified.



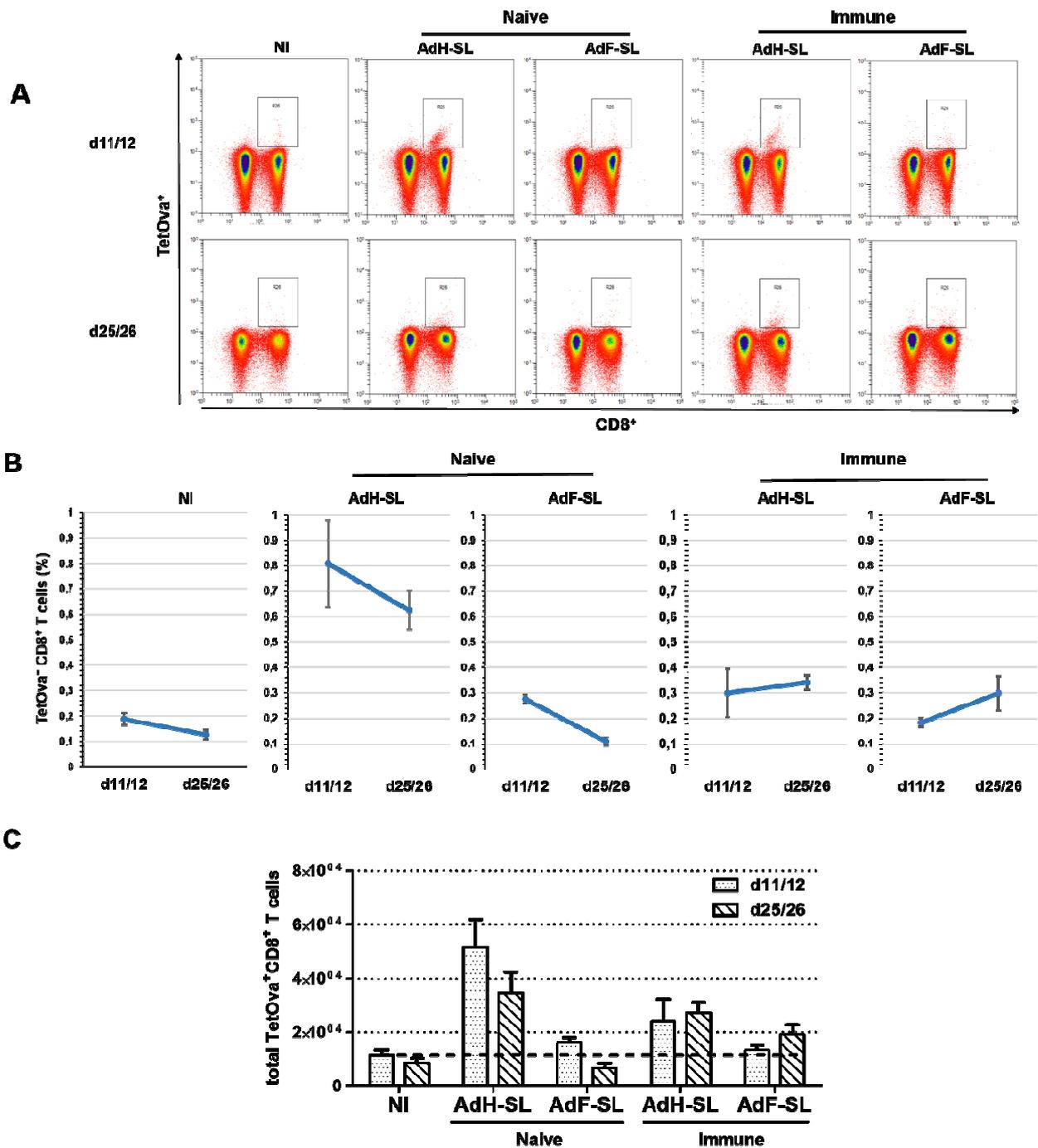
**Figure 2** Detection of SL, 5SL2 and 3SL2 epitopes on virions. ELISA plates were coated with 100 ng of denatured viruses and incubated with a rabbit polyclonal antibody against the Ova protein. The binding was detected with HRP-conjugated secondary antibody. One of two experiments is shown,  $n = 6$ ; means + SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus AdWT.



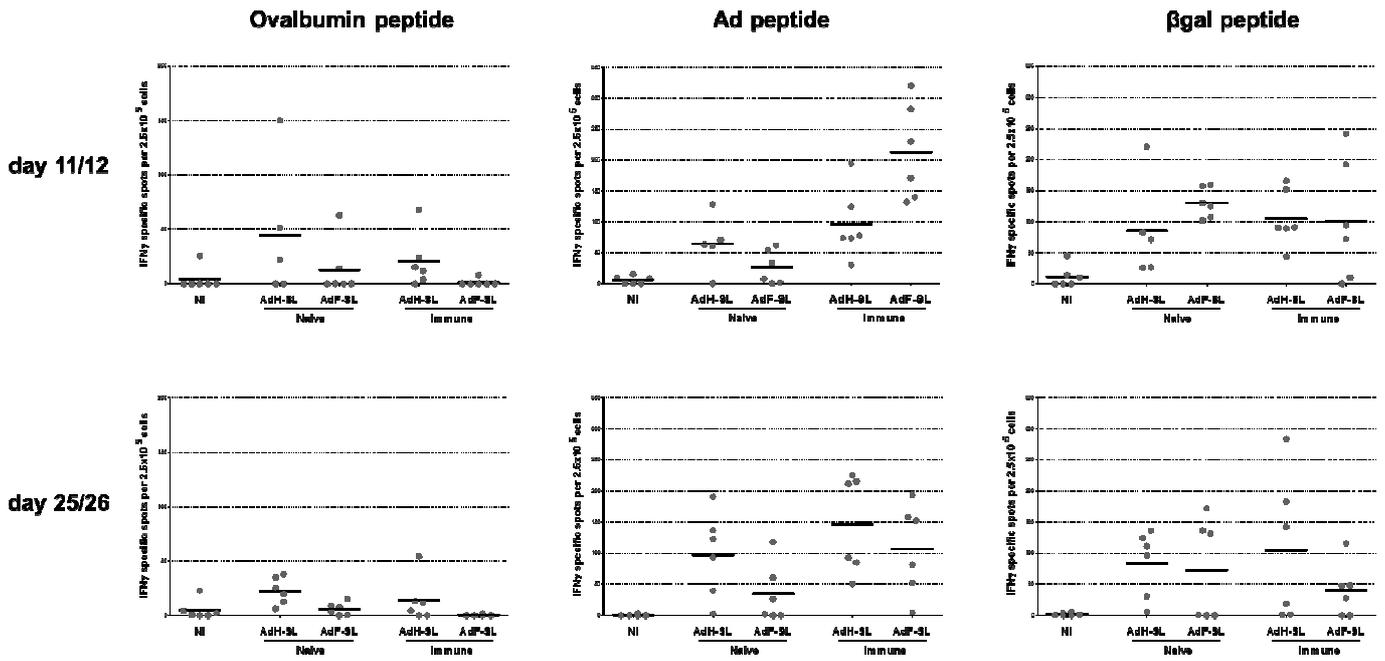
**Figure 3** Epitope presentation assay. **(A and B)** AdWT or capsid-modified Ads were incubated with FLDC for two hours at  $10^4$  vp/cell. Untreated FLDC (medium), FLDC pulsed with Ova protein or with SL peptide were used as controls. SL-specific hybridoma was then added and the amount of IL-2 released in the supernatant was measured two days later by ELISA. Saturated values were plotted as 100 pg/mL. One of two experiments is shown,  $n = 3$ ; means + SD. **(C)** Epitope presentation assay at same epitope copy number. Fiber-modified Ads were incubated with FLDC at  $2 \times 10^5$  vp/cell. AdWT and AdH-SL were incubated at  $10^4$  vp/cell.



**Figure 4** T cell immune responses elicited by Ad displaying Ova-derived SL T cell epitope. C57Bl/6 mice were injected with PBS or  $10^{10}$  vp of AdWT and two weeks after received intraperitoneal injection of  $10^{10}$  vp of Ad displaying SL epitope into hexon (AdH-SL) or fiber (AdF-SL) protein. Splenocytes harvested at day 10 p.i. were stimulated *in vitro* with Ova (SL), Ad or  $\beta$ gal peptide. Production of IFN- $\gamma$  in the supernatant was assessed by ELISA. One of two experiments is shown. Dots and bars represent specific IFN- $\gamma$  production of individual mice and the group, respectively.  $n = 5-7$ ; \*,  $P < 0.05$ .

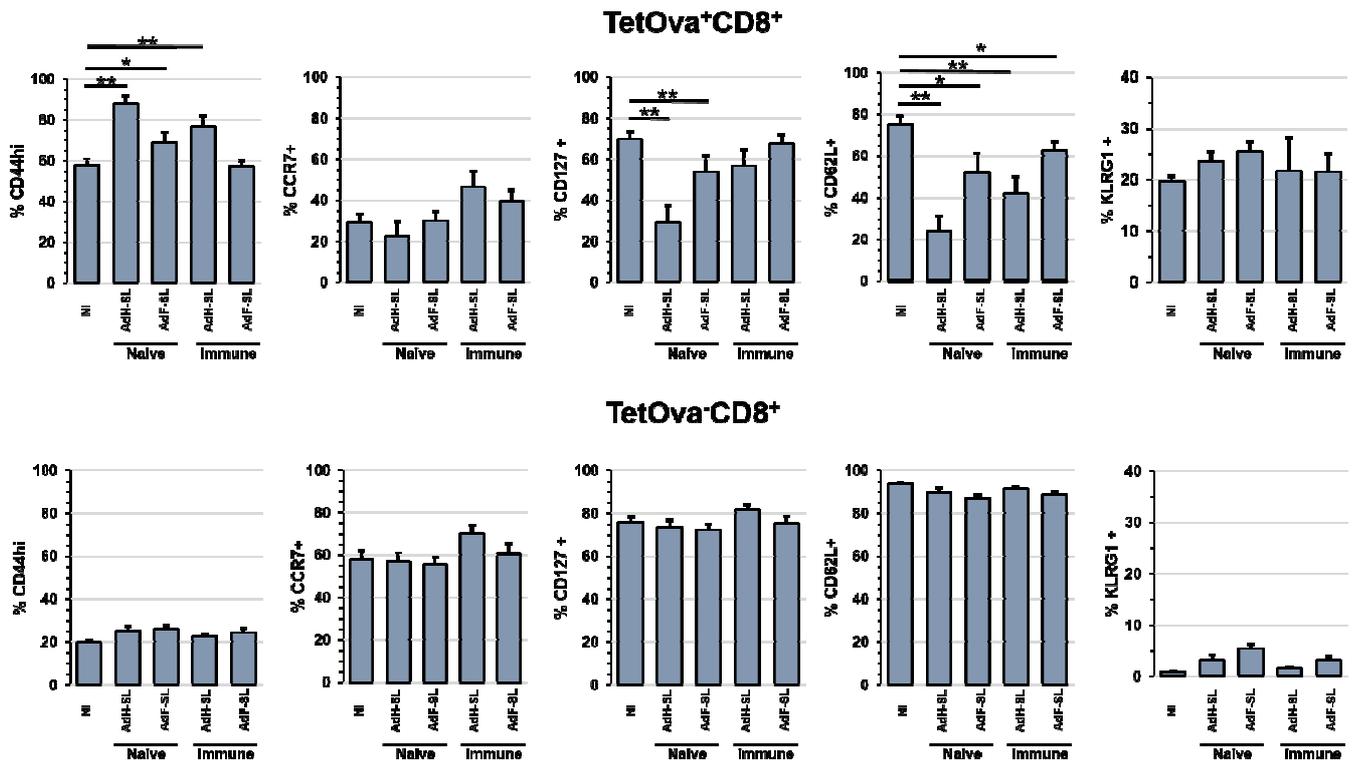


**Figure 5** Specific T cells elicited by Ad displaying Ova-derived SL epitope. C57Bl/6 mice were injected with PBS or  $10^{10}$  vp AdWT and after two weeks were injected intraperitoneally with  $10^{10}$  vp of Ad displaying SL epitope into hexon (AdH-SL) or fiber (AdF-SL) protein. Splenocytes were harvested at early (day 11-12) and late (day 25-26) time points and SL-specific  $CD8^+$  T cells were measured by tetramer staining. **(A)** Representative dot plots showing tetramers-positive cells in  $CD8^+$  T cell population. **(B)** Percentage of  $Tet^+Ova^+$  T cells and **(C)** total number of  $Tet^+Ova^+$  T cells per spleen.



**Figure 6** Functional capacity of T cells elicited by Ad displaying Ova-derived SL epitope. C57BL/6 mice were injected with PBS or  $10^{10}$  vp AdWT and after two weeks were injected intraperitoneally with  $10^{10}$  vp of Ad displaying SL epitope into hexon (AdH-SL) or fiber (AdF-SL) protein. Splenocytes were harvested at early (day 11-12) and late (day 25-26) time points after the second injection and their capacity to produce IFN- $\gamma$  was assessed after restimulation with Ova (SL), Ad and  $\beta$ gal peptides. Dots and bars represent the number of specific IFN- $\gamma$  spots per  $2.5 \times 10^5$  of splenocytes and the mean, respectively. Dots and bars represent specific IFN- $\gamma$  production of individual mice and the group, respectively. n= 5-6.

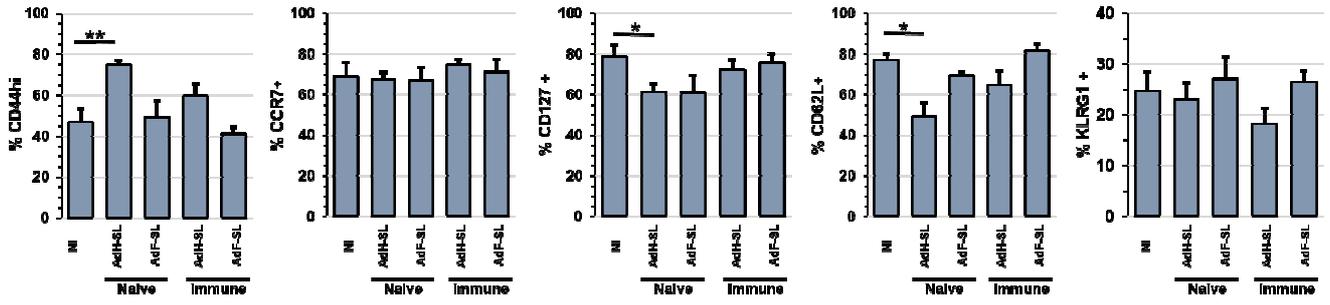
## Day 11-12



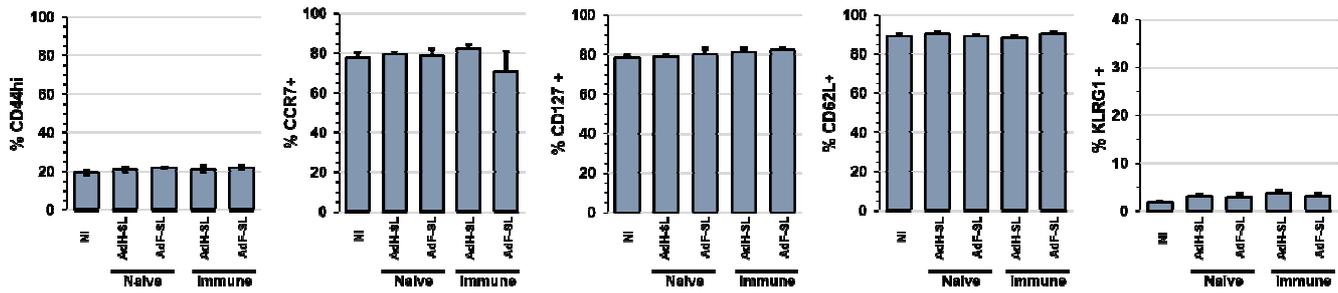
**Figure 7** Phenotypic characterization of TetOva<sup>+</sup>CD8<sup>+</sup> T cells. C57BL/6 mice were injected with PBS or 10<sup>10</sup> vp AdWT and after two weeks were injected intraperitoneally with 10<sup>10</sup> vp of Ad displaying SL epitope into hexon (AdH-SL) or fiber (AdF-SL) protein. Splenocytes were harvested at early (day 10-11) or late (day 25-26) time points and cells were labeled with a panel of antibodies and with (H-2Kb+SL) tetramer. Percentage of TetOva<sup>+</sup>CD8<sup>+</sup> cells expressing CD44, CCR7, CD127, CD62L and KLRG1 at early (**A**) or late (**B**) time point. (**C**) Representative dot plots showing CD6L<sup>+</sup> and CD127<sup>+</sup> TetOva<sup>+</sup>CD8<sup>+</sup> T cells. (**D**) Percentage of TetOva<sup>+</sup>CD8<sup>+</sup> T cells belonging to effectors (CD62L<sup>-</sup>CD127<sup>+</sup>), CD62L<sup>-</sup>CD127<sup>+</sup> (T<sub>EM</sub>) or CD62L<sup>+</sup>CD127<sup>+</sup> (T<sub>CM</sub>) populations.

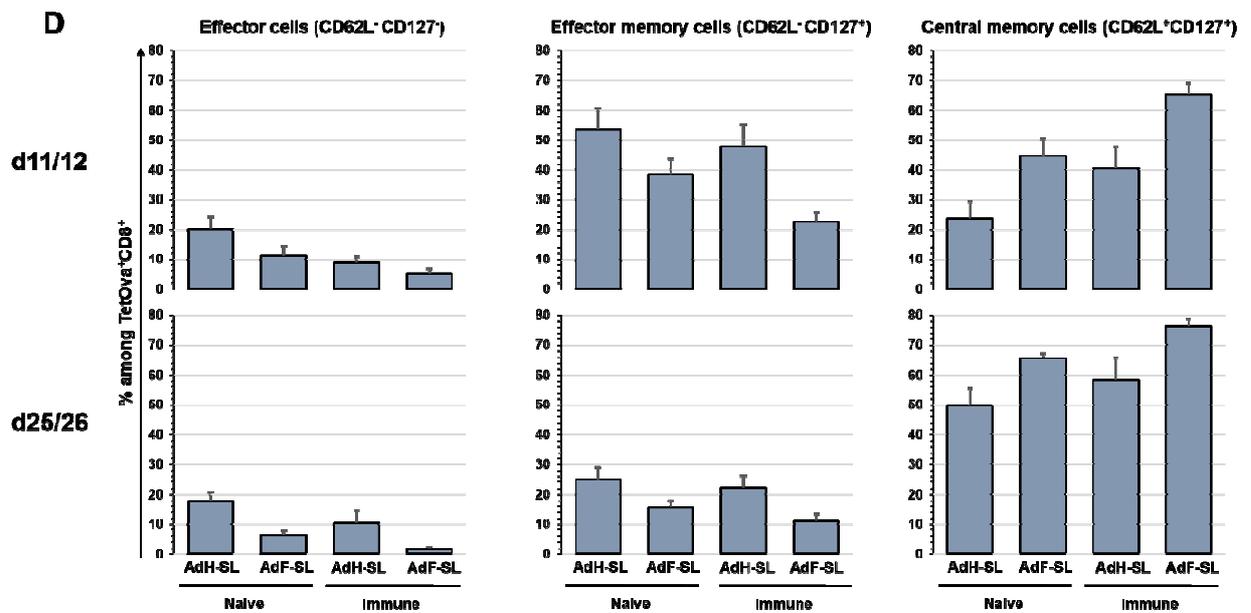
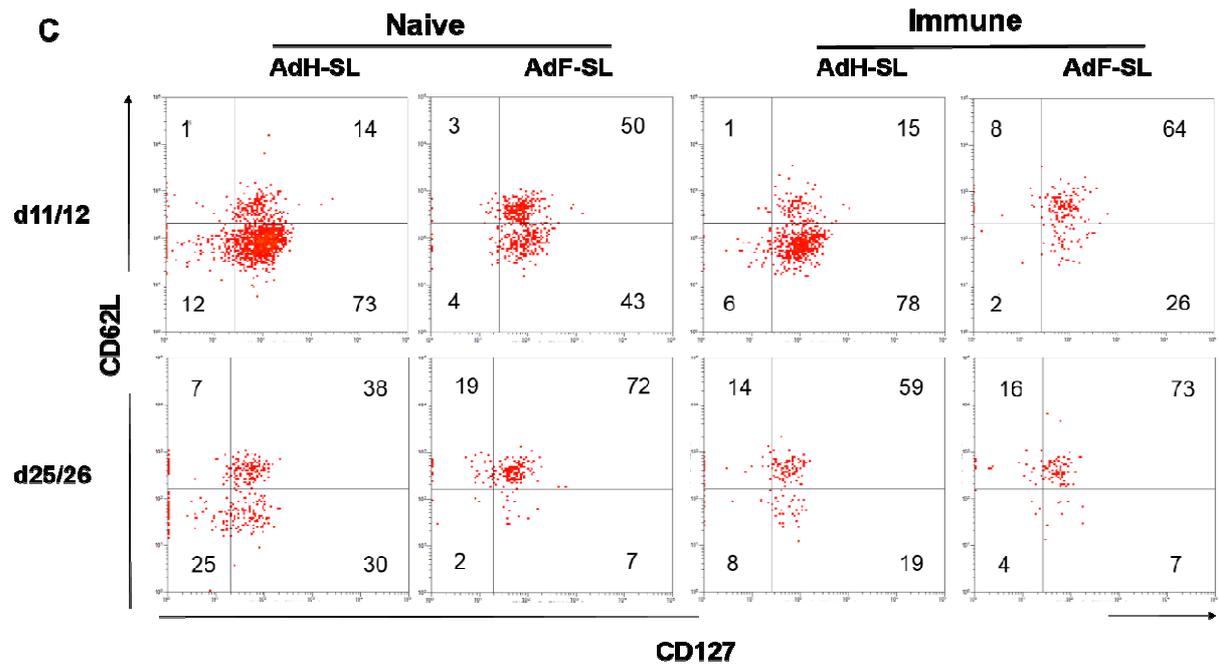
## Day 25-26

### TetOva<sup>+</sup>CD8<sup>+</sup>



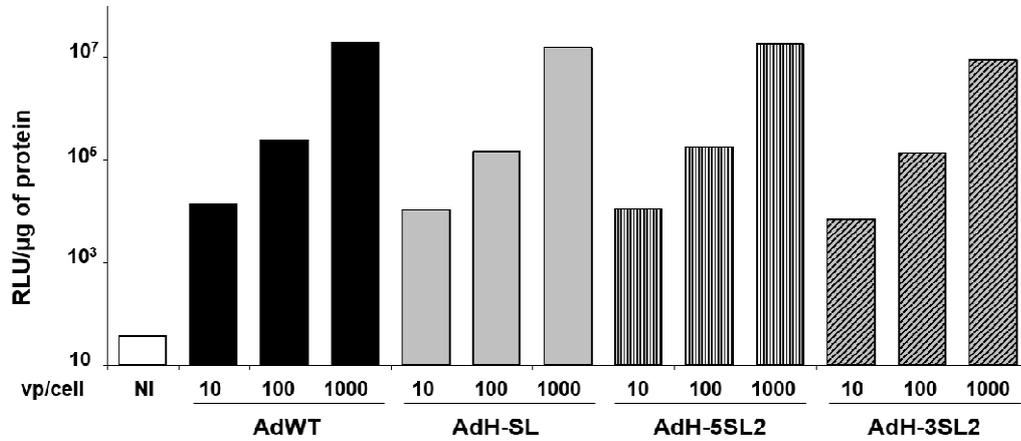
### TetOva<sup>-</sup>CD8<sup>+</sup>



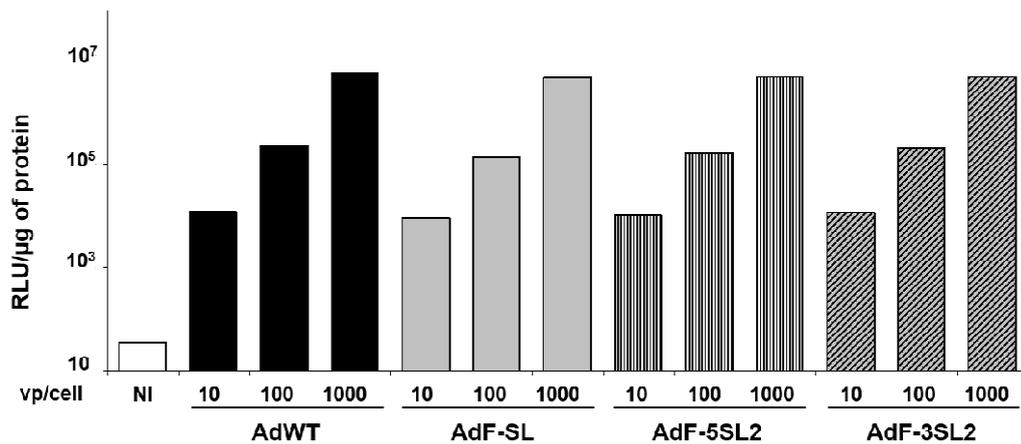


## Supplementary figures

**A**



**B**



**Supplementary figure 1** *In vitro* gene transfer by capsid-modified Ad. CHO-CAR were mock-infected (PBS) or infected with increasing MOI of *lacZ* recombinant AdWT, hexon-modified Ads **(a)** or fiber-modified Ads **(b)**.  $\beta$ gal activity, expressed as RLU/μg of protein, was measured in cell lysates 24 h later. Experiments run in duplicate were performed twice and representative results are shown.



## **PART 3**

### **Vaccination against HCV**



## **ARTICLE 3**

### **Immune responses triggered by adenovirus displaying HCV epitopes in their capsid proteins.**

#### **Abstract**

Hepatitis C virus (HCV) is a blood-borne pathogen estimated to chronically infect 3% of the worldwide population, significantly enhancing the risk of liver cancer development. Studies in infected individuals showed that vigorous cellular and humoral anti-HCV responses are correlated with resolution of acute infection. Hence, many strategies have been evaluated to propose a promising vaccine candidate. Herein we report the production of recombinant adenovirus (Ad) vectors displaying B cell or T cell epitopes of HCV into their capsid proteins. Our results indicate that vectors displaying T cell epitope are capable of triggering anti-HCV cellular responses, suggesting potential of this vaccination strategy in fighting this pathogen infection.

## 1. Introduction

Hepatitis C virus (HCV) infects 170 million people worldwide and is considered to be one of the major health burden. Due to its ability to escape the detection of the immune system, HCV can persist in the organism for years, leading to liver pathologies such as cirrhosis and hepatocellular carcinoma (HCC). Indeed, HCV infection is considered one of the main reasons of liver failure in the world. Until now, no effective vaccine is commercially available, and thus counteracting the HCV pathology relies exclusively on pharmacological approaches. For a long time the “golden standard” of anti-HCV therapy was a combination of IFN- $\alpha$  and ribavirin, both limiting virus infection in an unspecific manner. Recently, directly acting antivirals (DAAs), molecules which specifically target the virus proteins, were identified and developed. Combination of IFN- $\alpha$ /ribavirin with virus protease or polymerase inhibitors allowed to significantly increase the rate of sustained virologic response. However, treatment outcome is mostly depending on the virus genotype, as the majority of currently used DAAs do not have pan-genotypic efficacy. Also, new DAAs-based therapies are very expensive and associated with important secondary effects. This makes a discovery of an effective cross-reactive anti-HCV vaccine a high priority, both to prevent the initial infection and to counteract re-infection after successful cure with costly DAA-based [12].

HCV is a difficult target from the immunological point of view. Virus mutates quickly and thus six main genotypes can be distinguished, differing significantly in their genome sequence. In order to ensure cross-protection against all genotypes, a conserved epitopes must be selected in the pathogen sequence. It has been shown that humoral responses are mostly targeting surface glycoproteins, E1 and E2. Cellular branch of immunity, on the other hand, is directed against non-structural proteins performing important functions in virus life cycle, such as NS5A polymerase or NS3 helicase. Many strategies of immunization against HCV have been evaluated, such as recombinant proteins, synthetic peptides, DNA plasmids, virus-like particles or recombinant viruses [1]. Nevertheless, no candidate reached the market so far.

Epitope display strategy is a new vaccination approach developed in order to overcome pre-existing anti-Ad immunity. Numerous studies confirmed that insertion of antigenic peptide into the Ad capsid results in triggering humoral responses against the introduced epitope. This was observed for both model antigen ovalbumin [6] as well as for peptides coming from different pathogens, such as *P. aeruginosa* [13] or *B.anthraxis* [8]. With this regard, we aimed at the induction of anti-HCV immune responses by constructing Ad vectors displaying in their capsomers epitopes coming from the HCV (genotype 1B) proteins. Immunodominant conserved B cell epitopes from E2 glycoprotein and T cell epitopes from NS3 protein were introduced into Ad5 hexon or fiber protein. Our results show that Ad displaying T cell epitope are capable of inducing anti-HCV cellular immunity in HLA-A2 transgenic mice. On the contrary, vectors displaying B cell epitopes failed to mount detectable anti-E2 antibody responses.

## 2. Materials and methods

### 2.1. Virus construction and production

AdWT (described as AE18 in [11]) is based on Ad5 and possess deletions in E1 and E3 regions. The expression cassette containing the promoter/enhancer from the immediate early gene of human cytomegalovirus (CMV), lacZ gene *Escherichia coli* with a nuclear localization signal, and the SV40 late polyA signal were inserted in the place of E1 region. For the analysis of humoral responses, AdH-HVR, AdF-HVR and AdF-HCR vectors were constructed, all derived from AdWT, displaying epitopes from HCV genotype 1B. AdH-HVR and AdF-HVR contain E2 glycoprotein epitope HVR<sub>384-410</sub>: HTHVTGGRVASSTQSLVSWLSQGPSQK inserted into, respectively, the hexon protein in place of HVR5 region <sub>269</sub>TTEAAAGNGDNLT<sub>281</sub> or into the fiber protein knob in place of T<sub>539</sub>QETGDTTPS<sub>548</sub>. Similarly, AdF-HCR was obtained by inserting E2 glycoprotein epitope HCR1<sub>412-431</sub> QLVNTNGSWHINRTALNCND<sub>n</sub> into the fiber HI loop. For the assessment of cellular responses, AdH-NS3<sub>1073</sub> and AdH-NS3<sub>1406</sub> vectors were constructed, both derived from AdWT displaying epitopes from HCV genotype 1B. The HVR5 region <sub>269</sub>TTEAAAGNGDNLT<sub>281</sub> of hexon protein was replaced with HCV NS3<sub>1073-1081</sub> epitope <sub>1073</sub>CVNGVCWTV<sub>1081</sub> or NS3<sub>1406-1415</sub> epitope <sub>1406</sub>KLTGLGLNAV<sub>1415</sub> to generate AdH-NS3<sub>1073</sub> and AdH-NS3<sub>1406</sub>, respectively. AdE1E2 (kindly provided by Dr B.Bellier, UPMC) possess a wild-type capsid and encodes the entire E1 and E2 glycoproteins of HCV from the 1a genotype H77 strain in the E1 region [5]. All capsid-modified viruses (**Table 1 and 2**) were constructed in *E. coli* with the methods of molecular cloning [3]. Viruses were obtained using previously described procedures [7]. They were titrated by spectrophotometry ( $1 \text{ OD}_{260} = 1.1 \times 10^{12}$  viral particle (vp)/ml) and stored in PBS–7% glycerol at –80 °C.

## 2.2. Cell lines

The 293A human embryonic kidney cell line (R705-07, Invitrogen) was maintained in modified Eagle medium (MEM) supplemented with 10% FBS and 1% of non-essential amino acids (NEAA). CHO-k1-hCAR cells were kindly provided by J.M. Bergelson (School of Medicine, University of Pennsylvania) and were cultivated in F-12 Glutamax medium supplemented with 10% FBS and grown under G418 conditions. I8H4 is a stable Tcells-BW hybridoma cell line specific for HCV NS3<sub>1073-1081</sub> epitope (kindly provided by M.Chen, Karolinska Institut) [9]. Cells were maintained in modified Eagle medium (MEM), supplemented with 10% FBS, 1% NEAA, and 2- $\beta$ mercaptoethanol.

## 2.3. E2 glycoprotein

Truncated E2 glycoprotein, deprived of its transmembrane domain (kindly provided by K.Bienkowska-Szewczyk, Gdansk University), was produced using Bac-to-Bac™ system (Life Technologies. Inc.). Insect cells were infected with recombinant baculovirus encoding E2 protein. Medium from the cell culture was collected 72h post-infection and centrifuged 28 000 rpm for 2h to remove baculovirus particles. The supernatant was completed with glycerol (10% final concentration) and stored at -80°C.

## 2.4. Peptides

E2 glycoprotein HVR (QLVNTNGSWHINRTALNCND) and HCR (HTHVTGGRVASSTQSLVSWLSQGSPQK) botinylated peptides were purchased from GENECUST. NS3 protein peptide 1073-1081 (CVNGVCWTV) was ordered at Proimmune. NS3 peptide 1406-1415 (KLTGLGLNAV) and epitope 917-925 (YVLFEVFDV) from Ad hexon protein were synthesized by PolyPeptide. Purity of all peptides was > 90%.

### *2.5. Cell transduction*

In order to evaluate virus infectivity, CHO-k1-hCAR cells were plated out in 12-well plates ( $1 \times 10^5$  cells/well) 48h prior to infection. Then, Ad was added at different multiplicity of infection (MOI, ranging from  $10^2$  to  $10^4$  vp/cell) in 400 $\mu$ l of serum-free medium. After 1h of incubation in 37°C, an additional 2ml of medium supplemented with 10% FBS was added and cells were incubated for 24 hours at 37°C. Cells were then harvested and  $\beta$ gal activity was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Marnes-la-Coquette, France). Results were presented as relative light units (RLU) per microgram of proteins.

### *2.6. Epitope detection with ELISA*

To confirm the presence and accessibility of the epitopes on the capsid surface native viral particles were coated on 96-well plates (Nunc, Roskilde, Denmark). Viruses were inactivated by 30 min incubation at 56°C and subsequently denaturated with 0.1% SDS. Spectrophotometric measurements at 215nm and 225nm allowed determining protein concentrations, and quantities of non-denaturated viruses corresponding to 100ng of proteins were coated on 96-well plates. After overnight incubation at 4°C, wells were blocked with 5% milk PBS-Tween for 2h, then washed and incubated for 1 h with an anti-HCR rabbit monoclonal Ab (AP33, provided by A. Patel, Glasgow University). Upon washing, an anti-mouse IgG goat peroxidase-linked Ab (1030-05, Southern Biotechnology Associates, Birmingham, AL France) was added for 1 h and peroxidase activity was revealed by incubation with O-phenylenediamine dihydrochloride (Sigma–Aldrich, Lyon, France) for 30 min. The reaction was stopped by addition of 3N HCl and spectrophotometric readings were performed at 490 nm.

### *2.7. FLDC preparation*

FLDC (Flt3 ligand-dependent dendritic cells) were generated from bone marrow precursors. Bone marrow cells were harvested from tibias and femurs of HHD mice by flushing with complete RPMI medium (RPMI supplemented with 5% SVF, 1% non-essential amino acids, 1% glutamine, 1% pyruvate and  $5 \cdot 10^{-5}$  M  $\beta$ -mercaptoethanol), and filtered through a 100 $\mu$ m cell strainer. After removal of blood cells by ACK Lysing Buffer (Invitrogen, Cergy-Pontoise, France), cells were resuspended and the concentration was adjusted at  $2 \times 10^6$  cells/mL. Cells ( $5 \times 10^6$ /well) were plated in MW6 plates, and recombinant human Flt3 Ligand (Flt3L) was added at 250 ng/mL. Cultures were fed with fresh Flt3L-complemented RPMI medium every 2-3 days. At day 10, FLDCs were harvested from the cultures by vigorously pipetting and labelled with Pacific blue anti-CD11b (eBioscience, CA, USA), FITC anti-A<sup>b</sup>, PE anti-CD24, PE-Cy<sup>TM</sup>7 anti-CD11b and PerCP anti-CD45R/B220 (BD Bioscience, CA, USA) Abs. The diverse phenotypic characteristics of FLDC were confirmed using a FACS scan (BD Biosciences).

### *2.8. Antigen presentation in vitro*

FLDC were seeded on a 96-well culture plate at a density of  $10^5$  cells/well. Cells were incubated with control or capsid-modified Ads for 2 hours at  $10^2$ ,  $10^3$  or  $10^4$  vp/cell in a final volume of 100  $\mu$ L of complete RPMI medium. Controls included untreated FLDC or FLDC incubated for 2 hours with NS3<sub>1073-1081</sub> peptide CVNGVCWTV (10 $\mu$ g/mL and 20 $\mu$ g/mL). Then, I8H4 cells were added ( $10^5$  cells/well in 100  $\mu$ L) and co-cultured with FLDC during 2 days at 37°C. The response of stimulated hybridoma cells was assessed by determination of IL-2 concentration in the supernatants using the murine IL-2 ELISA Kit (BD Bioscience).

### 2.9. Determination of antigen-specific serum antibodies

Sera were analysed for the presence of E2-,  $\beta$ gal- and Ad-specific Ab by ELISA. In order to detect anti-E2 Ab, 0.25 $\mu$ g of *Galanthus Nivalis* lectin was coated on 96-well plates (Nunc) and incubated overnight. After washing plates with TBS supplemented with Tween20 0.05% (TBST) and blocking with 5% milk TBST, 100 $\mu$ l of supernatant containing E2, was added to each well for 2 hours. For the detection of anti- $\beta$ gal and anti-Ad Ab, 96-well plates (Nunc) were coated with 100ng of  $\beta$ gal (Sigma) or 100ng of denaturated AdWT viral particles per well, respectively, and blocked with 5% milk TBST. Subsequently, serial dilutions of the sera in 5% milk TBST were added. Then, bound Ab were detected as described above. Titers were defined as the reciprocal of the highest dilution giving an OD<sub>490</sub> 2-fold above background values.

Sera were also analysed for the presence 2 peptide-specific Abs. Biotinylated HCR (100ng/well) and HVR (500ng/well) peptides were added to 96-well plates coated with streptavidin (DUTSCHER) and incubated overnight. After washing plates with TBST 0.05% and blocking with 5% milk TBST, different sera dilutions were added to each well for 2 hours.

### 2.10. In vivo experiments

All *in vivo* experiments were approved by the Gustave Roussy Institutional Animal Care and Use Committee. C57BL/6 female mice (seven-week-old or at the age corresponding to obtained HHD mice) were purchased from Harlan (Gannat, France) and were conditioned for at least one week in the animal facilities before beginning of the experiments. DR3 HHD mice with C57BL/6 genetic background express a chimeric HLA-A2 molecule expressing  $\alpha$ 1 and  $\alpha$ 2 domains of the HLA-A2.1 fused to the  $\alpha$ 3 domain of murine H-2D<sup>b</sup> and to human  $\beta$ 2-microglobulin. In addition, they express HLA-DR3 molecule and are deficient in murine class-I and class-I genes.

Viruses ( $10^{10}$  vp) in PBS or PBS alone (200 $\mu$ l) as a negative control were injected intraperitoneally. Repeated injections were performed at 2 weeks intervals with total number of

injections ranging between 1 and 2. Blood samples were collected sub-mandibularly, before virus injection and at different time points thereafter. Mice sera were prepared and analyzed for the presence of anti-E2, anti- $\beta$ galactosidase ( $\beta$ gal), and anti-Ad Ab by ELISA as described previously.

For the experiments assessing cellular immune responses mice were sacrificed 10 days after the second injection. Spleen was harvested and after obtaining single-cell suspension it was filtered through a 100  $\mu$ m cell strainer. After removal of blood cells by ACK Lysing Buffer (Invitrogen, Cergy-Pontoise, France), the cells were resuspended in Supplemented RPMI and the concentration was adjusted at  $2.5 \times 10^6$  cells/mL. Then, splenocytes were restimulated *in vitro* to assess their function.

### *2.11. Statistical analysis*

For the statistical analysis a Mann–Whitney test was performed, recommended for groups fewer than 30 mice. Differences were considered significant when  $P < 0.05$ . All graphs and statistical data were obtained with the use of GraphPad Prism software.

### 3. Results

#### Production and characterization of Ad displaying HCV-derived epitopes

In order to evaluate the induction of anti-HCV humoral and cellular responses with the epitope display vaccination strategy, first, epitopes were selected from structural E2 protein (HVR1 and HCR1) or from non-structural NS3 protein (NS3<sub>1038-1047</sub>, NS3<sub>1073-1081</sub> and NS3<sub>1406-1415</sub>). The sequences encoding these epitopes were introduced by homologous recombination in *E. coli* in a lacZ recombinant Ad5. Of note, the sequence encoding NS3 epitopes was flanked or not with additional residues surrounding the epitope in NS3 protein. Among the 18 different viral genomes produced, only 6 were infectious (**Table 1 and 2**). AdH-HVR and AdF-HVR encoding HVR into hexon or fiber protein, respectively, were produced. AdF-HCR encoding HCR epitope in the fiber protein was produced while AdH-HCR was not rescued (**Table 2**). Regarding T cell NS3 epitopes, AdHNS3<sub>1073</sub> and AdHNS3<sub>1406</sub> bearing, respectively, NS3<sub>1073-1081</sub> and NS3<sub>1406-1415</sub> epitopes into hexon protein were successfully produced. AdF-NS3<sub>1035</sub> containing NS3<sub>1038-1047</sub> epitope surrounded by flanking residues and inserted into fiber protein was successfully produced but not its counterpart containing the same peptide in the hexon protein (**Table 1**). All 6 viruses were produced at titer comparable to AdWT bearing a wild-type capsid (**Table 1 and 2**).

SDS-PAGE analyses of the viruses showed similar capsid composition and integrity, with an additional band corresponding to the modified fiber visible for vectors displaying HVR or HCR epitopes in the fiber protein (**Figure 1A**). The presence of the epitope on AdF-HCR was confirmed by ELISA performed on denatured virions using AP33 monoclonal Ab (A.Patel, Glasgow University) (**Figure 1B**). All vectors displaying HCV epitopes were able to transduce CHO-k1-CAR cells expressing Ad5 primary receptor to the same extent as AdWT (**Figure 2**), thus demonstrating no modification of their infectivity.

### Humoral responses elicited by Ad displaying E2 epitopes

First, we sought to evaluate the potential of vectors displaying B cell epitopes to induce humoral anti-HCV responses. C57BL/6 mice were injected twice i.p. with AdH-HVR, AdH-HCR and AdF-HVR ( $10^{10}$  vp). Ab responses were analysed 2 weeks after each injection but no anti-E2 titers were detected for neither of the virus (data not shown). Of note, the presence of anti- $\beta$ gal Ab confirmed virus infectivity *in vivo*, ruling out any vector precipitation. Interestingly, immunized mice with AdE1E2 vector, encoding both HCV glycoproteins as a transgene (B. Bellier, UPMC) present significant anti-E2 Ab titers, indicating that C57BL/6 mice are capable of mounting E2-specific humoral responses. Then, we performed ELISA to analyse the ability of anti-E2 Abs produced in C57BL/6 mice to interact with HCR or HVR peptides. The results showed that HCR but not HVR peptide was recognised by anti-E2 mouse sera (data not shown). These results indicate that HVR epitope is not an immunodominant E2 epitope, at least in C57BL/6 background.

### Processing of NS3 epitope inserted into capsid Ad proteins

In order to confirm the presence of NS3<sub>1073-1081</sub> peptide on the surface of AdH-NS3<sub>1073</sub> and to evaluate epitope processing *in vitro*, we set up an *in vitro* antigen presentation assay based on the use of FLDC, dendritic cells derived from bone marrow precursors of HHD mice, and NS3<sub>1073-1081</sub>-specific T cell hybridoma I8H4 (M. Chen, Karolinska Institut). FLDCs were incubated with AdH-NS3<sub>1073</sub> or AdWT and I8H4 cells were added 2 hours later. Measurement of IL-2 production in the supernatant was used as a marker of hybridoma activation, indicating the recognition of NS3<sub>1073-1081</sub> peptide on HLA-2 molecules displayed by FLDC. Remarkably, IL-2 was produced in a dose-dependent manner when AdH-NS3<sub>1073</sub> was added in the co-culture but not in the presence of control AdWT (**Figure 3**). These results demonstrate that NS3<sub>1073-1081</sub> epitope embedded in hexon protein is efficiently processed and presented on HLA-A2 molecules of antigen-presenting cells.

### ***Cellular responses triggered by Ad displaying HCV T cell epitopes***

Having demonstrated efficient NS3 epitope processing from AdH-NS3<sub>1073</sub>, we evaluated the capacity of Ad bearing NS3 epitopes to trigger cellular immune responses in mice. HHD mice received two i.p. injections ( $10^{10}$  vp) of either AdH-NS3<sub>1073</sub> or AdH-NS3<sub>1406</sub> at an interval of 14 days and were sacrificed 10 days after the second administration. Spleens were harvested and single cell suspensions of splenocytes were restimulated *in vitro* in different conditions. Remarkably, IFN- $\gamma$  production in the supernatant of cells restimulated by NS3<sub>1073-1081</sub> peptide revealed the capacity of AdH-NS3<sub>1073</sub> to trigger CD8<sup>+</sup> T cell responses (**Figure 4**). More importantly, IFN- $\gamma$  production *in vitro* was higher upon restimulation with NS3<sub>1073-1081</sub> than with Ad peptide, emphasizing the capacity of AdH-NS3<sub>1073</sub> to induce anti-HCV T cell responses.

In contrast to AdH-NS3<sub>1073</sub>, AdH-NS3<sub>1406</sub> was unable to elicit *in vivo* cellular responses directed against NS3<sub>1406</sub> as documented by the absence of IFN- $\gamma$  production upon specific restimulation. This is not due to a general lack of immunogenicity of this vector, since *in vitro* restimulation revealed induction of potent anti-Ad cellular responses (**Figure 4**).

#### 4. Conclusions

HCV remains one of the most extensively studied human pathogens. Its capacity to induce chronic infections, leading to hepatocellular carcinoma as well as its variability strongly highlights the need for an effective cross-reactive vaccine. Among numerous vaccination strategies, a new approach based on genetic insertion of the selected epitopes into the Ad capsid is currently taking the lead. The efficiency of this strategy was already demonstrated for other pathogens, such as *B.anthraxis* or *P.aeruginosa*. Based on these results, we evaluated the potential of epitope display approach in counteracting HCV infection.

Several studies described E2 envelope glycoprotein as the main target for anti-HCV humoral responses. More precisely two regions named hyper conserved region (HCR) and hyper variable region (HVR) were described as immunodominant epitopes. We thus selected these sequences and inserted them into hexon or fiber protein. Vectors with HVR epitope in hexon and fiber were successfully rescued. As for HCR, only vector displaying this sequence in fiber was producible. Due to the limited antibody availability we were able to confirm only the presence of HCR epitope on the surface of AdF-HCR.

We encountered difficulties in inducing humoral anti-E2 responses with our vectors displaying HCR and HVR epitopes into their capsid. Further analysis with the use of positive control vector, AdE1E2 encoding E1 and E2 glycoproteins as a transgene, disclosed that HVR epitope is not recognized by anti-E2 Abs induced in C57BL/6 mice. HCR region, on the other hand was one of the targets of Abs induced upon immunization with the entire E2 protein. Thus it remains unclear why AdF-HCR vector failed to mount a detectable humoral response and further experiments will be needed (increased virus dose, addition of the adjuvants) in order to assess whether HCR epitope presented in the context of Ad capsid can be recognized by the immune system.

In case of T cell epitopes, we successfully produced Ad vectors displaying NS3 epitopes 1073-1081 and 1406-1415 inserted into the hexon protein and 1038-1047 (with flanking sequences) inserted into fiber protein. AdH-NS3<sub>1073</sub> was shown to efficiently stimulate IL-2 production by HCV-specific T cell hybridoma, demonstrating that epitope displayed on this vector can be processed and presented on antigen-presenting cells by HLA-A2 molecules.

AdH-NS3<sub>1073</sub> injected into HHD mice was capable of triggering anti-HCV responses as observed in *in vitro* splenocyte restimulation assay. Interestingly, these responses were more robust than those directed against one Ad hexon epitope. Studies are ongoing to better characterize these T cells responses.

## References

1. Benihoud, K., et al., Efficient, repeated adenovirus-mediated gene transfer in mice lacking both tumor necrosis factor alpha and lymphotoxin alpha. *J Virol*, 1998. 72(12): p. 9514-25.
2. Benihoud, K., P. Yeh, and M. Perricaudet, Adenovirus vectors for gene delivery. *Curr Opin Biotechnol*, 1999. 10(5): p. 440-7.
3. Crouzet, J., et al., Recombinational construction in *Escherichia coli* of infectious adenoviral genomes. *Proc Natl Acad Sci U S A*, 1997. 94(4): p. 1414-9.
4. Descamps, D. and K. Benihoud, Two key challenges for effective adenovirus-mediated liver gene therapy: innate immune responses and hepatocyte-specific transduction. *Curr Gene Ther*, 2009. 9(2): p. 115-27.
5. Desjardins, D., et al., Recombinant retrovirus-like particle forming DNA vaccines in prime-boost immunization and their use for hepatitis C virus vaccine development. *J Gene Med*, 2009. 11(4): p. 313-25.
6. Lanzi, A., et al., Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid. *Vaccine*, 2011. 29(7): p. 1463-71.
7. Martin, K., et al., Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism. *Mol Ther*, 2003. 8(3): p. 485-94.
8. McConnell, M.J., X. Danthinne, and M.J. Imperiale, Characterization of a permissive epitope insertion site in adenovirus hexon. *J Virol*, 2006. 80(11): p. 5361-70.
9. Pasetto, A., et al., Generation of T-cell receptors targeting a genetically stable and immunodominant cytotoxic T-lymphocyte epitope within hepatitis C virus non-structural protein 3. *J Gen Virol*, 2012. 93(Pt 2): p. 247-58.
10. Schweitzer, C.J. and T.J. Liang, Impact of host and virus genome variability on HCV replication and response to interferon. *Curr Opin Virol*, 2013. 3(5): p. 501-7.
11. Vigne, E., et al., RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol*, 1999. 73(6): p. 5156-61.
12. Walker, C.M. and A. Grakoui, Hepatitis C virus: why do we need a vaccine to prevent a curable persistent infection? *Curr Opin Immunol*, 2015. 35: p. 137-43.
13. Worgall, S., et al., Protection against *P. aeruginosa* with an adenovirus vector containing an OprF epitope in the capsid. *J Clin Invest*, 2005. 115(5): p. 1281-9.



**Table 1. Ad vectors displaying T cell epitopes from HCV NS3 protein.**

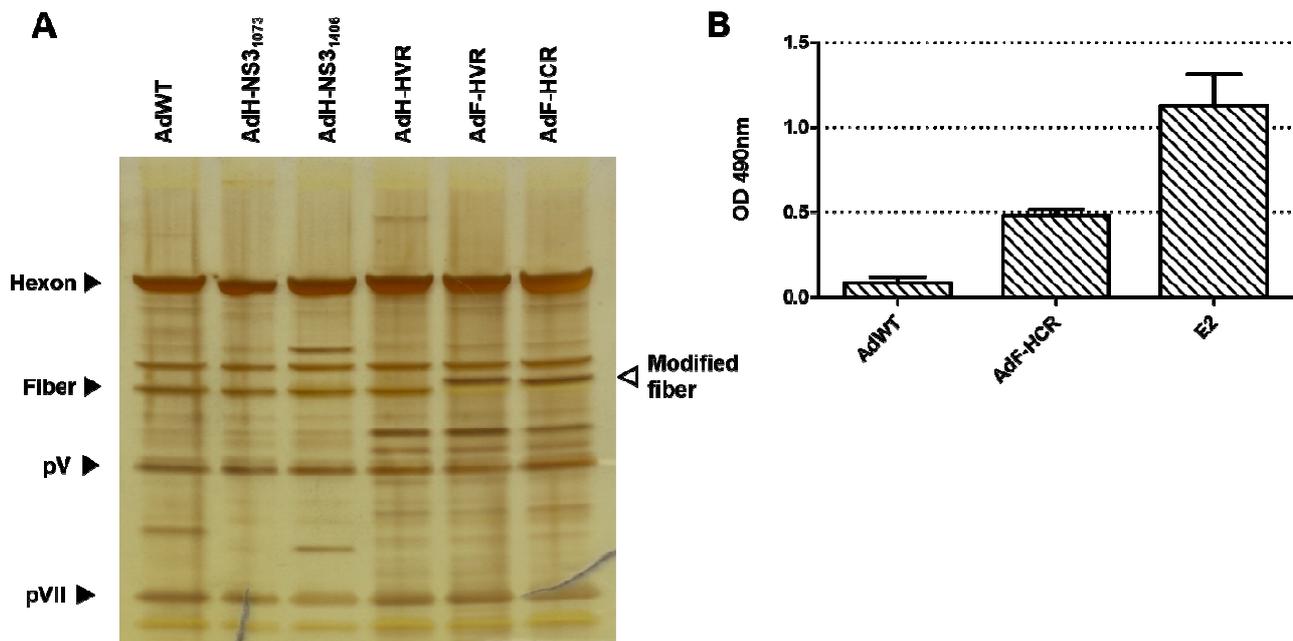
Virus	Transgene	Modified protein	Epitope	Inserted sequence	Titer <sup>a</sup> ( $\times 10^{12}$ vp/ml)
AdWT	$\beta$ gal	-	-	-	<b>4.4 <math>\pm</math> 0.8</b>
AdH-NS3 <sub>1035</sub>	$\beta$ gal	Hexon	<b>NS3 (1038-1047)</b>	QRTGLLGCIITSLTG	n.i. <sup>b</sup>
AdF-NS3 <sub>1035</sub>	$\beta$ gal	Fiber		QRTGLLGCIITSLTG	<b>2.6</b>
AdH-NS3 <sub>1038</sub>	$\beta$ gal	Hexon		GLLGCIITS	-
AdF-NS3 <sub>1038</sub>	$\beta$ gal	Fiber		GLLGCIITS	-
AdH-NS3 <sub>1068</sub>	$\beta$ gal	Hexon	<b>NS3 (1073-1081)</b>	SFLATCVNGVCWTVYHGAG	-
AdF-NS3 <sub>1068</sub>	$\beta$ gal	Fiber		SFLATCVNGVCWTVYHGAG	-
AdH-NS3 <sub>1070</sub>	$\beta$ gal	Hexon		LATCVNGVCWTVYHG	-
AdF-NS3 <sub>1070</sub>	$\beta$ gal	Fiber		LATCVNGVCWTVYHG	-
AdH-NS3 <sub>1073</sub>	$\beta$ gal	Hexon	<b>CVNGVCWTV</b>	<b>CVNGVCWTV</b>	<b>8.2</b>
AdF-NS3 <sub>1073</sub>	$\beta$ gal	Fiber		<b>CVNGVCWTV</b>	-
AdH-NS3 <sub>1403</sub>	$\beta$ gal	Hexon	<b>NS3 (1406-1415)</b>	LAAKLTGLGLNAVAYY	-
AdF-NS3 <sub>1403</sub>	$\beta$ gal	Fiber		LAAKLTGLGLNAVAYY	-
AdH-NS3 <sub>1406</sub>	$\beta$ gal	Hexon		<b>KLTGLGLNAV</b>	<b>6.8</b>
AdF-NS3 <sub>1406</sub>	$\beta$ gal	Fiber		<b>KLTGLGLNAV</b>	-

<sup>a</sup> Mean  $\pm$  SD; <sup>b</sup> n.i. – non infectious, - non producible

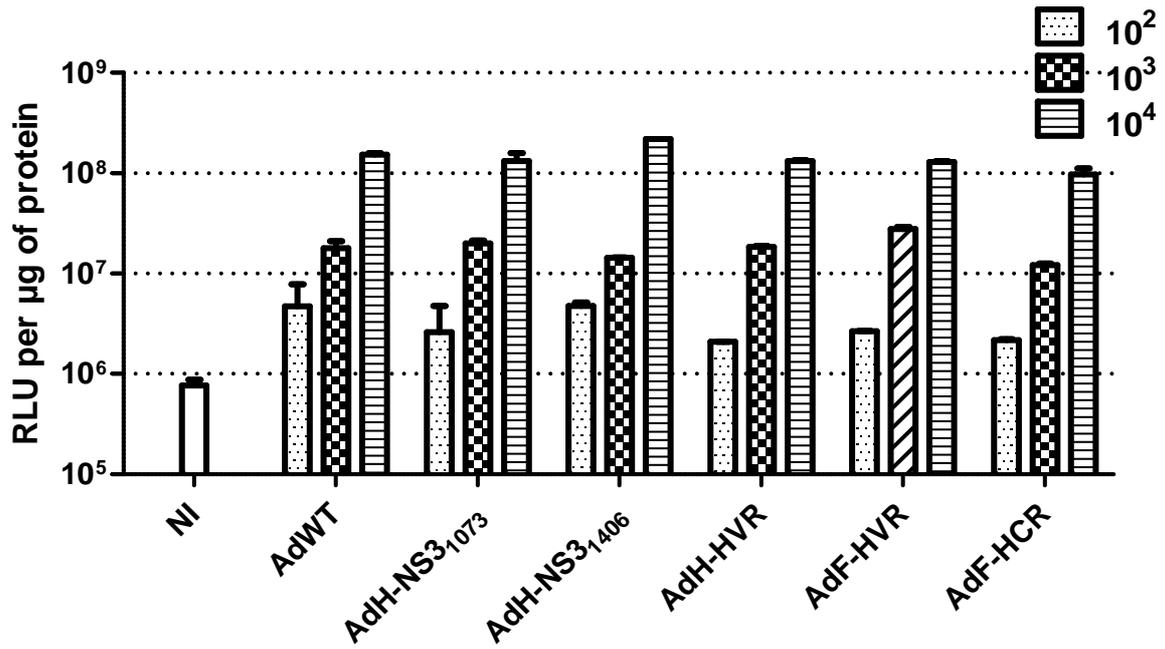
**Table 2. Ad vectors displaying B cell epitopes from HCV E2 protein.**

<b>Virus</b>	<b>Transgene</b>	<b>Modified protein</b>	<b>Epitope</b>	<b>Inserted sequence</b>	<b>Titer <sup>a</sup> (<math>\times 10^{12}</math> vp/ml)</b>
AdWT	$\beta$ gal	-	-	-	<b>4.4 <math>\pm</math> 0.8</b>
AdE1E2	E1 and E2	-	-	-	<b>2.2</b>
AdH-HVR	$\beta$ gal	Hexon	<b>HVR (384-410)</b>	<b>HTHVTGGRVASSTQSLVSWLSQGPSQK</b>	<b>4.5<math>\pm</math>0.4</b>
AdF-HVR	$\beta$ gal	Fiber			<b>1.6<math>\pm</math>0.9</b>
AdH-HCR	$\beta$ gal	Hexon	<b>HCR (412-431)</b>	<b>QLVNTNGSWHINRTALNCND</b>	-
AdF-HCR	$\beta$ gal	Fiber			<b>3.8<math>\pm</math>0.4</b>

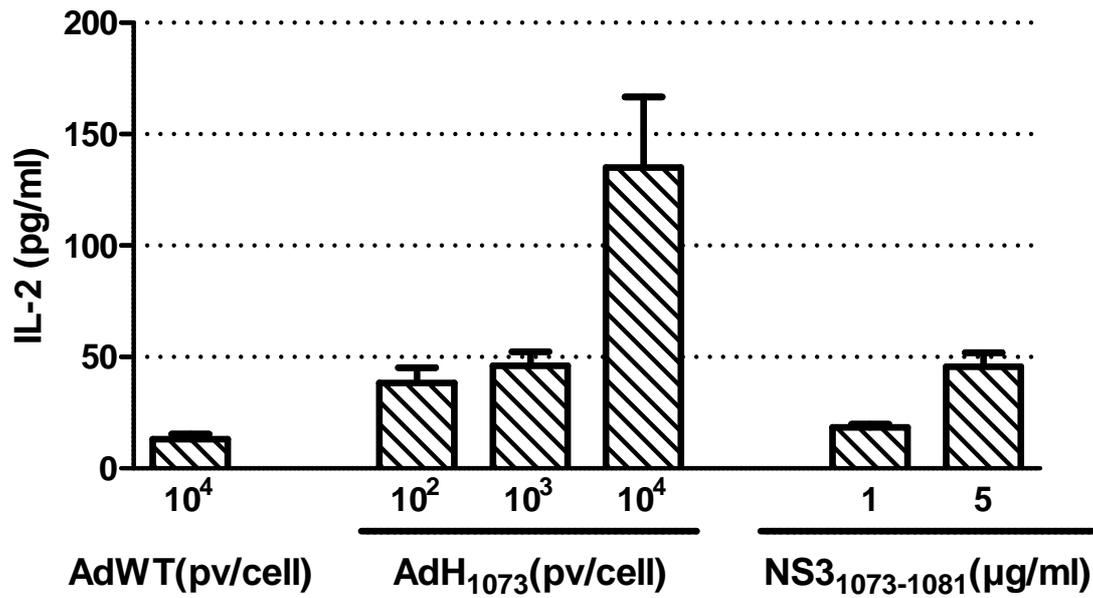
<sup>a</sup>Mean  $\pm$  SD



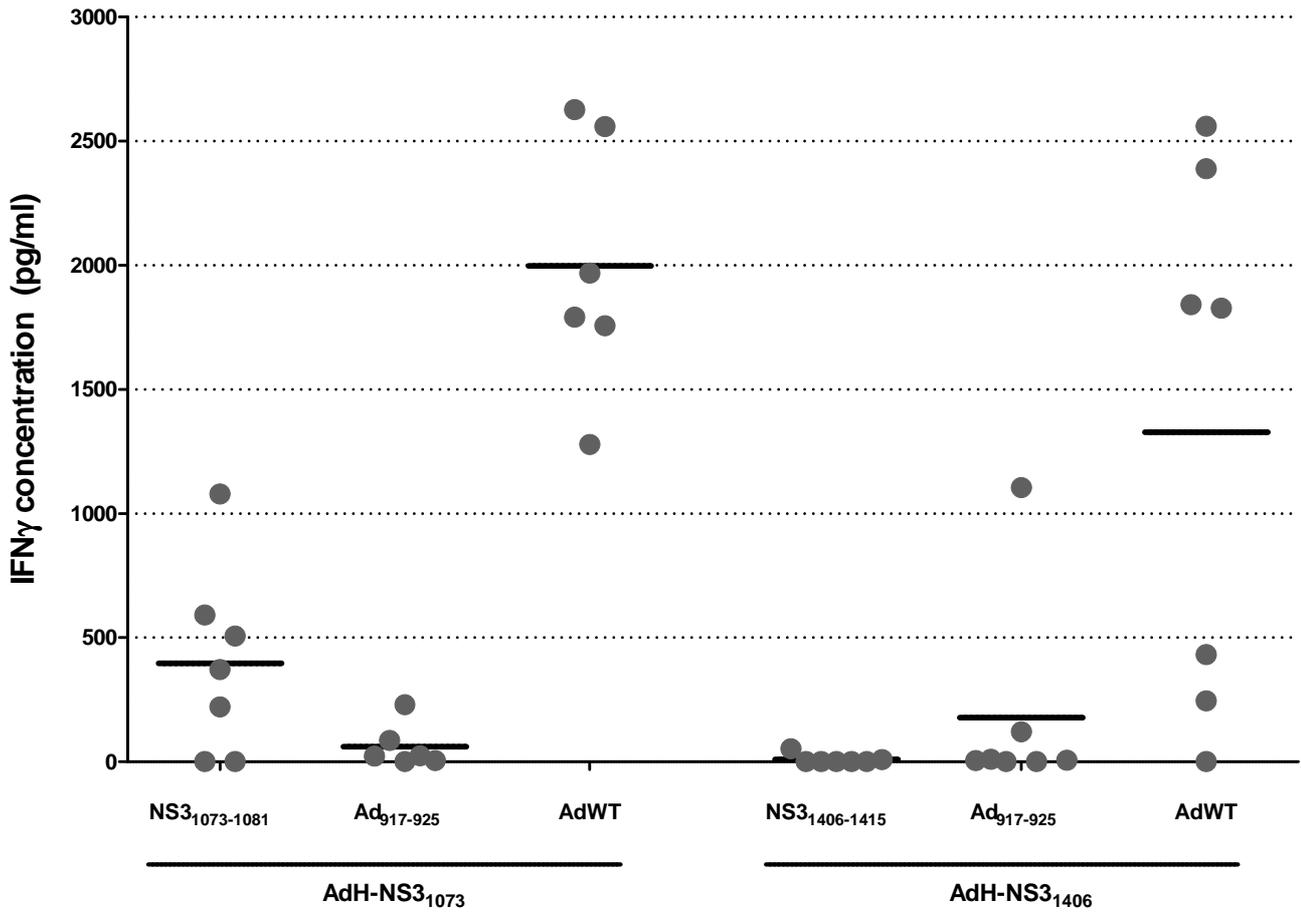
**Fig. 1** Vector characterization *in vitro*. **(A)** Silver staining of capsid-modified Ad.  $10^{10}$  vp of either a control Ad (AdWT) or a capsid-modified Ad (AdH-NS3<sub>1073</sub>, AdH-NS3<sub>1406</sub>, AdH-HVR, AdF-HVR, AdF-HCR) were separated on a 10% polyacrylamide gel. Major capsid components (black arrow) and modified fiber (white arrow) are identified. **(B)** Detection of HCR epitope on the surface of AdF-HCR. ELISA plates were coated with 100 ng of denatured virus or E2 glycoprotein and incubated with AP33 monoclonal antibody against the HCR epitope. The binding was detected with HRP-conjugated secondary antibody. One of two experiments is shown, means+SD.



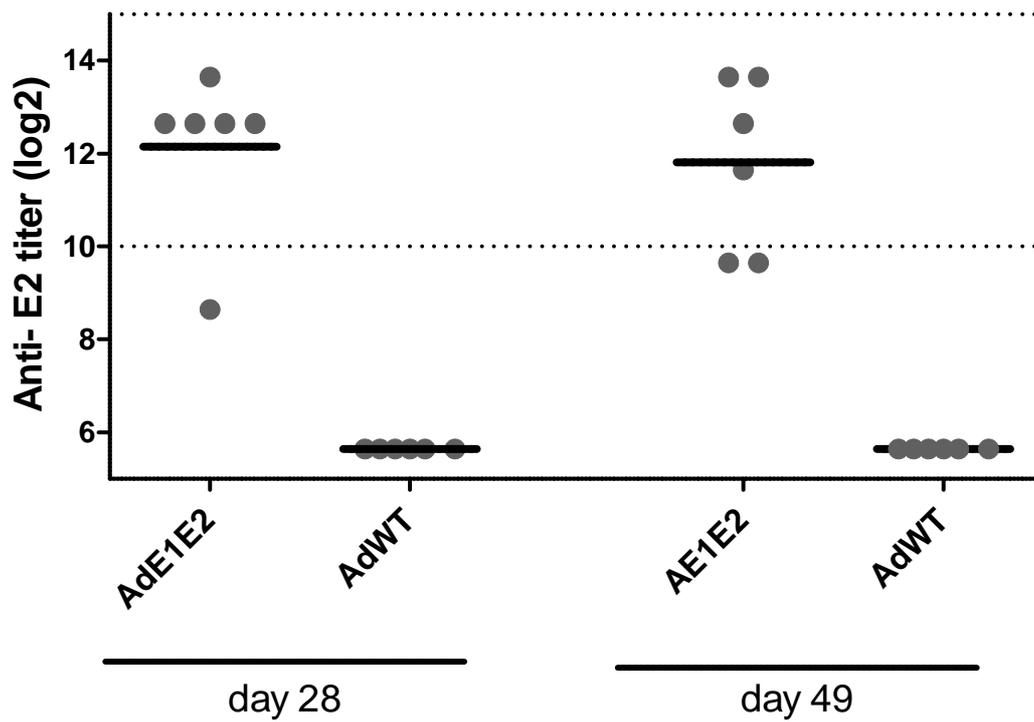
**Figure 2** *In vitro* infectivity of capsid modified vectors. CHO-k1-hCAR cells were mock-infected (NI) or infected with different MOI of AdWT or capsid-modified Ad (AdH-NS3<sub>1073</sub>, AdH-NS3<sub>1405</sub>, AdH-HVR, AdF-HVR, AdF-HCR).  $\beta$ gal activity was measured in cells harvested 24 h post-infection. ( $\beta$ gal activity expressed as relative light unit (RLU) per  $\mu$ g of protein. The representative results of at least 2 experiments are shown.



**Figure 3** Epitope presentation assay. AdWT (10<sup>4</sup> vp/cell) or AdH-NS3<sub>1073</sub> (10<sup>2</sup> -10<sup>4</sup> vp/cell) were incubated with FLDC for two hours. FLDC incubated with different concentrations of NS3<sub>1073-1081</sub> peptide were used as positive controls. Anti- NS3<sub>1073-1081</sub> T cells were then added and the amount of IL-2 released in the supernatant was measured two days later by ELISA. One of two experiments is shown, n=3; means+SD.



**Figure 4** T cell immune responses elicited by Ad displaying HCV-derived T cell epitopes. HHD mice at two week distance received two intraperitoneal injections of  $10^{10}$  vp of Ad displaying NS3<sub>1073-1081</sub> or NS3<sub>1406-1415</sub> epitope into their hexon protein (AdH-NS3<sub>1073</sub> or AdH-NS3<sub>1406</sub>, respectively). Splenocytes harvested at day 10 p.i. were stimulated *in vitro* with corresponding NS3 epitope, Ad epitope or AdWT. Production of IFN-γ in the supernatant was assessed by ELISA. Dots and bars represent specific IFN-γ production of individual mice and the group, respectively. n = 4-6



**Figure 5.** Humoral responses elicited by Ad encoding E1 and E2 glycoproteins in the form of transgene. C57BL/6 mice were immunized intraperitoneally twice in a 2 week distance with  $10^{10}$  vp of AdE1E2 od AdWT. Anti-E2 titers were determined by ELISA at day 28 and 49 after the first administration. Titers below 100 were plotted as 50. One of two experiments is shown, dots and bars represent results form individual mice ( $n = 5-6$  mice) and means, respectively.



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# ***Discussion***

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Progresses in vaccine development allowed to select the most immunogenic elements in the pathogen structure, reducing antigen size to a single protein or peptide. However, administration of highly purified, non-vectorised molecules fails to trigger adaptive immune responses. These limits underpinned a need for a potent vaccine platform and Adenovirus (Ad) -derived vectors are among the few candidates. Unfortunately, high prevalence of this virus results in pre-existing anti-Ad immunity, detected in the majority of population (50-90%). Various strategies aiming at overcoming this limitation were proposed (described in paragraph 2.3.1.), among them a new vaccination approach called Epitope Display, based on the genetic insertion of heterologous epitopes into Ad capsid. In a previous work of our lab we evaluated the influence of the epitope insertion site and the host status towards Ad on the vaccination potency of epitope display strategy (Lanzi *et al.*, 2011). The results indicated that in Ad-immune mice vector displaying ovalbumin (Ova) B cell epitope (Ova<sub>323-339</sub>) into its fiber protein was by far more potent in eliciting anti-Ova antibody upon the second injection than Ad with Ova<sub>323-339</sub> inserted into hexon. This boosting potential of fiber-displaying vector was owed to the presence of anti-Ad antibodies (Abs).

In the present work we pursued the investigation of the efficacy of epitope display strategy. The results presented above will be discussed in three paragraphs. Initially, the molecular bases controlling the induction of humoral responses by Ad displaying Ova B cell epitope will be evaluated, with an emphasize on virus and host factors shaping the vaccination efficacy. Subsequently, in the second paragraph, I will comment on our data obtained with Ad vectors displaying Ova-derived CD8<sup>+</sup> T cell epitope. In the following paragraph our attempts to apply epitope display strategy into the vaccination against HCV will be discussed, and finally I will finish by describing the future perspectives of this vaccination approach.

### **1. Humoral responses elicited against a model antigen**

The results obtained by Lanzi *et al.*, showed that presence of anti-Ad Abs does not impede the boosting potential of Ad vectors displaying Ova<sub>323-339</sub> in fiber. This suggests that the virus infectivity process might not be a crucial factor influencing the efficacy of this vaccination strategy. Thus, in the first turn, we aimed at deciphering the molecular components, of both vector and host origin, dictating the remarkable vaccination potential of Ad vectors displaying heterologous B cell epitopes into their capsid.

## 1.1. Role of virus factors

### 1.1.1. Requirement of cell infection

In the first turn we decided to evaluate the capacity of vectors displaying Ova<sub>323-339</sub> B cell epitope in the fiber or hexon protein to induce anti-Ova humoral responses in naïve and Ad-immune mice. We showed that these vectors were not limited by pre-existing anti-Ad immunity and induced detectable anti-Ova antibody titers. Moreover, vector displaying epitope in fiber elicited more robust anti-ovalbumin humoral responses in Ad-immune mice than in naïve animal, and anti-ovalbumin Abs were detectable up to 7 months after the first injection.  $\beta$ -galactosidase ( $\beta$ gal), introduced as a transgene, served as a control of viral genome expression. The lack of anti- $\beta$ gal Abs in Ad-immune mice confirmed that these vectors were unable to efficiently transduce cells *in vivo* and to express  $\beta$ gal. The reduced anti-transgene humoral responses coinciding with unaltered levels of anti-Ova Abs clearly indicate that virus infectivity process and subsequent gene transfer are not indispensable steps in epitope display vaccination strategy.

Previous reports indicate that Ad gene expression is not needed to mount an innate immunity (Muruve *et al.*, 2004). Also, experiments with transcription-defective Ad induced inflammatory responses similar to the transcription-competent Ad viral particles, demonstrating that the activation of innate immunity is provided by Ad particle and not by products issued from viral genome expression (McCoy *et al.*, 1995). Another study using Ad vector with inactivated gene expression (through UV-induced DNA crosslinking) showed that this modification did not hamper the induction of humoral response against Ova protein fused to pIX capsid protein (Johrden *et al.*, 2013). All these reports support our results and confirm that gene transfer is dispensable, and suggest that intrinsic adjuvant capacity ensured by Ad particle provides sufficient stimulation of innate immunity sensors.

Another interesting point is the influence of pre-existing anti-Ad Abs on the induced immune responses. We observed that Ad displaying epitope in fiber benefits from pre-Ad immunity in its immunogenicity. Similar observations were obtained with the use of AAV viral like particles (VLPs, described below) displaying epitopes of HPV, where anti-HPV humoral response was increased in animals pre-immunized with AAV (Nieto *et al.*, 2012). A recent study found that anti-Ad immunity could potentially be advantageous in the vaccination approach, since Ad particles coated with Abs, while uptaken into the cells, can activate a cytoplasmic Fc-receptor TRIM21, leading to NF- $\kappa$ B-, IRF- and AP-1-dependent production of pro-inflammatory cytokines (McEwan *et al.*, 2013). Different studies also showed that Ad particles coated with Abs not only are better uptaken by DCs, but also cause a better maturation of these cells, as reflected by an increased expression of co-stimulatory molecules and more vigorous production of pro-inflammatory molecules (Perreau *et al.*, 2008). These

reports could explain the stronger anti-Ova titers induced by fiber-displaying vector in Ad-immune mice. They also further underline that the strategy of displaying heterologous epitopes in Ad fiber protein can be successfully used in both naïve and Ad-immune background.

### **1.1.2. Interaction with natural receptors**

To further investigate the minimal requirements that Ad vector needs to fulfill in order to elicit potent humoral responses against displayed epitopes, we evaluated the role of vector interactions with its natural receptors. We previously reported that Ad interactions with cellular receptors and host factors are shaping vector tropism and inflammatory profile (Vigant *et al.*, 2008; Raddi *et al.*, 2016). Others also described that Ad binding to these molecules not only influences the cell entry but also results in the activation of intracellular signaling (for a review see Descamps and Benihoud; 2009). In order to investigate the impact of these interactions on Ad vaccination potential, we constructed a panel of Ad vectors simultaneously displaying Ova<sub>323-339</sub> B cell epitope in the fiber protein and possessing receptor-detargeting mutations.

Interaction of fiber protein with CAR receptor triggers downstream signaling, resulting in NF- $\kappa$ B-mediated upregulation of IL-8 production (Tamanini *et al.*, 2006). We recently showed that Ad5 vector pseudotyped with Ad3 shaft induces reduced blood toxicity and cytokine production (Raddi *et al.*, 2016) as well as decreased liver uptake (Raddi, in revision). We thus pseudotyped previously described AdF-3OVA2 vector with Ad3 shaft in order to generate a vector with reduced potency to interact with CAR receptor and disabled in HSPG binding due to the lack of KKTK motif present in Ad5 shaft. We were able to show that this mutation significantly limits anti-transgene antibody titers but does not have an impact on anti-Ova humoral responses. Other molecules which play an important role in both cell entry and innate signaling are integrins. Di Paolo and colleagues found that interaction of RGD motifs with  $\beta_3$ integrins on the macrophage surface induces IL-1 $\alpha$ -mediated response (Di Paolo *et al.*, 2009). Another study reported that Ad generates reduced inflammatory profile in  $\beta_3$ integrin knock-out mice, confirming involvement of this molecule in the early innate immune responses (Browne *et al.*, 2015). Thus, basing on previous results of our laboratory showing that mutation in penton base (RGD deletion) ablates Ad5 interaction with integrins (Vigne *et al.*, 2003), a vector bearing this mutation was also evaluated in this part of my work. Similarly to the ablation of CAR and HSPG interactions, blocking the integrin binding did not diminished the potency of epitope display strategy. Another cell entry factor, FX, was more recently proposed as an element shaping Ad-induced immune responses. Doronin *et al.* claim that while bound to Ad, FX can stimulate TLR4, reinforcing innate immune responses in mice (Doronin *et al.*, 2012). However, a more recent study reporting no stimulation of human mononuclear phagocytes by Ad-FX questioned these results (Eichholz *et al.*, 2015). We took benefit from our earlier data, showing

that mutation in hexon (HVR5 deletion) prevents FX binding (Vigant *et al.*, 2008), and generated a vector displaying Ova<sub>323-339</sub> in fiber and crippled in FX binding. The ablation of Ad interactions with its natural cell entry factor did not have any influence on anti-Ova humoral responses which were as potent as for AdF-3OVA2 vector. Since Ad binding to FX was shown to be responsible for strong liver uptake (Parker *et al.*, 2006), our results could lead to the generation of vectors with reduced hepatocyte tropism and unaltered vaccination potential.

Altogether our results raise questions how severely Ad vector can be crippled before it loses its adjuvant properties. With this regard we decided to combine mutations altering FX and integrin binding on one virus particle. This vector displayed further reduction in its *in vivo* infectivity, nevertheless presented unaltered capacity to trigger anti-Ova humoral responses, further underpinning our previous observations. Krause and colleagues also reported that reducing CAR- and integrin-dependent interaction of Ad vector displaying epitope from hemagglutinin into its fiber protein had no impact on the humoral responses elicited *in vivo* by this vector (Krause *et al.*, 2006). After evaluating the role of reduced Ad gene transfer this would be interesting to analyze the outcome of vaccination with empty capsid (an Ad particle completely deprived of viral DNA) displaying Ova epitope. It is difficult to anticipate the outcome of such particles unable to drive *de novo* synthesis of viral proteins and disabled in stimulating DNA sensors such as TLR9. Some studies have investigated virus like particles (VLPs), generated from viral structural proteins upon *in vitro* self-assembly. In case of Ad, twelve Ad3 penton base proteins (with or without the fiber protein) can self-assemble into so-called dodecahedron (Dd) (Fender *et al.*, 1997). A few attempts were made to use these structures as a vaccine platform. Ova fragment was fused to the penton base protein and Dd immunization resulted in induction of anti-Ova humoral responses (Villegas-Mendez *et al.*, 2010). However, since full Ad particle was not included in this study, it is hard to compare the vaccination performance of Dd with entire Ad capsid. One could assume that since Ad hexon is the main source of CD4<sup>+</sup> T cell epitopes (Onion *et al.*, 2007), weaker stimulation of T cells responses by these structures based solely on penton base protein will limit the capacity of Ova-specific B cells to differentiate, resulting in weaker anti-Ova antibody titers. Other vaccination strategies use adeno-associated virus (AAV) or hepatitis B virus VLPs as an epitope display platforms, but when non-adjuvanted these constructs induce poor innate and adaptive anti-epitope responses (Nieto *et al.*, 2012; Sominskaya *al.*, 2010).

Altogether, our data indicate that the rupture in the signaling pathways activated upon Ad binding to its native receptor can be surrogated by numerous PAMPs associated with Ad particle. These results also further support the data obtained in our previous experiment, indicating that efficient infection is a dispensable factor in the process of triggering an adaptive immunity with epitope displaying Ad vectors.

## 1.2. Host factors

In parallel to the investigation of the role of Ad receptors, we also evaluated innate immunity pathways controlling the efficacy of epitope display vaccination. Ad is known to stimulate TLR2 and TLR9, both of which use MyD88 as an adaptor protein (Yamaguchi *et al.*, 2007; Appledorn *et al.*, 2008). Thus, in order to evaluate the impact of these signaling pathways on mounting anti-epitope humoral responses, we used mice deficient in MyD88, an adaptor molecule shared by all TLR with the exception of TLR3. Interestingly, anti-Ova humoral responses did not differ in MyD88<sup>-/-</sup> and wild-type mice, suggesting that other innate sensing pathways were involved in the induction of innate immunity. To the best of our knowledge, this is the first analysis of the impact of TLR/MyD88 pathway on epitope display strategy,. Nevertheless, some teams assessed the role of MyD88 signaling in innate as well as adaptive responses induced by transgene-encoding vectors, derived from Ad or other viruses. Rhee and colleagues evaluated the impact of MyD88 signaling ablation on Ad5-induced responses and reported significantly reduced anti-transgene CD8<sup>+</sup> cellular immunity (Rhee *et al.*, 2011). Interestingly, while the lack of MyD88 protein severely impairs immune responses, mice deficient in a single TLR develop unaltered cellular immunity. These findings indicate that these TLR are not critical individually but contribute together to the potent Ad innate immunity. Still, Yamaguchi and his team noticed that Ad reliance on TLR9 and MyD88 stimulation depends on the cell type, suggesting that Ad vectors can use both TLR9/ MyD88-dependent and -independent pathways in innate immunity induction (Yamaguchi *et al.*, 2007). Reduction of anti-transgene cellular responses observed by others and unaltered B cell responses in our experiment could be explained by the repetitive exposure of the epitope on the surface of Ad capsid. Indeed, a previous study suggested that, when present in a repetitive manner, epitopes can lead to the cross-linking of B cell receptors and induce T cell independent humoral responses (Fehr *et al.*, 1998). Also, Maglione and colleagues observed that while IgM responses against T-independent antigens are broadly impaired in MyD88-deficient patients, IgG antibody titers remain unaltered (Maglione *et al.*, 2014).

Other teams investigating the impact of MyD88 protein on vector-induced humoral immunity obtained results contradictory to ours. Humoral responses against Respiratory Syncytial virus were impaired in mice lacking MyD88 protein (Bhoj *et al.*, 2008). Similarly, Sudres *et al.* reported that antibody titers against AAV vector were significantly reduced in MyD88<sup>-/-</sup> (Sudres *et al.*, 2012). The discrepancy between these studies and our results could be explained by the differences in the vectors/viruses used, as divergent dependence on innate signaling pathways among the viruses have been underpinned by various studies. Still, an interesting aspect observed by Sudres *et al.* is the intrinsic B cell dependence on Myd88 signaling and ability of MyD88<sup>-/-</sup> mice to restore the reduced total IgG titers upon infusion with wild-type B cells. Analogical observations came from Guay

and colleagues who assessed responses to polyoma virus (Guay *et al.*, 2007). They proposed that while antiviral antibody responses are efficiently initiated in MyD88<sup>-/-</sup> mice (as measured 7 days after injection), the establishment of long lived plasma cells is strictly dependent on intrinsic B cell MyD88 signaling. Indeed, titers analyzed 1.5 years after the vector injection were significantly reduced for MyD88<sup>-/-</sup> mice. Similarly, it would be interesting to evaluate capacity of AdF-3OVA2 to induce long-lived, epitope-specific plasma cells in Myd88 deficient mice.

Interestingly, analysis of induced anti-Ova isotypes in our study revealed significant increase in IgG1 titers in Myd88<sup>-/-</sup> mice, indicating the development of adaptive response of Th2 type. Rogers *et al.* with his team compared the responses induced against vector capsid and transgene in MyD88<sup>-/-</sup> and wild type mice upon administration of AAV vector encoding Ova (Rogers *et al.*, 2015). While anti-Ova IgG1 levels were slightly reduced, in MyD88<sup>-/-</sup> mice, anti-AAV IgG1 titers were significantly elevated with regard to wild-type mice. Our results showing an increase of IgG1 titers in MyD88<sup>-/-</sup> mice could be explained by the fact that Ova<sub>323-339</sub> peptide integrated in the fiber protein is now an intrinsic capsid component. We also observed that presence of MyD88 was required for the production of anti-transgene IgG2a Abs. The exact molecular mechanism dictating these differences between anti-transgene and anti-vector responses remain to be elucidated but could be linked to the intrinsic nature of the antigen, its concentration or its localization. Nevertheless, one study already showed an impact of MyD88 signaling in B cells on Th-polarization and IgG isotype class switching (Rawlings *et al.*, 2012). Also, Myd88 signaling in DC results in the production of type-I IFN and this cytokine was shown to result in increased IgG2a and IgG2b production and reduced IgG1 levels (Heer *et al.*, 2007; Zhu *et al.*, 2009).

As far as TLR sensing is concerned, Ad particle coated with FX is also thought to be recognized by TLR4 (Doronin *et al.*, 2012). TLR4 can use both MyD88 and TRIF as its adaptor proteins and thus signaling through this receptor in MyD88<sup>-/-</sup> could be only partially disabled. The results obtained by Rhee in TRIF<sup>-/-</sup> mice showed that this adaptor molecule has little impact on the cellular responses induced by Ad vector. Nevertheless, in order to completely evaluate the influence of TLR sensing on Ad vector vaccination potential, double knock-out mice (MyD88- and TRIF-deprived) would need to be used.

Similarly to TLR signaling, we sought to investigate the impact of RLR sensing on the vaccination performance of epitope display Ad vectors. It has been shown that RNA associated with Ad particle is capable of stimulating the RIG-I signaling pathway (Minamitani *et al.*, 2011). Since RIG-I uses MAVS as an adaptor protein, we investigated the ability of MAVS<sup>-/-</sup> mice to mount humoral responses against the displayed epitope. Our observations indicate that, analogically to MyD88<sup>-/-</sup> mice, MAVS<sup>-/-</sup> mice immunized with AdF-3OVA2 displayed anti-Ova titers as robust as wild-type mice. Studies analyzing the role of RIG-I in virus-induced immune responses are less numerous than those

investigating MyD88 protein. One group evaluated the influence of RLRs signaling in the immune responses to Influenza virus. They observed, that MAVS<sup>-/-</sup> mice are capable of inducing both anti-transgene and anti-vector humoral responses as efficiently as wild-type mice. Additionally, a link between the stimulation of previously described TRIM21 (cytoplasmic antibody Fc-receptor) and RIG-I sensing was recently deciphered (Watkinson *et al.*, 2015). The authors proposed that TRIM21-induced premature Ad uncoating improves viral RNA and DNA recognition by RIG-I and cGAS/STING, respectively. With regard to these findings, an evaluation of Ad epitope display approach in Ad-immune MAVS<sup>-/-</sup> mice would bring an interesting insight into the way these two signaling pathways overlap.

Altogether, the results of other as well as our data suggest that innate immune responses to Ad vector do not rely on one particular receptor or signaling pathway and rather seem to be regulated in an orchestrated manner by various sensors. The complexity of diverse sensors provides Ad vector with numerous mechanisms of innate immunity activation. In addition to previously discussed TLR and RLR signaling, viral DNA could contribute to the induction of inflammatory profile by stimulating the cGAS/STING pathway. Herpes Simplex virus induced significantly attenuated inflammatory responses in cGAS<sup>-/-</sup> mice (Li *et al.*, 2013). Studies using Ad vector confirmed *in vitro* the influence of cGAS/STING viral DNA sensing on the activation of IRF3-responsive genes (Lam *et al.*, 2014). However, no study so far analyzed the influence cGAS/STING pathway *in vivo*. Evaluation of the vaccination performance of our vector in cGAS<sup>-/-</sup> or STING<sup>-/-</sup> mice could bring valuable results and generate a set of data complementing the observations of wild-type mice immunization with empty vectors. Another interesting aspect to evaluate would be the determination of humoral responses triggered in MyD88<sup>-/-</sup> and MAVS<sup>-/-</sup> mice by detargeted vectors. Determining the innate immune signaling pathways involved in mounting adaptive immune responses is critical in order to better address the safety issues and control the immunogenicity of the vector. Such increase in our knowledge would pave the way for the development of improved vaccine vectors.

## **2. Induction of cellular responses by Ad vectors displaying T cell epitopes into their capsid**

Beside the analysis of humoral responses elicited by Ad displaying cell epitopes, in the second part of my thesis we sought to evaluate the potential of epitope display strategy to induce cellular responses. Ad vectors displaying OVA class-I T cell epitope SIINFEKL (SL) into their hexon or fiber proteins were previously constructed. Inserted sequence was containing either epitope alone or flanked with additional amino acids residues from the original Ova. Altogether, six vectors were produced and evaluated *in vitro* for their ability to be uptaken and processed by DCs (thesis Anastasia Lanzi). In the frame of my thesis we sought to pursue their characterization *in vivo*. Two

main questions were investigated: the role of the insertion site of the epitope and the possible influence of pre-existing Ad immunity.

The results of splenocyte restimulation indicate that Ad displaying SL epitope into hexon protein was more potent in inducing epitope-specific T cells than AdF-SL. In parallel to *in vitro* restimulation of Ova-specific splenocytes, we also quantified by cytometry Ova-specific splenocytes using tetramers (H-2K<sup>b</sup>+SL peptide). Percentage of tetramer positive CD8<sup>+</sup> cells among the total pool of CD8<sup>+</sup> cells was the most elevated in naïve mice injected with AdH-SL. This tendency was observed at both early (11-12 days) and late (25-26 days) time points. Similarly, the analysis of total number of SL-specific CD8<sup>+</sup> cells in the spleen of naïve mice pointed at AdH-SL as the most immunogenic construct at the early and late stages of induced cellular responses. In order to complement our quantitative assessment of cellular immunity we additionally counted the SL-specific cells producing IFN- $\gamma$ . Results were underpinning the previous observations, with the most robust cellular responses detected at both time points in naïve mice immunized with AdH-SL. All these observation conjointly lead to the conclusion that induction of cellular responses strongly depends on the epitope insertion site.

The superiority of hexon display could potentially be explained by its increased abundance in the Ad particle composition. Indeed, while inserted into hexon protein, epitope is present in a total number of 720 (3 x 240) copies while fiber display provides 36 (3 x 12) epitopes copies on one Ad capsid. However, results previously obtained by Dr A. Lanzi during the analysis of epitope processing *in vitro* suggest otherwise. Vectors displaying epitopes into their hexons were more potently inducing production of IL-2 cytokine than Ad with epitopes inserted into fibers. Thus, it is probable that this capacity of hexon-located epitope to be more efficiently processed and/or presented on the surface of APC governs its immunization potential *in vivo*. Stronger levels of anti-ovalbumin CD8<sup>+</sup> T cells induced by AdH-SL while compared to AdF-SL could also be due to distinct processing pathways of hexon and fiber protein. After binding to a host cell and entry by endocytosis, Ad virion undergoes the uncoating step during which the fiber protein is separated from the capsid. Then, after endosome escape, the remaining Ad particle traffic to the nucleus using microtubules and finally a complex formed by hexon and viral DNA is able to enter the nucleus through the nuclear pore complex. Since the fiber and hexon protein traffic through endosomes, they should be processed through the endocytic pathway in antigen-presenting cells and presented by MHC class II molecules. The capacity of hexon protein to traffic into the cytosol on microtubules until the nucleus presumably makes it able to be processed and presented in antigen-presenting cells through the endogenous pathway. Altogether, the better CD8<sup>+</sup> T cell responses obtained after mice immunization with AdH-SL could be linked to the efficient processing of hexon protein by the endogenous processing pathway.

Both AdH-SL and AdF-SL are less efficient in Ad-immune mice, as confirmed by our results obtained with splenocyte restimulation, tetramer staining and ELISPOT assays. It is interesting to observe that pre-existing anti-Ad immunity hampers the induction of the cellular responses directed against displayed epitope. One could assume, that while coated with Abs, Ad particle would be better uptaken by antigen-presenting cells such as DCs through the Fc $\gamma$  receptor. Studies reported previously, that DCs take up immune complexes (Ad coated with Abs) more efficiently than the soluble antigens (Gil-Torregrosa *et al.*, 2004). Additionally, another study reports that in the presence of virus coated with Abs DCs display more mature phenotype, as shown by increased expression of co-stimulatory molecules and robust production of pro-inflammatory cytokines (Perreau *et al.*, 2008). Moreover, results of León *et al.* suggested that in the presence of immune complexes, DC continue to present the antigen to CD8<sup>+</sup> cells even after virus clearance. (León *et al.*, 2014). This prolonged presentation results in delayed contraction of primary CD8<sup>+</sup> and drive the establishment of memory CD8<sup>+</sup>. Additionally, upon the identification of cytosol Fc receptor TRIM21 and its capacity to stimulate the production of pro-inflammatory cytokines, it is tempting to speculate that cellular responses induced in Ad-immune setting should be more vigorous. Our results contradict this theory. Still, the previous analyses were usually investigating the responses induced against the encoded transgene or the Ad itself. To our knowledge, this is the first report of the epitope display strategy with class-I T cell epitopes on Ad capsid and thus our data cannot be directly compared to other studies. Nevertheless, few papers reported the use of VLPs as a platform for T cell epitopes. Influenza class-I epitope was inserted into the external positions of Ad dodecahedron and showed the induction of cellular responses in immunized chickens (Szurgot *et al.*, 2013). Another study, also selecting CD8<sup>+</sup> epitope from influenza, used VLPs of simian virus 40 (SV40) as a platform and observed induction of cellular responses in mice (Kawano *et al.*, 2014). Still, none of these studies tried to assess the influence of anti-vector Abs.

Numerous studies suggested that not only the number but also the phenotype of memory CD8<sup>+</sup> T cells influences their protective capacity (Wherry *et al.*, 2003). Thus, we decided to assess the expression level of various phenotypic markers in order to elucidate the properties of the cellular responses induced by our Ad vectors. More precisely, at both time points, we investigated the levels of CD44, CCR7, CD127, CD62L and KLRG1 molecules on the surface of harvested splenocytes. Analysis performed 11-12 days after the Ad vector immunization disclosed that, although some differences were observed between the experimental groups, SL-specific CD8<sup>+</sup> subpopulation (TetOva<sup>+</sup>CD8<sup>+</sup>) exhibited significant alterations in the marker expression levels in comparison to the remaining pool of CD8<sup>+</sup> cells (TetOva<sup>-</sup>CD8<sup>+</sup>). The levels of CD44 and KLRG1 molecules were significantly increased on the surface of SL-specific CD8<sup>+</sup> cells while reduction of surface expression of CD127, CCR7 and CD62L was observed. At the second time point, all groups continued to exhibit increased expression of CD44

and KLRG1 molecules compared to total CD8<sup>+</sup> T cells. Still, only SL-specific T cells isolated from naïve mice immunized with AdH-SL displayed reduced amounts of CD127 and CD62L molecules, suggesting persistence of effector T cells in this group.

In order to have a clearer picture of phenotypic features of SL-specific CD8<sup>+</sup> cells we decided to quantify the different phenotypes contributing to the total pool of induced memory cells. Memory T cells can be divided in two main lineages, named central (T<sub>CM</sub>) and memory (T<sub>EM</sub>) T cells. Briefly, T<sub>CM</sub> cells are known to reside in secondary lymphoid organs and through their proliferation capacity constitute a source of specific T cells, while T<sub>EM</sub> are found in peripheral tissues, providing quick response during antigen re-encounter. Thus, both subsets are important elements of efficient vaccination outcome. At the early time point (day 11-12), the biggest contribution of SL-specific effector cells to the total population of TetOva<sup>+</sup>CD8<sup>+</sup> was detected in naïve mice infected with AdH-SL. T<sub>EM</sub> lineage was induced more vigorously by AdH-SL than AdF-SL vector, regardless of host status towards Ad. Interestingly, cellular responses induced by AdH-SL displayed reduced pool of T<sub>CM</sub> cells in comparison to fiber display, in both naïve and immune mice. Two weeks later a strong increase in the pool of T<sub>CM</sub> cells was observed, especially in the case of AdH-SL in the naïve background. Interestingly, both naïve and immune groups immunized with AdF-SL vector still displayed significantly increased levels of T<sub>CM</sub>. Effector memory cells, on the other hand, decreased to the equal levels for all four groups. When drawing conclusions from these results, it should be pointed out that the experiment with the kinetic study was not yet repeated. If these results are to be confirmed, the underlying reasons of these differences in memory phenotypes between hexon and fiber display will have to be elucidated.

### **3. Immune responses induced with Ad vectors displaying HCV epitopes**

In the last part of this work we aimed at constructing Ad vectors displaying epitopes from a therapeutic target. Our choice of hepatitis C virus (HCV) as a pathogen was dictated by its severe pathogenicity and lack of available vaccine. Altogether, for both B cell and T cell epitopes, we envisaged 18 constructions but only 6 vectors were successfully produced and infectious. Interestingly, the HCV sequences we were trying to insert into the Ad capsid were comparable in their length to the fragments we previously selected in Ova protein. This suggests that not only the size of the epitope but also the sequence itself influences the insertion permissiveness of Ad capsomers. An interesting question to be addressed would be to try to understand at which steps of the viral cycle Ad production is blocked. We can anticipate some defects in multimerisation of capsomers or in assembly of capsomers. The difficulty in producing Ad bearing HCV epitopes points out a limit of epitope display strategy and underlines also the importance of developing methods in

order to predict whether a peptide inserted into this or that protein will allow efficient rescue of the virus.

We encountered difficulties during vector characterization *in vitro*. Commercially available anti-E2 Abs failed to recognize the HVR and HCR epitopes on the surface of produced vectors. Monoclonal anti-HCR Abs confirmed the presence of this epitope on the surface of AdF-HCR vector. In case of T cell epitopes, due to the lack of suitable T cell hybridoma availability, only one vector (AdH-NS3<sub>1073</sub>) was characterized in an antigen processing assay. Strong production of IL-2 cytokine confirmed efficient antigen presentation on the surface of DCs.

The *in vivo* studies with vectors displaying B cell epitopes, although repeated three times, failed to induce detectable anti-E2 humoral responses. Subsequent analyses of sera collected from mice immunized with AdE1E2 encoding E1 and E2 glycoproteins as a transgene, disclosed that anti-HCR but not anti-HVR Abs were produced upon immunization with full E2 protein. This questions the role of the immunogenicity of HVR epitope in C57BL/6 background. The lack of humoral responses upon mice immunization with the vector displaying HCR sequence remains to be elucidated. The epitope was detected on the capsid surface and we confirmed that this sequence is immunogenic in C57BL/6 mice. Detection of anti-βgal Abs excluded the possibility of vector precipitation. One of the possible explanation could be a change in epitope accessibility upon *in vivo* injection. Another possible solution might be the differences in epitope conformation while embedded in the natural E2 sequence and inserted into the Ad viral particle. For the future studies, it would be interesting to increase the dose of injected virus or add some additional adjuvant in order to stimulate immune responses more efficiently.

Assessment of the induction of cellular responses by our Ad vectors was performed in HHD mice. This strain possess HLA-A2 molecules, capable of presenting selected NS3 epitopes. Two vectors were included in the *in vivo* vaccination studies: AdH-NS3<sub>1073</sub> whose processing was confirmed *in vitro* and AdH-NS3<sub>1406</sub>. Splenocytes of animals immunized with AdH-NS3<sub>1073</sub> produced upon NS3 peptide restimulation quantities of IFN-γ more elevated than in the case of restimulation with Ad peptide. AdH-NS3<sub>1406</sub> vector failed to trigger detectable cellular responses. Still, since epitope presence and its processing was never assessed for this vector, we cannot conclude whether absence of IFN-γ production relies on the lack of vector immunogenicity or is caused by the problems in the epitope insertion/accessibility and its processing by DCs. The evaluation of cellular responses induced by HCV vectors was strongly impaired by the limited availability of HHD mice. Nevertheless, the results we obtained are very encouraging and the vaccination studies with AdH-NS3<sub>1073</sub> vector will be continued in order to better characterize anti-HCV cellular responses.

## 4. Perspectives

### 4.1. Vector re-targeting

Our result shed a new light on the epitope display strategy of vaccination. Conclusions drawn from the first part of this work imply that Ad capsid can be modified in the sites involved in the interaction with the cellular receptors and host factors without losing its capacity to mount efficient humoral responses gives a possibility of re-designing Ad vector biodistribution *in vivo*. In our study we produced vector crippled in both integrin and FX interactions and observed a significant reduction in its *in vivo* infectivity without altering its vaccination efficacy. Thus, a combination of multiple detargeting mutations on one viral particle is a very compelling perspective.

From the vaccination point of view it would be interesting to increase virus uptake by DCs. A few studies already explored this possibility by inserting into the fiber protein RGD motif, capable of binding integrins present on the DC surface (Okada *et al.*, 2001; Krause *et al.*, 2013). Another team relied on CD40 targeting in order to increase Ad uptake by DC and their maturation (Huang *et al.*, 2008). Nevertheless, Ad vector used in these studies were encoding antigen as a transgene. Thus it would be interesting to see how such modifications would impact the immunization outcome with Ad displaying epitope on the capsid. Eventually, with regard to the fact that both integrins and CD40 molecules are present on numerous cell types, targeting a molecule whose expression is more exclusive to DC surface would allow to reduce the vector-related toxicity and dose. Another tempting perspective is to combine the insertion of specific targeting motifs with the various detargeting mutations.

### 4.2. Crippled vectors

Another intriguing aspect is the generation of further crippled vectors. We already showed that interaction with cell receptors and host factors is not indispensable for the efficient vaccination. A very interesting strategy would be the development of empty capsid Ad vectors displaying heterologous epitopes on their surface. This would allow us to address the question of the role of virus DNA sensing. Although this aspect was partially investigated by immunizing MyD88<sup>-/-</sup>, cGAS<sup>-/-</sup> or STING<sup>-/-</sup> mice, to our knowledge no study so far obtained double deficient mice background with ruptures in both TLR9/MyD88 and cGAS/STING signaling pathways, both capable of sensing foreign DNA. AIM2/ASC is another DNA-sensing pathway that needs to be investigated. A few interesting studies were made with Ad dodecahedrons (Szurgot *et al.*, 2013), but due to its less complex structure, this particles could exhibit different adjuvant properties. Indeed, in the Ad particle hexon protein is the major source of CD4<sup>+</sup> epitopes.

#### **4.3. Combining numerous epitopes on the vector surface**

Many studies describe various Ad capsomers permissive for the insertion of heterologous epitopes. Among them the hexon and fiber proteins are the most frequently used, but penton base and pIX have also turned out to be a potential insertion sites. Additionally, in the sequences of these proteins diverse regions capable of accommodating epitope insertion were identified (various HVR in hexon protein, different loops in fiber protein). It is thus tempting to combine various epitopes on one viral particle. This could either consist in the insertion of the same epitope in many different sites or alternatively in the insertion of various B cell or T cell epitopes of the same pathogen. Since, in the case of therapeutic vaccination, it is preferable to induce both humoral and cellular responses considering the results we obtained it would be especially interesting to construct vectors displaying B cell epitope into the fiber protein and T cell epitope into the hexon protein.

#### **4.4. Combining epitope display with transgene**

Combination of B cell epitope display and the same antigen in the form of transgene was already evaluated by others (Worgall *et al.*, 2007; Zhou *et al.*, 2013) and results suggested that although the antibody titers are not significantly increased, the antibody avidity is enhanced. This is related to the presence of additional CD4<sup>+</sup> epitopes. However, nobody addressed the possibility of combining class-I T cell epitope display with the antigen in the form of transgene. Comparing this dual vector with the classic recombinant vector and Ad displaying solely the epitope could shed a new light on the induction of cellular immunity with epitope display approach. Evaluation of the kinetics and phenotype of induced cellular responses would bring valuable informations, allowing to design effective vaccine vectors.



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# *References*

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1. Abe, S., et al., Adenovirus type 5 with modified hexons induces robust transgene-specific immune responses in mice with pre-existing immunity against adenovirus type 5. *J Gene Med*, 2009. 11(7): p. 570-9.
2. Abel, B., et al., The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am J Respir Crit Care Med*, 2010. 181(12): p. 1407-17.
3. Abou El Hassan, M.A., et al., Conditionally replicating adenoviruses kill tumor cells via a basic apoptotic machinery-independent mechanism that resembles necrosis-like programmed cell death. *J Virol*, 2004. 78(22): p. 12243-51.
4. Ago, H., et al., Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Structure*, 1999. 7(11): p. 1417-26.
5. Akira, S., S. Uematsu, and O. Takeuchi, Pathogen recognition and innate immunity. *Cell*, 2006. 124(4): p. 783-801.
6. Akiyama, M., et al., Ablating CAR and integrin binding in adenovirus vectors reduces nontarget organ transduction and permits sustained bloodstream persistence following intraperitoneal administration. *Mol Ther*, 2004. 9(2): p. 218-30.
7. Alba, R., A. Bosch, and M. Chillon, Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther*, 2005. 12 Suppl 1: p. S18-27.
8. Alba, R., et al., Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood*, 2010. 116(15): p. 2656-64.
9. Alba, R., et al., Identification of coagulation factor (F)X binding sites on the adenovirus serotype 5 hexon: effect of mutagenesis on FX interactions and gene transfer. *Blood*, 2009. 114(5): p. 965-71.
10. Alemany, R. and D.T. Curiel, CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther*, 2001. 8(17): p. 1347-53.
11. Allen, I.C., et al., The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity*, 2009. 30(4): p. 556-65.
12. Amalfitano, A., et al., Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. *J Virol*, 1998. 72(2): p. 926-33.
13. Ambrosch, F., et al., Immunogenicity and protectivity of a new liposomal hepatitis A vaccine. *Vaccine*, 1997. 15(11): p. 1209-13.
14. Andre, P., et al., Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol*, 2002. 76(14): p. 6919-28.
15. Andre, P., et al., Hepatitis C virus particles and lipoprotein metabolism. *Semin Liver Dis*, 2005. 25(1): p. 93-104.
16. Appaiahgari, M.B. and S. Vрати, Adenoviruses as gene/vaccine delivery vectors: promises and pitfalls. *Expert Opin Biol Ther*, 2015. 15(3): p. 337-51.
17. Appay, V., et al., Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med*, 2002. 8(4): p. 379-85.
18. Appledorn, D.M., et al., Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 in vivo. *J Immunol*, 2008. 181(3): p. 2134-44.
19. Auffermann-Gretzinger, S., E.B. Keeffe, and S. Levy, Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood*, 2001. 97(10): p. 3171-6.
20. Averill, L., W.M. Lee, and N.J. Karandikar, Differential dysfunction in dendritic cell subsets during chronic HCV infection. *Clin Immunol*, 2007. 123(1): p. 40-9.

21. Babitt, J., et al., Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. *J Biol Chem*, 1997. 272(20): p. 13242-9.
22. Baden, L.R., et al., First-in-human evaluation of the safety and immunogenicity of a recombinant adenovirus serotype 26 HIV-1 Env vaccine (IPCAVD 001). *J Infect Dis*, 2013. 207(2): p. 240-7.
23. Bagai, R., et al., Mouse endothelial cells cross-present lymphocyte-derived antigen on class I MHC via a TAP1- and proteasome-dependent pathway. *J Immunol*, 2005. 174(12): p. 7711-5.
24. Bakr, I., et al., Higher clearance of hepatitis C virus infection in females compared with males. *Gut*, 2006. 55(8): p. 1183-7.
25. Balandya, E., et al., Adenovirus serotype 26 and 35 vectors induce simian immunodeficiency virus-specific T lymphocyte responses in foreskin in rhesus monkeys. *J Virol*, 2014. 88(7): p. 3756-65.
26. Bangari, D.S. and S.K. Mittal, Development of nonhuman adenoviruses as vaccine vectors. *Vaccine*, 2006. 24(7): p. 849-62.
27. Barba, G., et al., Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A*, 1997. 94(4): p. 1200-5.
28. Barlan, A.U., et al., Adenovirus membrane penetration activates the NLRP3 inflammasome. *J Virol*, 2011. 85(1): p. 146-55.
29. Barnes, E., et al., Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. *Sci Transl Med*, 2012. 4(115): p. 115ra1.
30. Bartenschlager, R., M. Frese, and T. Pietschmann, Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res*, 2004. 63: p. 71-180.
31. Bartosch, B., et al., Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem*, 2003. 278(43): p. 41624-30.
32. Batista, F.D. and N.E. Harwood, The who, how and where of antigen presentation to B cells. *Nat Rev Immunol*, 2009. 9(1): p. 15-27.
33. Bergelson, J.M., et al., Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, 1997. 275(5304): p. 1320-3.
34. Bergman, P.J., et al., Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine*, 2006. 24(21): p. 4582-5.
35. Berk, A.J., Functions of adenovirus E1A. *Cancer Surv*, 1986. 5(2): p. 367-87.
36. Berk, A.J., Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene*, 2005. 24(52): p. 7673-85.
37. Bernhardt, S.L., et al., Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: A dose escalating phase I/II study. *Br J Cancer*, 2006. 95(11): p. 1474-82.
38. Bett, A.J., L. Prevec, and F.L. Graham, Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol*, 1993. 67(10): p. 5911-21.
39. Bevan, M.J., Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med*, 1976. 143(5): p. 1283-8.
40. Bhoj, V.G., et al., MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. *Proc Natl Acad Sci U S A*, 2008. 105(37): p. 14046-51.
41. Blanchard, E., et al., Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol*, 2006. 80(14): p. 6964-72.

42. Blum, J.S., P.A. Wearsch, and P. Cresswell, Pathways of antigen processing. *Annu Rev Immunol*, 2013. 31: p. 443-73.
43. Boulant, S., P. Targett-Adams, and J. McLauchlan, Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. *J Gen Virol*, 2007. 88(Pt 8): p. 2204-13.
44. Bowie, A. and L.A. O'Neill, The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J Leukoc Biol*, 2000. 67(4): p. 508-14.
45. Bradley, R.R., et al., Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. *J Virol*, 2012. 86(1): p. 625-9.
46. Bradshaw, A.C., et al., Biodistribution and inflammatory profiles of novel penton and hexon double-mutant serotype 5 adenoviruses. *J Control Release*, 2012. 164(3): p. 394-402.
47. Bradshaw, A.C., et al., Requirements for receptor engagement during infection by adenovirus complexed with blood coagulation factor X. *PLoS Pathog*, 2010. 6(10): p. e1001142.
48. Bremner, K.H., et al., Adenovirus transport via direct interaction of cytoplasmic dynein with the viral capsid hexon subunit. *Cell Host Microbe*, 2009. 6(6): p. 523-35.
49. Brenndorfer, E.D. and M. Sallberg, Hepatitis C virus-mediated modulation of cellular immunity. *Arch Immunol Ther Exp (Warsz)*, 2012. 60(5): p. 315-29.
50. Brichacek, B., et al., Increased plasma human immunodeficiency virus type 1 burden following antigenic challenge with pneumococcal vaccine. *J Infect Dis*, 1996. 174(6): p. 1191-9.
51. Brimacombe, C.L., et al., Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. *J Virol*, 2011. 85(1): p. 596-605.
52. Browne, A., et al., Pharmacological Inhibition of beta3 Integrin Reduces the Inflammatory Toxicities Caused by Oncolytic Adenovirus without Compromising Anticancer Activity. *Cancer Res*, 2015. 75(14): p. 2811-21.
53. Brunsvig, P.F., et al., Telomerase peptide vaccination in NSCLC: a phase II trial in stage III patients vaccinated after chemoradiotherapy and an 8-year update on a phase I/II trial. *Clin Cancer Res*, 2011. 17(21): p. 6847-57.
54. Buchbinder, S.P., et al., Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet*, 2008. 372(9653): p. 1881-93.
55. Calcedo, R., et al., Host immune responses to chronic adenovirus infections in human and nonhuman primates. *J Virol*, 2009. 83(6): p. 2623-31.
56. Casimiro, D.R., et al., Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol*, 2003. 77(11): p. 6305-13.
57. Castellanos, M., et al., Immunization with a DNA vaccine candidate in chronic hepatitis C patients is safe, well tolerated and does not impair immune response induction after anti-hepatitis B vaccination. *J Gene Med*, 2010. 12(1): p. 107-16.
58. Cerny, A., et al., Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. *J Clin Invest*, 1995. 95(2): p. 521-30.
59. Chakraborty, A.A. and W.P. Tansey, Adenoviral E1A function through Myc. *Cancer Res*, 2009. 69(1): p. 6-9.
60. Chang, C.H., et al., Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. *Immunity*, 1996. 4(2): p. 167-78.

61. Chang, K.M., et al., Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest*, 1997. 100(9): p. 2376-85.
62. Chaplin, D.D., Overview of the immune response. *J Allergy Clin Immunol*, 2010. 125(2 Suppl 2): p. S3-23.
63. Chen, J., N. Morral, and D.A. Engel, Transcription releases protein VII from adenovirus chromatin. *Virology*, 2007. 369(2): p. 411-22.
64. Chen, P.H., D.A. Ornelles, and T. Shenk, The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J Virol*, 1993. 67(6): p. 3507-14.
65. Chirmule, N., et al., Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther*, 1999. 6(9): p. 1574-83.
66. Chmielewska, A.M., et al., Combined adenovirus vector and hepatitis C virus envelope protein prime-boost regimen elicits T cell and neutralizing antibody immune responses. *J Virol*, 2014. 88(10): p. 5502-10.
67. Chung, N.S. and K.M. Wasan, Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. *Adv Drug Deliv Rev*, 2004. 56(9): p. 1315-34.
68. Collier, K.E., et al., RNA interference and single particle tracking analysis of hepatitis C virus endocytosis. *PLoS Pathog*, 2009. 5(12): p. e1000702.
69. Collier, K.E., et al., Molecular determinants and dynamics of hepatitis C virus secretion. *PLoS Pathog*, 2012. 8(1): p. e1002466.
70. Contreras, A.M., et al., Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. *J Virol*, 2002. 76(17): p. 8505-17.
71. Cox, A.L., et al., Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology*, 2005. 42(1): p. 104-12.
72. Crawford-Miksza, L. and D.P. Schnurr, Analysis of 15 adenovirus hexon proteins reveals the location and structure of seven hypervariable regions containing serotype-specific residues. *J Virol*, 1996. 70(3): p. 1836-44.
73. Croyle, M.A., et al., Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice. *PLoS One*, 2008. 3(10): p. e3548.
74. Danthinne, X. and M.J. Imperiale, Production of first generation adenovirus vectors: a review. *Gene Ther*, 2000. 7(20): p. 1707-14.
75. Davis, B.K., H. Wen, and J.P. Ting, The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*, 2011. 29: p. 707-35.
76. Davis, B.S., et al., West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. *J Virol*, 2001. 75(9): p. 4040-7.
77. Davison, A.J., M. Benko, and B. Harrach, Genetic content and evolution of adenoviruses. *J Gen Virol*, 2003. 84(Pt 11): p. 2895-908.
78. De Maria, A., et al., Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol*, 2007. 37(2): p. 445-55.
79. De Nardo, D., Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*, 2015. 74(2): p. 181-9.

80. Dehecchi, M.C., et al., Heparan sulfate glycosaminoglycans are involved in adenovirus type 5 and 2-host cell interactions. *Virology*, 2000. 268(2): p. 382-90.
81. Deddouche, S., et al., Identification of an LGP2-associated MDA5 agonist in picornavirus-infected cells. *Elife*, 2014. 3: p. e01535.
82. Deleersnyder, V., et al., Formation of native hepatitis C virus glycoprotein complexes. *J Virol*, 1997. 71(1): p. 697-704.
83. Denby, L., et al., Adenoviral serotype 5 vectors pseudotyped with fibers from subgroup D show modified tropism in vitro and in vivo. *Hum Gene Ther*, 2004. 15(11): p. 1054-64.
84. Deng, L., et al., Activation of the I $\kappa$ B kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell*, 2000. 103(2): p. 351-61.
85. Di Bisceglie, A.M. and J.H. Hoofnagle, Optimal therapy of hepatitis C. *Hepatology*, 2002. 36(5 Suppl 1): p. S121-7.
86. Di Paolo, N.C., O. Kalyuzhniy, and D.M. Shayakhmetov, Fiber shaft-chimeric adenovirus vectors lacking the KKTK motif efficiently infect liver cells in vivo. *J Virol*, 2007. 81(22): p. 12249-59.
87. Di Paolo, N.C., et al., Virus binding to a plasma membrane receptor triggers interleukin-1 alpha-mediated proinflammatory macrophage response in vivo. *Immunity*, 2009. 31(1): p. 110-21.
88. Dixit, E., et al., Peroxisomes are signaling platforms for antiviral innate immunity. *Cell*, 2010. 141(4): p. 668-81.
89. Dolganiuc, A., et al., Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology*, 2004. 127(5): p. 1513-24.
90. Dolganiuc, A., et al., Myeloid dendritic cells of patients with chronic HCV infection induce proliferation of regulatory T lymphocytes. *Gastroenterology*, 2008. 135(6): p. 2119-27.
91. Donati, C. and R. Rappuoli, Reverse vaccinology in the 21st century: improvements over the original design. *Ann N Y Acad Sci*, 2013. 1285: p. 115-32.
92. Donnelly, J.J., B. Wahren, and M.A. Liu, DNA vaccines: progress and challenges. *J Immunol*, 2005. 175(2): p. 633-9.
93. Doronin, K., et al., Coagulation factor X activates innate immunity to human species C adenovirus. *Science*, 2012. 338(6108): p. 795-8.
94. Doumba, P.P., et al., Phenotypic and functional alterations of primary human PBMCs induced by HCV non-enveloped capsid-like particles uptake. *Cell Mol Life Sci*, 2013. 70(18): p. 3463-74.
95. Drane, D., et al., Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers. *Hum Vaccin*, 2009. 5(3): p. 151-7.
96. Dubuisson, J. and F.L. Cosset, Virology and cell biology of the hepatitis C virus life cycle: an update. *J Hepatol*, 2014. 61(1 Suppl): p. S3-S13.
97. Dubuisson, J., et al., Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol*, 1994. 68(10): p. 6147-60.
98. Edwards, M.S., Group B streptococcal conjugate vaccine: a timely concept for which the time has come. *Hum Vaccin*, 2008. 4(6): p. 444-8.
99. Egger, D., et al., Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol*, 2002. 76(12): p. 5974-84.
100. Eichholz, K., F.J. Mennechet, and E.J. Kremer, Human coagulation factor X-adenovirus type 5 complexes poorly stimulate an innate immune response in human mononuclear phagocytes. *J Virol*, 2015. 89(5): p. 2884-91.

101. Einfeld, D.A., et al., Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J Virol*, 2001. 75(23): p. 11284-91.
102. Elazar, M., et al., Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J Virol*, 2003. 77(10): p. 6055-61.
103. Epstein, J.E., et al., Malaria vaccines: are we getting closer? *Curr Opin Mol Ther*, 2007. 9(1): p. 12-24.
104. Evans, M.J., et al., Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*, 2007. 446(7137): p. 801-5.
105. Fabregat, I., Dysregulation of apoptosis in hepatocellular carcinoma cells. *World J Gastroenterol*, 2009. 15(5): p. 513-20.
106. Farci, P., et al., A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N Engl J Med*, 1991. 325(2): p. 98-104.
107. Farci, P., et al., Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A*, 1996. 93(26): p. 15394-9.
108. Fehr, T., et al., T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles. *Proc Natl Acad Sci U S A*, 1998. 95(16): p. 9477-81.
109. Fender, P., et al., Adenovirus dodecahedron, a new vector for human gene transfer. *Nat Biotechnol*, 1997. 15(1): p. 52-6.
110. Fessler, S.P. and C.S. Young, The role of the L4 33K gene in adenovirus infection. *Virology*, 1999. 263(2): p. 507-16.
111. Firbas, C., et al., Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: a randomized, placebo controlled trial for dose optimization in 128 healthy subjects. *Vaccine*, 2006. 24(20): p. 4343-53.
112. Fitzgerald, J.C., et al., A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J Immunol*, 2003. 170(3): p. 1416-22.
113. Fitzgerald, K.A., et al., IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol*, 2003. 4(5): p. 491-6.
114. Fitzgerald, K.A., et al., Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature*, 2001. 413(6851): p. 78-83.
115. Fitzgerald, K.A., et al., LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med*, 2003. 198(7): p. 1043-55.
116. Fitzwater, S.P., et al., The worldwide impact of the seven-valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J*, 2012. 31(5): p. 501-8.
117. Flingai, S., et al., Synthetic DNA vaccines: improved vaccine potency by electroporation and co-delivered genetic adjuvants. *Front Immunol*, 2013. 4: p. 354.
118. Folgori, A., et al., A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nat Med*, 2006. 12(2): p. 190-7.
119. Fontalba, A., O. Gutierrez, and J.L. Fernandez-Luna, NLRP2, an inhibitor of the NF-kappaB pathway, is transcriptionally activated by NF-kappaB and exhibits a nonfunctional allelic variant. *J Immunol*, 2007. 179(12): p. 8519-24.
120. Fournillier, A., et al., An accelerated vaccine schedule with a poly-antigenic hepatitis C virus MVA-based candidate vaccine induces potent, long lasting and in vivo cross-reactive T cell responses. *Vaccine*, 2007. 25(42): p. 7339-53.

121. Franck, N., et al., Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. *J Virol*, 2005. 79(5): p. 2700-8.
122. Freimuth, P., et al., Coxsackievirus and adenovirus receptor amino-terminal immunoglobulin V-related domain binds adenovirus type 2 and fiber knob from adenovirus type 12. *J Virol*, 1999. 73(2): p. 1392-8.
123. Frey, S.E., et al., Safety and immunogenicity of HCV E1E2 vaccine adjuvanted with MF59 administered to healthy adults. *Vaccine*, 2010. 28(38): p. 6367-73.
124. Gahery-Segard, H., et al., Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. *J Virol*, 1998. 72(3): p. 2388-97.
125. Gall, J., et al., Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J Virol*, 1996. 70(4): p. 2116-23.
126. Garver, K.A., S.E. LaPatra, and G. Kurath, Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook *Oncorhynchus tshawytscha* and sockeye *O. nerka* salmon. *Dis Aquat Organ*, 2005. 64(1): p. 13-22.
127. Ge, D., et al., Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*, 2009. 461(7262): p. 399-401.
128. Geisbert, T.W., et al., Recombinant adenovirus serotype 26 (Ad26) and Ad35 vaccine vectors bypass immunity to Ad5 and protect nonhuman primates against ebolavirus challenge. *J Virol*, 2011. 85(9): p. 4222-33.
129. Gerlach, J.T., et al., Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology*, 1999. 117(4): p. 933-41.
130. Ghasemi, F., S. Rostami, and Z. Meshkat, Progress in the development of vaccines for hepatitis C virus infection. *World J Gastroenterol*, 2015. 21(42): p. 11984-2002.
131. Ghebremedhin, B., Human adenovirus: Viral pathogen with increasing importance. *Eur J Microbiol Immunol (Bp)*, 2014. 4(1): p. 26-33.
132. Gil-Torregrosa, B.C., et al., Control of cross-presentation during dendritic cell maturation. *Eur J Immunol*, 2004. 34(2): p. 398-407.
133. Ginsberg, H.S., et al., A proposed terminology for the adenovirus antigens and virion morphological subunits. *Virology*, 1966. 28(4): p. 782-3.
134. Girardin, S.E., et al., Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science*, 2003. 300(5625): p. 1584-7.
135. Goffard, A., et al., Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. *J Virol*, 2005. 79(13): p. 8400-9.
136. Goubau, D., et al., Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature*, 2014. 514(7522): p. 372-5.
137. Grakoui, A., et al., HCV persistence and immune evasion in the absence of memory T cell help. *Science*, 2003. 302(5645): p. 659-62.
138. Grand, R.J., et al., Adenovirus early region 1A protein binds to mammalian SUG1-a regulatory component of the proteasome. *Oncogene*, 1999. 18(2): p. 449-58.
139. Green, N.M., et al., Evidence for a repeating cross-beta sheet structure in the adenovirus fibre. *EMBO J*, 1983. 2(8): p. 1357-65.
140. Gruener, N.H., et al., Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol*, 2001. 75(12): p. 5550-8.

141. Guay, H.M., et al., MyD88 is required for the formation of long-term humoral immunity to virus infection. *J Immunol*, 2007. 178(8): p. 5124-31.
142. Guermonprez, P., et al., ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature*, 2003. 425(6956): p. 397-402.
143. Gutcher, I. and B. Becher, APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest*, 2007. 117(5): p. 1119-27.
144. Habersetzer, F., T.F. Baumert, and F. Stoll-Keller, GI-5005, a yeast vector vaccine expressing an NS3-core fusion protein for chronic HCV infection. *Curr Opin Mol Ther*, 2009. 11(4): p. 456-62.
145. Hager-Braun, C. and K.B. Tomer, Determination of protein-derived epitopes by mass spectrometry. *Expert Rev Proteomics*, 2005. 2(5): p. 745-56.
146. Hall, K., M.E. Blair Zajdel, and G.E. Blair, Unity and diversity in the human adenoviruses: exploiting alternative entry pathways for gene therapy. *Biochem J*, 2010. 431(3): p. 321-36.
147. Hammer, S.M., et al., Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. *N Engl J Med*, 2013. 369(22): p. 2083-92.
148. Hanlon, C.A., et al., First North American field release of a vaccinia-rabies glycoprotein recombinant virus. *J Wildl Dis*, 1998. 34(2): p. 228-39.
149. Harman, C., et al., A view of the E2-CD81 interface at the binding site of a neutralizing antibody against hepatitis C virus. *J Virol*, 2015. 89(1): p. 492-501.
150. Haruki, H., et al., Ternary complex formation between DNA-adenovirus core protein VII and TAF-Ibeta/SET, an acidic molecular chaperone. *FEBS Lett*, 2003. 555(3): p. 521-7.
151. Hasson, T.B., D.A. Ornelles, and T. Shenk, Adenovirus L1 52- and 55-kilodalton proteins are present within assembling virions and colocalize with nuclear structures distinct from replication centers. *J Virol*, 1992. 66(10): p. 6133-42.
152. Haut, L.H. and H.C. Ertl, Obstacles to the successful development of an efficacious T cell-inducing HIV-1 vaccine. *J Leukoc Biol*, 2009. 86(4): p. 779-93.
153. He, L.F., et al., Determining the size of non-A, non-B hepatitis virus by filtration. *J Infect Dis*, 1987. 156(4): p. 636-40.
154. He, Q.Q., et al., Hepatocyte transformation and tumor development induced by hepatitis C virus NS3 c-terminal deleted protein. *World J Gastroenterol*, 2003. 9(3): p. 474-8.
155. He, X.S., et al., Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci U S A*, 1999. 96(10): p. 5692-7.
156. Heer, A.K., et al., TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J Immunol*, 2007. 178(4): p. 2182-91.
157. Helle, F., G. Duverlie, and J. Dubuisson, The hepatitis C virus glycan shield and evasion of the humoral immune response. *Viruses*, 2011. 3(10): p. 1909-32.
158. Hendrickx, R., et al., Innate immunity to adenovirus. *Hum Gene Ther*, 2014. 25(4): p. 265-84.
159. Hezode, C. and J.P. Bronowicki, Ideal oral combinations to eradicate HCV: The role of ribavirin. *J Hepatol*, 2016. 64(1): p. 215-25.
160. Hezode, C., et al., Effectiveness of telaprevir or boceprevir in treatment-experienced patients with HCV genotype 1 infection and cirrhosis. *Gastroenterology*, 2014. 147(1): p. 132-142 e4.
161. Hijikata, M., et al., Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J Virol*, 1993. 67(4): p. 1953-8.
162. Hilleman, M.R., Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. *J Hum Virol*, 2000. 3(2): p. 63-76.

163. Hilleman, M.R., et al., Live, attenuated mumps-virus vaccine. *N Engl J Med*, 1968. 278(5): p. 227-32.
164. Hilleman, M.R., et al., Quality and safety of human hepatitis B vaccine. *Dev Biol Stand*, 1983. 54: p. 3-12.
165. Hiltbold, E.M., N.J. Poloso, and P.A. Roche, MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. *J Immunol*, 2003. 170(3): p. 1329-38.
166. Hofherr, S.E., et al., Modification of adenoviral vectors with polyethylene glycol modulates in vivo tissue tropism and gene expression. *Mol Ther*, 2008. 16(7): p. 1276-82.
167. Holterman, L., et al., Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. *J Virol*, 2004. 78(23): p. 13207-15.
168. Hong, S.S., et al., Identification of adenovirus (ad) penton base neutralizing epitopes by use of sera from patients who had received conditionally replicative ad (add1520) for treatment of liver tumors. *J Virol*, 2003. 77(19): p. 10366-75.
169. Hong, S.S., et al., The 100K-chaperone protein from adenovirus serotype 2 (Subgroup C) assists in trimerization and nuclear localization of hexons from subgroups C and B adenoviruses. *J Mol Biol*, 2005. 352(1): p. 125-38.
170. Horner, S.M., et al., Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci U S A*, 2011. 108(35): p. 14590-5.
171. Horng, T., et al., The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature*, 2002. 420(6913): p. 329-33.
172. Horng, T., G.M. Barton, and R. Medzhitov, TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol*, 2001. 2(9): p. 835-41.
173. Hou, F., et al., MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell*, 2011. 146(3): p. 448-61.
174. Houde, M., et al., Phagosomes are competent organelles for antigen cross-presentation. *Nature*, 2003. 425(6956): p. 402-6.
175. Huang, D., et al., Significant alterations of biodistribution and immune responses in Balb/c mice administered with adenovirus targeted to CD40(+) cells. *Gene Ther*, 2008. 15(4): p. 298-308.
176. Huang, H., et al., Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc Natl Acad Sci U S A*, 2007. 104(14): p. 5848-53.
177. Huang, X., et al., Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. *Mol Cell Biol*, 2000. 20(3): p. 755-9.
178. Huppa, J.B. and M.M. Davis, T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol*, 2003. 3(12): p. 973-83.
179. Hurez, V., et al., Efficient adenovirus-mediated gene transfer into primary T cells and thymocytes in a new coxsackie/adenovirus receptor transgenic model. *BMC Immunol*, 2002. 3: p. 4.
180. Hutnick, N.A., et al., Adenovirus-specific human T cells are pervasive, polyfunctional, and cross-reactive. *Vaccine*, 2010. 28(8): p. 1932-41.
181. Iwasaki, A. and R. Medzhitov, Control of adaptive immunity by the innate immune system. *Nat Immunol*, 2015. 16(4): p. 343-53.
182. Janeway, C.A., Jr. and R. Medzhitov, Innate immune recognition. *Annu Rev Immunol*, 2002. 20: p. 197-216.

183. Jernigan, J.A., et al., Adenovirus type 8 epidemic keratoconjunctivitis in an eye clinic: risk factors and control. *J Infect Dis*, 1993. 167(6): p. 1307-13.
184. Ji, H., et al., Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. *Proc Natl Acad Sci U S A*, 2004. 101(49): p. 16990-5.
185. Jiang, H., et al., Adenovirus's last trick: you say lysis, we say autophagy. *Autophagy*, 2008. 4(1): p. 118-20.
186. Jinushi, M., et al., Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J Immunol*, 2004. 173(10): p. 6072-81.
187. Joffre, O.P., et al., Cross-presentation by dendritic cells. *Nat Rev Immunol*, 2012. 12(8): p. 557-69.
188. Joshi, M.D., et al., Targeting tumor antigens to dendritic cells using particulate carriers. *J Control Release*, 2012. 161(1): p. 25-37.
189. Jung, S., et al., In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*, 2002. 17(2): p. 211-20.
190. Kagan, J.C., V.G. Magupalli, and H. Wu, SMOCs: supramolecular organizing centres that control innate immunity. *Nat Rev Immunol*, 2014. 14(12): p. 821-6.
191. Kalams, S.A., et al., Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults. *PLoS One*, 2012. 7(1): p. e29231.
192. Kanerva, A., et al., Gene transfer to ovarian cancer versus normal tissues with fiber-modified adenoviruses. *Mol Ther*, 2002. 5(6): p. 695-704.
193. Kang, S.M., et al., Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-alpha-mediated apoptosis. *Cancer Lett*, 2009. 279(2): p. 230-7.
194. Kanto, T., et al., Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. *Hepatology*, 1994. 19(2): p. 296-302.
195. Kaplan, J.M., et al., Novel role for E4 region genes in protection of adenovirus vectors from lysis by cytotoxic T lymphocytes. *J Virol*, 1999. 73(5): p. 4489-92.
196. Kaplan, J.M., et al., Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J Immunol*, 1999. 163(2): p. 699-707.
197. Karen, K.A. and P. Hearing, Adenovirus core protein VII protects the viral genome from a DNA damage response at early times after infection. *J Virol*, 2011. 85(9): p. 4135-42.
198. Kato, N., et al., Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J Virol*, 1993. 67(7): p. 3923-30.
199. Kawano, M., et al., Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. *Virology*, 2014. 448: p. 159-67.
200. Kawase, H., et al., NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. *Genes Cells*, 1996. 1(12): p. 1045-56.
201. Keck, Z.Y., et al., Definition of a conserved immunodominant domain on hepatitis C virus E2 glycoprotein by neutralizing human monoclonal antibodies. *J Virol*, 2008. 82(12): p. 6061-6.

202. Keefer, M.C., et al., A phase I double blind, placebo-controlled, randomized study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults. *PLoS One*, 2012. 7(8): p. e41936.
203. Khare, R., et al., Identification of adenovirus serotype 5 hexon regions that interact with scavenger receptors. *J Virol*, 2012. 86(4): p. 2293-301.
204. Kim, J., et al., Enhancing the therapeutic efficacy of adenovirus in combination with biomaterials. *Biomaterials*, 2012. 33(6): p. 1838-50.
205. Kim, J.J., et al., Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J Interferon Cytokine Res*, 1999. 19(1): p. 77-84.
206. Kim, Y.K., J.S. Shin, and M.H. Nahm, NOD-Like Receptors in Infection, Immunity, and Diseases. *Yonsei Med J*, 2016. 57(1): p. 5-14.
207. Kirby, I., et al., Identification of contact residues and definition of the CAR-binding site of adenovirus type 5 fiber protein. *J Virol*, 2000. 74(6): p. 2804-13.
208. Klein, J. and A. Sato, The HLA system. First of two parts. *N Engl J Med*, 2000. 343(10): p. 702-9.
209. Klein, J. and A. Sato, The HLA system. Second of two parts. *N Engl J Med*, 2000. 343(11): p. 782-6.
210. Kohyama, S., et al., Efficient induction of cytotoxic T lymphocytes specific for severe acute respiratory syndrome (SARS)-associated coronavirus by immunization with surface-linked liposomal peptides derived from a non-structural polyprotein 1a. *Antiviral Res*, 2009. 84(2): p. 168-77.
211. Koizumi, N., et al., Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alphav integrin, and heparan sulfate binding reduce in vivo tissue transduction and toxicity. *Hum Gene Ther*, 2006. 17(3): p. 264-79.
212. Koizumi, N., et al., Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J Virol*, 2003. 77(24): p. 13062-72.
213. Koizumi, N., et al., Fiber-modified adenovirus vectors decrease liver toxicity through reduced IL-6 production. *J Immunol*, 2007. 178(3): p. 1767-73.
214. Kolesanova, E.F., M.A. Sanzhakov, and O.N. Kharybin, Development of the schedule for multiple parallel "difficult" Peptide synthesis on pins. *Int J Pept*, 2013. 2013: p. 197317.
215. Komatsu, T., H. Haruki, and K. Nagata, Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. *Nucleic Acids Res*, 2011. 39(3): p. 889-901.
216. Kosuga, M., et al., Adenovirus-mediated gene therapy for mucopolysaccharidosis VII: involvement of cross-correction in wide-spread distribution of the gene products and long-term effects of CTLA-4Ig coexpression. *Mol Ther*, 2000. 1(5 Pt 1): p. 406-13.
217. Kowalinski, E., et al., Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell*, 2011. 147(2): p. 423-35.
218. Krause, A., et al., Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity. *J Virol*, 2006. 80(11): p. 5523-30.
219. Krause, A., et al., RGD capsid modification enhances mucosal protective immunity of a non-human primate adenovirus vector expressing *Pseudomonas aeruginosa* OprF. *Clin Exp Immunol*, 2013. 173(2): p. 230-41.
220. Kreppel, F. and S. Kochanek, Modification of adenovirus gene transfer vectors with synthetic polymers: a scientific review and technical guide. *Mol Ther*, 2008. 16(1): p. 16-29.

221. Kurokohchi, K., et al., Use of recombinant protein to identify a motif-negative human cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. *J Virol*, 1996. 70(1): p. 232-40.
222. Lam, B.P., et al., The changing landscape of hepatitis C virus therapy: focus on interferon-free treatment. *Therap Adv Gastroenterol*, 2015. 8(5): p. 298-312.
223. Lam, E., S. Stein, and E. Falck-Pedersen, Adenovirus detection by the cGAS/STING/TBK1 DNA sensing cascade. *J Virol*, 2014. 88(2): p. 974-81.
224. Lambrecht, B.N., et al., Mechanism of action of clinically approved adjuvants. *Curr Opin Immunol*, 2009. 21(1): p. 23-9.
225. Lan, K.H., et al., HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene*, 2002. 21(31): p. 4801-11.
226. Langley, W.A., et al., Induction of neutralizing antibody responses to anthrax protective antigen by using influenza virus vectors: implications for disparate immune system priming pathways. *J Virol*, 2010. 84(16): p. 8300-7.
227. Lanzi, A., et al., Anti-adenovirus humoral responses influence on the efficacy of vaccines based on epitope display on adenovirus capsid. *Vaccine*, 2011. 29(7): p. 1463-71.
228. Law, J.L., et al., A hepatitis C virus (HCV) vaccine comprising envelope glycoproteins gpE1/gpE2 derived from a single isolate elicits broad cross-genotype neutralizing antibodies in humans. *PLoS One*, 2013. 8(3): p. e59776.
229. Lechner, F., et al., Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med*, 2000. 191(9): p. 1499-512.
230. Leen, A.M., et al., Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific CD8+ T cells. *Blood*, 2004. 104(8): p. 2432-40.
231. Lemckert, A.A., et al., Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol*, 2005. 79(15): p. 9694-701.
232. Leon, B., et al., Prolonged antigen presentation by immune complex-binding dendritic cells programs the proliferative capacity of memory CD8 T cells. *J Exp Med*, 2014. 211(8): p. 1637-55.
233. Leroux-Roels, G., Unmet needs in modern vaccinology: adjuvants to improve the immune response. *Vaccine*, 2010. 28 Suppl 3: p. C25-36.
234. Leroux-Roels, G., et al., A candidate vaccine based on the hepatitis C E1 protein: tolerability and immunogenicity in healthy volunteers. *Vaccine*, 2004. 22(23-24): p. 3080-6.
235. Leroux-Roels, G., et al., Vaccine Adjuvant Systems containing monophosphoryl lipid A and QS-21 induce strong humoral and cellular immune responses against hepatitis B surface antigen which persist for at least 4 years after vaccination. *Vaccine*, 2015. 33(8): p. 1084-91.
236. Li, H., et al., Hepatitis C virus NS5A hijacks ARFGAP1 to maintain a phosphatidylinositol 4-phosphate-enriched microenvironment. *J Virol*, 2014. 88(11): p. 5956-66.
237. Li, S., et al., IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A*, 2002. 99(8): p. 5567-72.
238. Li, W., et al., Peptide Vaccine: Progress and Challenges. *Vaccines (Basel)*, 2014. 2(3): p. 515-36.
239. Li, X.D., et al., Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science*, 2013. 341(6152): p. 1390-4.
240. Liu, H., et al., Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science*, 2010. 329(5995): p. 1038-43.

241. Liu, H., L. Wu, and Z.H. Zhou, Model of the trimeric fiber and its interactions with the pentameric penton base of human adenovirus by cryo-electron microscopy. *J Mol Biol*, 2011. 406(5): p. 764-74.
242. Liu, M.A., Immunologic basis of vaccine vectors. *Immunity*, 2010. 33(4): p. 504-15.
243. Liu, Y., et al., Evolution of human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitopes: fitness-balanced escape. *J Virol*, 2007. 81(22): p. 12179-88.
244. Lochmuller, H., et al., Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum Gene Ther*, 1994. 5(12): p. 1485-91.
245. Loomba, R., et al., The natural history of acute hepatitis C: clinical presentation, laboratory findings and treatment outcomes. *Aliment Pharmacol Ther*, 2011. 33(5): p. 559-65.
246. Lusky, M., et al., In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J Virol*, 1998. 72(3): p. 2022-32.
247. Ma, Y., et al., Hepatitis C virus NS2 protein serves as a scaffold for virus assembly by interacting with both structural and nonstructural proteins. *J Virol*, 2011. 85(1): p. 86-97.
248. Maag, D., et al., Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J Biol Chem*, 2001. 276(49): p. 46094-8.
249. Maglione, P.J., et al., IRAK-4 and MyD88 deficiencies impair IgM responses against T-independent bacterial antigens. *Blood*, 2014. 124(24): p. 3561-71.
250. Magombedze, G., et al., Cellular and population plasticity of helper CD4(+) T cell responses. *Front Physiol*, 2013. 4: p. 206.
251. Maizel, J.V., Jr., D.O. White, and M.D. Scharff, The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology*, 1968. 36(1): p. 115-25.
252. Mangel, W.F. and C. San Martin, Structure, function and dynamics in adenovirus maturation. *Viruses*, 2014. 6(11): p. 4536-70.
253. Manns, M.P., et al., Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet*, 2001. 358(9286): p. 958-65.
254. Manzano-Szalai, K., et al., Adeno-associated virus-like particles as new carriers for B-cell vaccines: testing immunogenicity and safety in BALB/c mice. *Viral Immunol*, 2014. 27(9): p. 438-48.
255. Martin, K., et al., Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism. *Mol Ther*, 2003. 8(3): p. 485-94.
256. McCaffrey, K., et al., The variable regions of hepatitis C virus glycoprotein E2 have an essential structural role in glycoprotein assembly and virion infectivity. *J Gen Virol*, 2011. 92(Pt 1): p. 112-21.
257. McCoy, R.D., et al., Expression of human interleukin-1 receptor antagonist in mouse lungs using a recombinant adenovirus: effects on vector-induced inflammation. *Gene Ther*, 1995. 2(7): p. 437-42.
258. McEwan, W.A., et al., Intracellular antibody-bound pathogens stimulate immune signaling via the Fc receptor TRIM21. *Nat Immunol*, 2013. 14(4): p. 327-36.
259. McHutchison, J.G. and T. Poynard, Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. *Semin Liver Dis*, 1999. 19 Suppl 1: p. 57-65.
260. McKelvey, T., et al., T-cell response to adenovirus hexon and DNA-binding protein in mice. *Gene Ther*, 2004. 11(9): p. 791-6.

261. McMahan, R.H., et al., Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest*, 2010. 120(12): p. 4546-57.
262. Medzhitov, R., Origin and physiological roles of inflammation. *Nature*, 2008. 454(7203): p. 428-35.
263. Meier, O., et al., Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol*, 2002. 158(6): p. 1119-31.
264. Mengshol, J.A., et al., A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection. *PLoS One*, 2010. 5(3): p. e9504.
265. Meunier, J.C., et al., Isolation and characterization of broadly neutralizing human monoclonal antibodies to the e1 glycoprotein of hepatitis C virus. *J Virol*, 2008. 82(2): p. 966-73.
266. Meyer, K., et al., Inhibition of hepatitis C virus core protein expression in immortalized human hepatocytes induces cytochrome c-independent increase in Apaf-1 and caspase-9 activation for cell death. *Virology*, 2005. 336(2): p. 198-207.
267. Meylan, E., et al., RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol*, 2004. 5(5): p. 503-7.
268. Minamitani, T., D. Iwakiri, and K. Takada, Adenovirus virus-associated RNAs induce type I interferon expression through a RIG-I-mediated pathway. *J Virol*, 2011. 85(8): p. 4035-40.
269. Miyanari, Y., et al., The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol*, 2007. 9(9): p. 1089-97.
270. Moffatt, S., et al., Circumvention of vector-specific neutralizing antibody response by alternating use of human and non-human adenoviruses: implications in gene therapy. *Virology*, 2000. 272(1): p. 159-67.
271. Moradpour, D., F. Penin, and C.M. Rice, Replication of hepatitis C virus. *Nat Rev Microbiol*, 2007. 5(6): p. 453-63.
272. Morefield, G.L., et al., Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. *Vaccine*, 2005. 23(13): p. 1588-95.
273. Moriya, K., et al., The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med*, 1998. 4(9): p. 1065-7.
274. Moriya, K., et al., Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol*, 1997. 78 ( Pt 7): p. 1527-31.
275. Motta, V., et al., NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev*, 2015. 95(1): p. 149-78.
276. Munakata, T., et al., Hepatitis C virus induces E6AP-dependent degradation of the retinoblastoma protein. *PLoS Pathog*, 2007. 3(9): p. 1335-47.
277. Muruve, D.A., The innate immune response to adenovirus vectors. *Hum Gene Ther*, 2004. 15(12): p. 1157-66.
278. Nagata, T., et al., Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses. *Vaccine*, 2007. 25(26): p. 4914-21.
279. Nakamoto, N., et al., Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog*, 2009. 5(2): p. e1000313.
280. Nalbantoglu, J., et al., Muscle-specific overexpression of the adenovirus primary receptor CAR overcomes low efficiency of gene transfer to mature skeletal muscle. *J Virol*, 2001. 75(9): p. 4276-82.

281. Nemerow, G.R., et al., Insights into adenovirus host cell interactions from structural studies. *Virology*, 2009. 384(2): p. 380-8.
282. Neutra, M.R. and P.A. Kozlowski, Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol*, 2006. 6(2): p. 148-58.
283. Nieto, K., et al., Development of AAVLP(HPV16/31L2) particles as broadly protective HPV vaccine candidate. *PLoS One*, 2012. 7(6): p. e39741.
284. Nociari, M., et al., Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. *J Virol*, 2007. 81(8): p. 4145-57.
285. Norrby, E., The relationship between the soluble antigens and the virion of adenovirus type 3. I. Morphological characteristics. *Virology*, 1966. 28(2): p. 236-48.
286. O'Hagan, D.T., et al., The mechanism of action of MF59 - an innately attractive adjuvant formulation. *Vaccine*, 2012. 30(29): p. 4341-8.
287. Okada, N., et al., Efficient antigen gene transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. *Cancer Res*, 2001. 61(21): p. 7913-9.
288. Okuwaki, M., et al., Identification of nucleophosmin/B23, an acidic nucleolar protein, as a stimulatory factor for in vitro replication of adenovirus DNA complexed with viral basic core proteins. *J Mol Biol*, 2001. 311(1): p. 41-55.
289. Olive, M., et al., The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. *Hum Gene Ther*, 2002. 13(10): p. 1167-78.
290. Onion, D., et al., The CD4+ T-cell response to adenovirus is focused against conserved residues within the hexon protein. *J Gen Virol*, 2007. 88(Pt 9): p. 2417-25.
291. Onomoto, K., et al., Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity. *PLoS One*, 2012. 7(8): p. e43031.
292. O'Riordan, C.R. and A. Song, PEGylated adenovirus for targeted gene therapy. *Methods Mol Biol*, 2008. 434: p. 133-60.
293. Oshiumi, H., et al., TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol*, 2003. 4(2): p. 161-7.
294. O'Sullivan, T.E., J.C. Sun, and L.L. Lanier, Natural Killer Cell Memory. *Immunity*, 2015. 43(4): p. 634-45.
295. Otto, G.A. and J.D. Puglisi, The pathway of HCV IRES-mediated translation initiation. *Cell*, 2004. 119(3): p. 369-80.
296. Panina-Bordignon, P., et al., Study on the immunogenicity of human class-II-restricted T-cell epitopes: processing constraints, degenerate binding, and promiscuous recognition. *Cold Spring Harb Symp Quant Biol*, 1989. 54 Pt 1: p. 445-51.
297. Parker, A.L., et al., Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood*, 2006. 108(8): p. 2554-61.
298. Parks, R.J. and F.L. Graham, A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol*, 1997. 71(4): p. 3293-8.
299. Paul, D., et al., Morphological and biochemical characterization of the membranous hepatitis C virus replication compartment. *J Virol*, 2013. 87(19): p. 10612-27.
300. Pearse, M.J. and D. Drane, ISCOMATRIX adjuvant for antigen delivery. *Adv Drug Deliv Rev*, 2005. 57(3): p. 465-74.

301. Peisley, A., et al., Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature*, 2014. 509(7498): p. 110-4.
302. Penin, F., et al., Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J Virol*, 2001. 75(12): p. 5703-10.
303. Penin, F., et al., Structural biology of hepatitis C virus. *Hepatology*, 2004. 39(1): p. 5-19.
304. Pereira, H.G., R.C. Valentine, and W.C. Russell, Crystallization of an adenovirus protein (the hexon). *Nature*, 1968. 219(5157): p. 946-7.
305. Perotti, M., et al., Identification of a broadly cross-reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. *J Virol*, 2008. 82(2): p. 1047-52.
306. Perreau, M., G. Pantaleo, and E.J. Kremer, Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. *J Exp Med*, 2008. 205(12): p. 2717-25.
307. Pestka, J.M., et al., Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A*, 2007. 104(14): p. 6025-30.
308. Petit, J.M., et al., Hepatitis C virus-associated hypobetalipoproteinemia is correlated with plasma viral load, steatosis, and liver fibrosis. *Am J Gastroenterol*, 2003. 98(5): p. 1150-4.
309. Philpott, N.J., et al., Adenovirus-induced maturation of dendritic cells through a PI3 kinase-mediated TNF-alpha induction pathway. *Proc Natl Acad Sci U S A*, 2004. 101(16): p. 6200-5.
310. Pichla-Gollon, S.L., et al., Effect of preexisting immunity on an adenovirus vaccine vector: in vitro neutralization assays fail to predict inhibition by antiviral antibody in vivo. *J Virol*, 2009. 83(11): p. 5567-73.
311. Pileri, P., et al., Binding of hepatitis C virus to CD81. *Science*, 1998. 282(5390): p. 938-41.
312. Pinto, A.R., et al., Induction of CD8+ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol*, 2003. 171(12): p. 6774-9.
313. Ploss, A., et al., Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature*, 2009. 457(7231): p. 882-6.
314. Plotkin, S., History of vaccination. *Proc Natl Acad Sci U S A*, 2014. 111(34): p. 12283-7.
315. Plotkin, S.A. and S.L. Plotkin, The development of vaccines: how the past led to the future. *Nat Rev Microbiol*, 2011. 9(12): p. 889-93.
316. Pluddemann, A., C. Neyen, and S. Gordon, Macrophage scavenger receptors and host-derived ligands. *Methods*, 2007. 43(3): p. 207-17.
317. Podda, A., The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. *Vaccine*, 2001. 19(17-19): p. 2673-80.
318. Qin, J., O. Vinogradova, and E.F. Plow, Integrin bidirectional signaling: a molecular view. *PLoS Biol*, 2004. 2(6): p. e169.
319. Raddi, N., et al., Pseudotyping Serotype 5 Adenovirus with the Fiber from Other Serotypes Uncovers a Key Role of the Fiber Protein in Adenovirus 5-Induced Thrombocytopenia. *Hum Gene Ther*, 2016. 27(2): p. 193-201.
320. Radziewicz, H., et al., Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol*, 2007. 81(6): p. 2545-53.

321. Raschperger, E., et al., The coxsackie- and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. *Exp Cell Res*, 2006. 312(9): p. 1566-80.
322. Rawlings, D.J., et al., Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat Rev Immunol*, 2012. 12(4): p. 282-94.
323. Ray, R.B. and R. Ray, Hepatitis C virus core protein: intriguing properties and functional relevance. *FEMS Microbiol Lett*, 2001. 202(2): p. 149-56.
324. Reddy, V.S., et al., Crystal structure of human adenovirus at 3.5 Å resolution. *Science*, 2010. 329(5995): p. 1071-5.
325. Reiss, S., et al., Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell Host Microbe*, 2011. 9(1): p. 32-45.
326. Rekosh, D.M., et al., Identification of a protein linked to the ends of adenovirus DNA. *Cell*, 1977. 11(2): p. 283-95.
327. Reyes-Sandoval, A., et al., Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. *J Virol*, 2004. 78(14): p. 7392-9.
328. Rhee, E.G., et al., Multiple innate immune pathways contribute to the immunogenicity of recombinant adenovirus vaccine vectors. *J Virol*, 2011. 85(1): p. 315-23.
329. Riezu-Boj, J.I., et al., Hepatitis C virus induces the expression of CCL17 and CCL22 chemokines that attract regulatory T cells to the site of infection. *J Hepatol*, 2011. 54(3): p. 422-31.
330. Roberts, D.M., et al., Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature*, 2006. 441(7090): p. 239-43.
331. Roberts, M.M., et al., Three-dimensional structure of the adenovirus major coat protein hexon. *Science*, 1986. 232(4754): p. 1148-51.
332. Rock, K.L., I.A. York, and A.L. Goldberg, Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nat Immunol*, 2004. 5(7): p. 670-7.
333. Rogers, G.L., et al., Unique Roles of TLR9- and MyD88-Dependent and -Independent Pathways in Adaptive Immune Responses to AAV-Mediated Gene Transfer. *J Innate Immun*, 2015. 7(3): p. 302-14.
334. Rollier, C.S., et al., Vaccine-induced early control of hepatitis C virus infection in chimpanzees fails to impact on hepatic PD-1 and chronicity. *Hepatology*, 2007. 45(3): p. 602-13.
335. Ross, P.J., et al., Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol*, 2011. 85(8): p. 3950-8.
336. Rowe, W.P., Propagation of group A Coxsackie viruses in denervated adult mouse muscle. *Science*, 1953. 117(3052): p. 710.
337. Roy, S., et al., Circumvention of immunity to the adenovirus major coat protein hexon. *J Virol*, 1998. 72(8): p. 6875-9.
338. Rubino, S.J., et al., Nod-like receptors in the control of intestinal inflammation. *Curr Opin Immunol*, 2012. 24(4): p. 398-404.
339. Russell, W.C., Adenoviruses: update on structure and function. *J Gen Virol*, 2009. 90(Pt 1): p. 1-20.
340. Rux, J.J., P.R. Kuser, and R.M. Burnett, Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J Virol*, 2003. 77(17): p. 9553-66.

341. Sabin, A.B., Perspectives on rapid elimination and ultimate global eradication of paralytic poliomyelitis caused by polioviruses. *Eur J Epidemiol*, 1991. 7(2): p. 95-120.
342. Salk, D. and J. Salk, Vaccinology of poliomyelitis. *Vaccine*, 1984. 2(1): p. 59-74.
343. Sallberg, M., L. Frelin, and O. Weiland, DNA vaccine therapy for chronic hepatitis C virus (HCV) infection: immune control of a moving target. *Expert Opin Biol Ther*, 2009. 9(7): p. 805-15.
344. Santantonio, T., J. Wiegand, and J.T. Gerlach, Acute hepatitis C: current status and remaining challenges. *J Hepatol*, 2008. 49(4): p. 625-33.
345. Scarselli, E., et al., The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J*, 2002. 21(19): p. 5017-25.
346. Scheel, T.K. and C.M. Rice, Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat Med*, 2013. 19(7): p. 837-49.
347. Schirmbeck, R., et al., The immunogenicity of adenovirus vectors limits the multispecificity of CD8 T-cell responses to vector-encoded transgenic antigens. *Mol Ther*, 2008. 16(9): p. 1609-16.
348. Schluns, K.S. and L. Lefrancois, Cytokine control of memory T-cell development and survival. *Nat Rev Immunol*, 2003. 3(4): p. 269-79.
349. Seeff, L.B. and J.H. Hoofnagle, National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. *Hepatology*, 2002. 36(5 Suppl 1): p. S1-2.
350. Seifert, U., et al., Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest*, 2004. 114(2): p. 250-9.
351. Semmo, N., et al., Maintenance of HCV-specific T-cell responses in antibody-deficient patients a decade after early therapy. *Blood*, 2006. 107(11): p. 4570-1.
352. Seth, R.B., et al., Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, 2005. 122(5): p. 669-82.
353. Sharma, A., et al., Adenovirus-based vaccine with epitopes incorporated in novel fiber sites to induce protective immunity against *Pseudomonas aeruginosa*. *PLoS One*, 2013. 8(2): p. e56996.
354. Sharma, S.D., Hepatitis C virus: molecular biology & current therapeutic options. *Indian J Med Res*, 2010. 131: p. 17-34.
355. Shayakhmetov, D.M., et al., Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol*, 2005. 79(12): p. 7478-91.
356. Sheehy, S.H., et al., ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. *Mol Ther*, 2012. 20(12): p. 2355-68.
357. Shott, J.P., et al., Adenovirus 5 and 35 vectors expressing *Plasmodium falciparum* circumsporozoite surface protein elicit potent antigen-specific cellular IFN-gamma and antibody responses in mice. *Vaccine*, 2008. 26(23): p. 2818-23.
358. Sin, J.I., et al., Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocyte-macrophage colony-stimulating factor expression cassettes. *Eur J Immunol*, 1998. 28(11): p. 3530-40.
359. Smith, A.C., K.L. Poulin, and R.J. Parks, DNA genome size affects the stability of the adenovirus virion. *J Virol*, 2009. 83(4): p. 2025-8.
360. Smith, A.D., et al., Human rhinovirus type 14:human immunodeficiency virus type 1 (HIV-1) V3 loop chimeras from a combinatorial library induce potent neutralizing antibody responses against HIV-1. *J Virol*, 1998. 72(1): p. 651-9.
361. Smith, J.S., Z. Xu, and A.P. Byrnes, A quantitative assay for measuring clearance of adenovirus vectors by Kupffer cells. *J Virol Methods*, 2008. 147(1): p. 54-60.

362. Smith, J.S., et al., Interaction of systemically delivered adenovirus vectors with Kupffer cells in mouse liver. *Hum Gene Ther*, 2008. 19(5): p. 547-54.
363. Smith, T.A., et al., Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum Gene Ther*, 2003. 14(8): p. 777-87.
364. Snijder, J., et al., Integrin and defensin modulate the mechanical properties of adenovirus. *J Virol*, 2013. 87(5): p. 2756-66.
365. Solares, A.M., et al., Safety and Immunogenicity of a Human Papillomavirus Peptide Vaccine (CIGB-228) in Women with High-Grade Cervical Intraepithelial Neoplasia: First-in-Human, Proof-of-Concept Trial. *ISRN Obstet Gynecol*, 2011. 2011: p. 292951.
366. Sominskaya, I., et al., Construction and immunological evaluation of multivalent hepatitis B virus (HBV) core virus-like particles carrying HBV and HCV epitopes. *Clin Vaccine Immunol*, 2010. 17(6): p. 1027-33.
367. Staehli, F., et al., NLRC5 deficiency selectively impairs MHC class I- dependent lymphocyte killing by cytotoxic T cells. *J Immunol*, 2012. 188(8): p. 3820-8.
368. Stanekova, Z., et al., Heterosubtypic protective immunity against influenza A virus induced by fusion peptide of the hemagglutinin in comparison to ectodomain of M2 protein. *Acta Virol*, 2011. 55(1): p. 61-7.
369. Staprans, S.I., et al., Activation of virus replication after vaccination of HIV-1-infected individuals. *J Exp Med*, 1995. 182(6): p. 1727-37.
370. Stein, S.C. and E. Falck-Pedersen, Sensing adenovirus infection: activation of interferon regulatory factor 3 in RAW 264.7 cells. *J Virol*, 2012. 86(8): p. 4527-37.
371. Steinmann, E., et al., Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog*, 2007. 3(7): p. e103.
372. Strunze, S., et al., Kinesin-1-mediated capsid disassembly and disruption of the nuclear pore complex promote virus infection. *Cell Host Microbe*, 2011. 10(3): p. 210-23.
373. Sudres, M., et al., MyD88 signaling in B cells regulates the production of Th1-dependent antibodies to AAV. *Mol Ther*, 2012. 20(8): p. 1571-81.
374. Sumida, S.M., et al., Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol*, 2005. 174(11): p. 7179-85.
375. Sun, Z., et al., Phosphorylation of RIG-I by casein kinase II inhibits its antiviral response. *J Virol*, 2011. 85(2): p. 1036-47.
376. Sung, R.S., L. Qin, and J.S. Bromberg, TNFalpha and IFNgamma induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression. *Mol Ther*, 2001. 3(5 Pt 1): p. 757-67.
377. Swadling, L., et al., A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci Transl Med*, 2014. 6(261): p. 261ra153.
378. Takahashi, K., et al., p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts. *Virology*, 1992. 191(1): p. 431-4.
379. Takaoka, A., et al., Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature*, 2005. 434(7030): p. 243-9.
380. Takeuchi, O. and S. Akira, Pattern recognition receptors and inflammation. *Cell*, 2010. 140(6): p. 805-20.

381. Tamanini, A., et al., Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells. *J Virol*, 2006. 80(22): p. 11241-54.
382. Tan, L.K., G.M. Carlone, and R. Borrow, Advances in the development of vaccines against *Neisseria meningitidis*. *N Engl J Med*, 2010. 362(16): p. 1511-20.
383. Tanaka, Y., et al., Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. *Gastroenterology*, 2006. 130(3): p. 703-14.
384. Tang, D.C., M. DeVit, and S.A. Johnston, Genetic immunization is a simple method for eliciting an immune response. *Nature*, 1992. 356(6365): p. 152-4.
385. Tang, J., et al., Adenovirus hexon T-cell epitope is recognized by most adults and is restricted by HLA DP4, the most common class II allele. *Gene Ther*, 2004. 11(18): p. 1408-15.
386. Tang, J., et al., Human CD8+ cytotoxic T cell responses to adenovirus capsid proteins. *Virology*, 2006. 350(2): p. 312-22.
387. Tao, W., et al., A single point mutation in E2 enhances hepatitis C virus infectivity and alters lipoprotein association of viral particles. *Virology*, 2009. 395(1): p. 67-76.
388. Tatsis, N., et al., Adenoviral vectors persist in vivo and maintain activated CD8+ T cells: implications for their use as vaccines. *Blood*, 2007. 110(6): p. 1916-23.
389. Tatsis, N., et al., Adenovirus vector-induced immune responses in nonhuman primates: responses to prime boost regimens. *J Immunol*, 2009. 182(10): p. 6587-99.
390. Thimme, R., et al., Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A*, 2002. 99(24): p. 15661-8.
391. Thomssen, R., et al., Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol*, 1992. 181(5): p. 293-300.
392. Thorner, A.R., et al., Immunogenicity of heterologous recombinant adenovirus prime-boost vaccine regimens is enhanced by circumventing vector cross-reactivity. *J Virol*, 2006. 80(24): p. 12009-16.
393. Thurner, C., et al., Conserved RNA secondary structures in Flaviviridae genomes. *J Gen Virol*, 2004. 85(Pt 5): p. 1113-24.
394. Tollefson, A.E., et al., The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. *Virology*, 1996. 220(1): p. 152-62.
395. Tollefson, A.E., et al., The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol*, 1996. 70(4): p. 2296-306.
396. Travassos, L.H., et al., Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*, 2010. 11(1): p. 55-62.
397. Troesch, M., et al., Study of a novel hypervariable region in hepatitis C virus (HCV) E2 envelope glycoprotein. *Virology*, 2006. 352(2): p. 357-67.
398. Tsai, S.L., et al., Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology*, 1997. 25(2): p. 449-58.
399. Tsuji, T., et al., Immunomodulatory effects of a plasmid expressing B7-2 on human immunodeficiency virus-1-specific cell-mediated immunity induced by a plasmid encoding the viral antigen. *Eur J Immunol*, 1997. 27(3): p. 782-7.
400. Uebelhoer, L., et al., Stable cytotoxic T cell escape mutation in hepatitis C virus is linked to maintenance of viral fitness. *PLoS Pathog*, 2008. 4(9): p. e1000143.

401. Uematsu, S., et al., Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon- $\alpha$  induction. *J Exp Med*, 2005. 201(6): p. 915-23.
402. Uprichard, S.L., et al., Replication of a hepatitis C virus replicon clone in mouse cells. *Virology*, 2006. 3: p. 89.
403. Urbani, S., et al., Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology*, 2006. 44(1): p. 126-39.
404. Vajanto, I., et al., Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. *J Gene Med*, 2002. 4(4): p. 371-80.
405. Valentine, R.C. and H.G. Pereira, Antigens and structure of the adenovirus. *J Mol Biol*, 1965. 13(1): p. 13-20.
406. van Oostrum, J. and R.M. Burnett, Molecular composition of the adenovirus type 2 virion. *J Virol*, 1985. 56(2): p. 439-48.
407. van Raaij, M.J., et al., Structure of the human adenovirus serotype 2 fiber head domain at 1.5 Å resolution. *Virology*, 1999. 262(2): p. 333-43.
408. Vayda, M.E., A.E. Rogers, and S.J. Flint, The structure of nucleoprotein cores released from adenovirions. *Nucleic Acids Res*, 1983. 11(2): p. 441-60.
409. Vertuani, S., et al., Effect of interferon- $\alpha$  therapy on epitope-specific cytotoxic T lymphocyte responses in hepatitis C virus-infected individuals. *Eur J Immunol*, 2002. 32(1): p. 144-54.
410. Vigant, F., et al., Substitution of hexon hypervariable region 5 of adenovirus serotype 5 abrogates blood factor binding and limits gene transfer to liver. *Mol Ther*, 2008. 16(8): p. 1474-80.
411. Vigne, E., et al., Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation. *Gene Ther*, 2003. 10(2): p. 153-62.
412. Villegas-Mendez, A., et al., In vivo delivery of antigens by adenovirus dodecahedron induces cellular and humoral immune responses to elicit antitumor immunity. *Mol Ther*, 2010. 18(5): p. 1046-53.
413. Vogt, M., et al., Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. *N Engl J Med*, 1999. 341(12): p. 866-70.
414. Vyas, J.M., A.G. Van der Veen, and H.L. Ploegh, The known unknowns of antigen processing and presentation. *Nat Rev Immunol*, 2008. 8(8): p. 607-18.
415. Waddington, S.N., et al., Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*, 2008. 132(3): p. 397-409.
416. Walker, C.M. and A. Grakoui, Hepatitis C virus: why do we need a vaccine to prevent a curable persistent infection? *Curr Opin Immunol*, 2015. 35: p. 137-43.
417. Walters, R.W., et al., Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell*, 2002. 110(6): p. 789-99.
418. Wang, C., et al., TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*, 2001. 412(6844): p. 346-51.
419. Wang, Y., Scotomas in molecular virology and epidemiology of hepatitis C virus. *World J Gastroenterol*, 2013. 19(44): p. 7910-21.
420. Warren, J.C. and L. Cassimeris, The contributions of microtubule stability and dynamic instability to adenovirus nuclear localization efficiency. *Cell Motil Cytoskeleton*, 2007. 64(9): p. 675-89.

421. Watkinson, R.E., et al., TRIM21 Promotes cGAS and RIG-I Sensing of Viral Genomes during Infection by Antibody-Opsonized Virus. *PLoS Pathog*, 2015. 11(10): p. e1005253.
422. Watkinson, R.E., et al., TRIM21 Promotes cGAS and RIG-I Sensing of Viral Genomes during Infection by Antibody-Opsonized Virus. *PLoS Pathog*, 2015. 11(10): p. e1005253.
423. Weber, M., et al., Incoming RNA virus nucleocapsids containing a 5'-triphosphorylated genome activate RIG-I and antiviral signaling. *Cell Host Microbe*, 2013. 13(3): p. 336-46.
424. Wedemeyer, H., et al., Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol*, 2002. 169(6): p. 3447-58.
425. Wherry, E.J., et al., Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol*, 2003. 4(3): p. 225-34.
426. White, D.L., V. Ratziu, and H.B. El-Serag, Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. *J Hepatol*, 2008. 49(5): p. 831-44.
427. Wickham, T.J., et al., Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*, 1993. 73(2): p. 309-19.
428. Wieland, S., et al., Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. *Hepatology*, 2014. 59(6): p. 2121-30.
429. Wies, E., et al., Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. *Immunity*, 2013. 38(3): p. 437-49.
430. Wiethoff, C.M., et al., Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J Virol*, 2005. 79(4): p. 1992-2000.
431. Windheim, M., A. Hilgendorf, and H.G. Burgert, Immune evasion by adenovirus E3 proteins: exploitation of intracellular trafficking pathways. *Curr Top Microbiol Immunol*, 2004. 273: p. 29-85.
432. Wolff, J.A., et al., Direct gene transfer into mouse muscle in vivo. *Science*, 1990. 247(4949 Pt 1): p. 1465-8.
433. Wood, M., et al., Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther*, 1999. 6(4): p. 367-72.
434. Worgall, S., et al., Protective immunity to *Pseudomonas aeruginosa* induced with a capsid-modified adenovirus expressing *P. aeruginosa* OprF. *J Virol*, 2007. 81(24): p. 13801-8.
435. Wu, S.C., et al., Hepatitis C virus NS5A protein down-regulates the expression of spindle gene *Aspm* through PKR-p38 signaling pathway. *J Biol Chem*, 2008. 283(43): p. 29396-404.
436. Wunschmann, S., et al., Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. *J Virol*, 2000. 74(21): p. 10055-62.
437. Xia, D., et al., Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. *Structure*, 1994. 2(12): p. 1259-70.
438. Xiang, Z. and H.C. Ertl, Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity*, 1995. 2(2): p. 129-35.
439. Xiang, Z.Q., et al., Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. *J Virol*, 2003. 77(20): p. 10780-9.
440. Xu, Z., et al., Coagulation factor X shields adenovirus type 5 from attack by natural antibodies and complement. *Nat Med*, 2013. 19(4): p. 452-7.
441. Xu, Z., et al., Clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies, and complement. *J Virol*, 2008. 82(23): p. 11705-13.
442. Yamaguchi, T., et al., Role of MyD88 and TLR9 in the innate immune response elicited by serotype 5 adenoviral vectors. *Hum Gene Ther*, 2007. 18(8): p. 753-62.

443. Yamamoto, M., et al., TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol*, 2003. 4(11): p. 1144-50.
444. Yamamoto, M., et al., Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol*, 2002. 169(12): p. 6668-72.
445. Yang, T.C., et al., Detailed analysis of the CD8+ T-cell response following adenovirus vaccination. *J Virol*, 2003. 77(24): p. 13407-11.
446. Yang, W.Y., et al., Two-step transformation for highly efficient construction of a replication-defective recombinant adenoviral vector. *Di Yi Jun Yi Da Xue Xue Bao*, 2004. 24(2): p. 164-7.
447. Yoneyama, M., et al., Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol*, 2005. 175(5): p. 2851-8.
448. Yoneyama, M., et al., The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*, 2004. 5(7): p. 730-7.
449. Yoneyama, M., et al., Viral RNA detection by RIG-I-like receptors. *Curr Opin Immunol*, 2015. 32: p. 48-53.
450. Youil, R., et al., Hexon gene switch strategy for the generation of chimeric recombinant adenovirus. *Hum Gene Ther*, 2002. 13(2): p. 311-20.
451. Yuasa, T., et al., The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. *J Gen Virol*, 1991. 72 ( Pt 8): p. 2021-4.
452. Yutani, S., et al., Phase I clinical study of a personalized peptide vaccination for patients infected with hepatitis C virus (HCV) 1b who failed to respond to interferon-based therapy. *Vaccine*, 2007. 25(42): p. 7429-35.
453. Zaiss, A.K., et al., Hepatocyte Heparan Sulfate Is Required for Adeno-Associated Virus 2 but Dispensable for Adenovirus 5 Liver Transduction In Vivo. *J Virol*, 2015. 90(1): p. 412-20.
454. Zeisel, M.B., F.L. Cosset, and T.F. Baumert, Host neutralizing responses and pathogenesis of hepatitis C virus infection. *Hepatology*, 2008. 48(1): p. 299-307.
455. Zhang, P., et al., Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. *Proc Natl Acad Sci U S A*, 2007. 104(20): p. 8449-54.
456. Zhang, P., et al., Depletion of interfering antibodies in chronic hepatitis C patients and vaccinated chimpanzees reveals broad cross-genotype neutralizing activity. *Proc Natl Acad Sci U S A*, 2009. 106(18): p. 7537-41.
457. Zhou, D., et al., Hexon-modified recombinant E1-deleted adenovirus vectors as dual specificity vaccine carriers for influenza virus. *Mol Ther*, 2013. 21(3): p. 696-706.
458. Zhou, J., et al., Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology*, 1991. 185(1): p. 251-7.
459. Zhu, J., X. Huang, and Y. Yang, The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *J Clin Invest*, 2009. 119(8): p. 2388-98.
460. Zubieta, C., et al., The structure of the human adenovirus 2 penton. *Mol Cell*, 2005. 17(1): p. 121-35.