Adenoviral vectors: a possible road to an HIV vaccine

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CHAPTER 1

Introduction

1.1 Human immunodeficiency virus

1.2 Challenges in the search for an HIV vaccine

1.3 Replication-incompetent adenoviral vectors for vaccination

1.4 Outline of this thesis
1.1 Human immunodeficiency virus

Introduction
In 1981, unusual high amounts of opportunistic infections and Kaposi sarcoma were observed among homosexual men in New York and California (147, 209, 304). It was established that these men suffered from a common immunological disorder, termed as acquired immune deficiency syndrome (AIDS). Few years later, a new member of the Retroviridae family, human immunodeficiency virus (HIV), was discovered and assessed to be the cause of AIDS (28, 101).

The Retroviridae family is divided into two subfamilies: Orthoretrovirinae and Spumaretrovirinae. The Orthoretrovirinae subfamily is further divided into six genera: Alpharetroviruses, Betaretroviruses, Gammaretroviruses, Deltaretroviruses, Lentiviruses and Epsilonretroviruses (148). HIV belongs to the Lentivirus genus and two different types are known, HIV-1 and HIV-2. Based on genetic similarities, HIV-1 is classified into three groups, M, N and O. The group N and O viruses, like HIV-2, are essentially only found in West Africa. The group M viruses, however, are the cause of the global HIV-1 pandemic. To date, an estimated amount of 39.5 million people are living with HIV worldwide and the majority (approximately 63%) are living in sub-Saharan Africa (http://www.unaids.org).

HIV-1 genome and proteins
The genome of HIV-1 consists of two identical strands of RNA and is approximately 9.8 kb in size. Both ends of genome are flanked by long terminal repeats (LTR), which are important for HIV replication. The genomic structure of HIV-1 is depicted in Fig. 2a. The structural proteins derived from the gag, pol and env genes make up the HIV-1 virion (see Fig. 2a and b). From the gag gene, a precursor protein called p55 is transcribed. Upon HIV maturation p55 is cleaved by the viral protease (Pol p10) into the structural intra-viral proteins: matrix protein (p17), capsid protein (p24), nucleocapsid protein (p9) and p6 (320)(see Fig. 2a and b). The pol gene encodes for the viral enzymes that are essential for the HIV maturation, HIV’ DNA synthesis and HIV’ DNA integration in the host genome. The viral enzymes are always obtained through an initial generation of a Gag-Pol fusion protein (p160). During HIV maturation Pol is first cleaved away from p55 by the viral protease, which than cleaves Pol further into the protease (p10), reverse transcriptase (p66, p51) and integrase (p32) (320)(see Fig. 2a and b). The env gene encodes for a precursor protein gp160, that is cleaved by a cellular protease into the viral envelope glycoproteins: transmembrane protein (gp41) and surface protein (gp120) (see Fig. 2a)(320). These two proteins make up the outer part of HIV particle, and the surface protein gp120 is essential for binding of the HIV particle to its cellular receptor CD4 and co-receptors CXCR4 and CCR5 (146).

The HIV-1 genome contains in addition to the gag, pol and env genes, six more genes that encode for the regulatory and accessory proteins. From the tat and rev genes the two regulatory proteins, Tat and Rev, are derived. The Tat protein functions as the viral transcriptional transactivator, which is essential for the replication cycle of HIV-1 (104). The Rev protein induces the transition of early to late phase HIV gene expression, by transporting unspliced or partially spliced viral mRNAs from the nucleus to the cytoplasm to be translated (256). In the absence of Rev, no progeny HIV-1 virions are produced. The last
group of HIV-1 proteins are the accessory proteins, which are transcribed from the nef, vpr, vpu and vif genes. The accessory proteins are not pivotal for HIV-1 replication in vitro, however they have proven to be essential for the HIV-1 life cycle in vivo by counteracting the host anti-viral response (Nef (64), Vpu (358) and Vif (379)) and by playing an important role in the HIV-1 replication and infectivity (Nef (64), Vpr (176), Vpu (167)).

Figure 2: HIV-1 particle and genomic organization. 
(a) Schematic representation of the HIV-1 genome, showing the structural genes (gag, pol and env) and the proteins derived thereof, the regulatory genes (tat and rev) and the accessory genes (vif, vpr, vpu and nef). (b) Schematic representation of the HIV particle: the HIV-1 structural proteins and RNA are displayed.
Chapter 1

1.2

Challenges in the search for an HIV vaccine

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Introduction

The development of a safe and effective human immunodeficiency virus (HIV) vaccine has proven a major scientific challenge. Given the rapidly expanding HIV pandemic, the need for a prophylactic HIV vaccine is paramount, particularly in the developing world. In the Western world, antiretroviral therapy is responsible for major declines in AIDS-related morbidity and mortality. However, despite recent advances in bringing antiretroviral therapy to resource-poor settings, these drugs are still not available to the vast majority of individuals in these areas where they are needed most.

The most effective way to control the spread of the HIV epidemic will be the development of a prophylactic vaccine. It is generally agreed that an effective vaccine will likely need to elicit both humoral and cellular immune responses. Neutralizing antibodies bind free virus particles and eliminate them through various effector mechanisms. CD⁸⁺ cytotoxic T lymphocytes (CTL) kill HIV-infected cells, and CD⁴⁺ helper T lymphocytes serve a critical role in orchestrating the immune response. The precise immune correlates of protection, however, have not yet been defined.

The ultimate goal of an HIV vaccine is to achieve sterilizing immunity. However, this level of protection is not even achieved with most clinically licensed vaccines. A more realistic goal may be to develop a vaccine that lowers viral loads and prevents clinical disease progression. Recent clinical and pre-clinical studies have demonstrated that virus-specific adaptive immune responses are critical for immune control of viral replication. For example, people who are highly exposed to HIV but remain uninfected have been reported to have HIV-specific cellular immune responses (92, 282). Moreover, potent CTL responses have been observed in long-term non-progressors (345). Studies in non-human primates have shown that passively transferred neutralizing antibodies can provide complete protection against infection (78, 206, 260, 261). Vaccine-induced cellular immune responses have been shown to suppress viral replication but not provide sterilizing immunity against pathogenic viral challenges (7, 17, 23, 302).

HIV neutralizing antibodies

HIV-specific neutralizing antibodies likely play a role in controlling viral replication, but the development of immunogens capable of eliciting broadly reactive neutralizing antibodies has proven extraordinarily difficult. HIV-infected patients generate antibodies to a variety of HIV proteins following infection. However, these antibodies are often not neutralizing and are often directed against viral debris (250). Neutralizing antibodies to HIV are mainly directed against the envelope proteins, gp120 and gp41. These antibodies often recognize
Challenges in the search for an HIV vaccine

epitopes in the third variable region (V3 loop) or the CD4 binding domain, but antibodies directed against the V3 loop more efficiently neutralize laboratory strains of HIV-1 than primary HIV isolates (224). Moreover, it has been shown that HIV is very efficient at escaping neutralizing antibodies by accumulating mutations in the envelope gene. Shielding of the conserved regions of the envelope protein by the other two variable loops (V1 and V2 loop) (251) and by extensive glycosylation (52) limits the accessibility of neutralizing antibodies. Interestingly, modification of glycosylation patterns has been reported as a novel neutralization escape mechanism (352).

Overall, the ability of HIV to escape neutralizing antibody responses and to shield conserved epitopes makes it difficult to develop a vaccine that is able to elicit neutralizing antibodies. Moreover, the genetic variability of HIV-1 is an enormous challenge that needs to be overcome. At present, no vaccine has been developed that reliably elicits neutralizing antibodies that recognize a broad diversity of primary isolates. Whether it is possible to elicit such broadly reactive neutralizing antibodies by vaccines is not known, although a wide variety of approaches are currently being explored in pre-clinical studies.

HIV CTL responses

Accumulating evidence has confirmed the importance of virus-specific CTL in controlling HIV replication in humans and SIV replication in rhesus monkeys. In particular, it has been convincingly shown that CTL are critical in controlling primary viremia in both human and monkeys (38, 289). High levels of HIV-specific CTL have also been observed in long-term non-progressors (45). These data suggest that vaccine strategies should elicit potent HIV-specific CTL responses. Recent non-human primate challenge studies have suggested that CTL-based vaccines will likely not protect against HIV infection, but they may have the ability to control viral replication and slow clinical disease progression (7, 23, 302).

It is possible that these CTL-based vaccines may slow disease progression and reduce the rate of HIV transmission (339). However, these vaccines may be limited by a lack of durability of immune control. For example, HIV might eventually escape dominant CTL responses by viral mutations within CTL epitopes (111, 170, 259). In fact, it has already been shown that viral escape from CTL can result in eventual AIDS vaccine failure in the rhesus monkey model (17). Viral escape from CTL has also been shown to result in eventual progression towards AIDS in humans (170, 259).

The loss of HIV-specific CD4+ T cells in HIV-1 infected patients may also contribute to the eventual failure of CTL-based vaccines. High frequency CD4+ T lymphocyte responses are typically observed in long-term non-progressors (279). In contrast the majority of HIV patients have poor CD4+ T lymphocyte responses (216). These data suggest that HIV-specific CD4+ T helper cells are essential for the functionality and survival of CTL, and that maintaining these cells will be critical for vaccine-mediated immune control of HIV-1.

Vaccine modalities

A large number of vaccine modalities are currently being explored in both pre-clinical and clinical trials. Recombinant envelope proteins have been the most extensively studied candidate HIV vaccines. It was hoped that these vaccines would elicit neutralizing antibodies that would be able to prevent infection. However, these envelope proteins have different conformational structures than the native HIV envelope trimers on the surface of virions. The challenge of developing effective gp120-based vaccines has recently been highlighted by the failure of recombinant gp120 in two recent phase III human trials (60). This was not surprising, however, since recombinant gp120 does not elicit broadly reactive neutralizing antibodies or virus-specific CTL responses.
Plasmid DNA vaccines and recombinant live viral vectors, including Semliki Forest virus (226), Venezuelan equine encephalitis (65), adeno-associated virus, poxvirus and adenovirus, are currently being developed to elicit virus-specific CTL responses. CTL responses are frequently detected against internal proteins such as gag and pol and accessory proteins such as nef in long-term non-progressors (165, 257, 338), suggesting that these genes should be included in an HIV vaccine. The use of plasmid DNA vaccines has shown great promise in rodents (reviewed in (74)). However in humans, the level of CTLs induced has thus far been disappointing (44, 177, 199, 349). Considerable effort is therefore being spent currently on improving the immunogenicity of these carriers. Research focuses on improving the level of expression in eukaryotic cells through codon optimization and on identification of cytokines or costimulatory molecules that further prime the host immune system.

Although many live recombinant vector-based vaccines are being explored, most pre-clinical and clinical experience has been obtained with live recombinant viral vectors based on poxviruses or adenoviruses. Attenuated poxvirus vectors have been developed that have the capacity to insert multiple HIV genes such as env, gag, pol and rev (2, 294). MVA, NYVAC and fowlpox vectors have been shown to elicit CTL responses, either alone (22) or in combination with DNA vaccines (7, 158). The canarypox vector ALVAC has been shown to induce low frequency but detectable HIV-specific CTLs in phase I/II clinical trials (30, 81, 119), but the immunogenicity of this vector was not potent enough to proceed with phase III clinical testing as a single-modality vaccine. Currently, a phase III clinical HIV vaccine trial evaluating a replication-defective ALVAC vector prime with an alum-adjuvated gp120 protein boost is ongoing in Thailand (58).

The adenoviral vector has been tested as vaccine carrier against a variety of both veterinary and human pathogens and the number of diseases targeted via an adenovirus-based vaccine approach is rapidly growing. Two clinical phase I studies using replication incompetent adenovirus serotype 5 (Ad5) have been completed (50, 76). These phase I clinical trials have clearly illustrated the ability of Ad5-based vaccine vectors to induce potent T lymphocyte mediated immune responses, which warranted further testing of these candidate vaccines in phase II clinical trials (59, 76). However, these trials also demonstrated that pre-existing immunity against Ad5, which is highly prevalent among human populations, might interfere with vaccine potency and dose control. Section 1.3 details the knowledge accumulated over two decades of basic adenovirus research and the generation of safe replication incompetent adenoviral vectors.
Replication-incompetent adenoviral vectors

1.3 Replication-incompetent adenoviral vectors for vaccination

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Adenovirus biology

Classification, disease association, particle structure and life cycle

Human adenovirus (Ad) was first isolated from adenoidal tissues in 1953 (281), hence the name adenovirus. To date, 51 human adenovirus serotypes are identified (68) and classified into 6 subgroups A to F (Table I). Classification of adenovirus serotypes is based on (i) the ability of the adenovirus serotype to either or not hemagglutinate with erythrocytes from different animal species, (ii) an unique antigenic fingerprint which means that sera raised in animals against one serotype do not cross-react with any other adenovirus serotype, and (iii) DNA restriction analysis on the double stranded adenoviral DNA genome (93). Clinical disease associations have been identified for many of the 51 human adenovirus serotypes (see also Table I). Adenoviral infections are normally self-limiting and do not require specific antiviral therapy. However, in immunocompromised hosts, such as HIV patients or transplant recipients, adenoviruses can cause severe illness which upon disseminated disease can become fatal (130, 132, 160, 171).

Table I: Classification of human adenoviruses and possible disease association.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Serotypes</th>
<th>Possible disease association</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>Central nervous system (CNS) disease,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Febrile respiratory disease</td>
</tr>
<tr>
<td>B</td>
<td>3, 7, 11, 14, 16, 21, 34, 35, 50</td>
<td>Hemorrhagic cystitis, Febrile respiratory disease</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>Febrile respiratory disease, Hepatitis, CNS disease</td>
</tr>
<tr>
<td>D</td>
<td>8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51</td>
<td>Keratoconjunctivitis, Febrile respiratory disease, CNS disease, Gastroenteritis</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>Febrile respiratory disease</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>Gastroenteritis</td>
</tr>
</tbody>
</table>
The adenoviral genome ranges from 25 to 36 kilobases (kb) depending on the serotype and is remarkably conserved among the adenoviridae. The adenoviral genome is always flanked on both ends by inverted terminal repeats (ITR), which are essential for DNA replication. Attached to the ITRs at both ends of the viral genome is the so-called terminal protein (TP), which is considered to be essential to ensure early transcription and onset of DNA replication (351). Three other viral proteins are directly associated with the viral DNA and are called, V, VII and X (also called mu (8)) (54). These core proteins are essential for folding the DNA genome such that it can be packaged into the adenoviral particle. Adenoviruses are non-enveloped viruses with an icosahedral capsid (see Fig. 1), which is predominantly made up from three proteins: hexon (in total 240 trimeric proteins), penton-base (in total 12 pentameric proteins) and fiber (in total 12 trimeric proteins and associated with penton base). So-called minor capsid proteins, called IIIa and IX are located near the outer surface of the particle and interact with the hexon protein thereby increasing the strengths of the particle. Likewise, minor capsid proteins called VI and VIII which are located more at the interior, ensure DNA genome binding to the capsid and regulate particle size by interacting with hexon respectively (341, 342).

The life cycle of adenovirus (Fig. 2) is both fascinating and complex starting with binding of the adenovirus particle to a specific cellular receptor. Thereafter the adenovirus particle is internalized via coated pits and escapes from the cellular endosome. Subsequently the adenoviral particle is transported to the nucleus (see paragraph Tropism and internalization). Once the adenoviral DNA is delivered to the nucleus a tightly controlled process of adenoviral transcription, replication and particle assembly follows. During this process the adenovirus takes complete control of the cellular transcription and translation machinery. Eventually, this leads to cell death and subsequent escape of hundreds of thousands of newly synthesized adenoviral particles (see paragraph replication cycle). Since the first adenoviruses studies were based on serotypes 2 and 5 most of the knowledge regarding the adenovirus life cycle is based on these two viruses. It is therefore that the processes described below are based predominantly on these viruses unless indicated otherwise.

**Figure 1:** The adenoviral capsid.
Schematic representation of the adenoviral capsid. Displayed are the major capsid proteins (hexon, penton and fiber) and minor capsid proteins (IIIa, IX, VI and VIII).
Tropism and internalization

The fiber protein has a characteristic structure, which consists of a tail domain located at the N-terminus, a central shaft and a knob domain at the C-terminus (Fig. 1). The first thirty amino acids of the fiber-tail are pivotal for the attachment of the fiber to the penton base (56). The fiber and penton base are necessary for both cellular attachment and internalization of the adenovirus particle during infection. The fiber knob interacts with a specific cellular receptor to initiate cellular attachment. To date, several cellular receptors are identified that are employed by adenoviruses for cellular attachment. The coxsackievirus-adenovirus receptor (CAR) is used by most serotypes (33, 275), except for those from subgroup B and some from subgroup D. The serotypes from subgroup B and some from subgroup D can utilize the membrane cofactor protein CD46 as primary receptor (1, 96, 178, 293, 305, 366). However, several other molecules like sialic acid (13), dipalmitoyl phosphatidylethanolamines (DPPC) (16), vascular cell adhesion molecule 1 (VCAM1) (57), CD80 and CD86 (303), heparan sulfate glycosaminoglycans (71), αMβ2 and αLβ2 integrins (144), major histocompatibility complex I (MHC-I) (138) and lactoferrin (151) may also function as adenoviral cellular attachment molecules for certain serotypes. Despite the knowledge gathered to date, it is likely that the list of adenoviral receptors is to increase over time since for some adenoviral serotypes the cellular receptor has not yet been identified.

After initial adenoviral attachment to its cellular receptor, particle internalization via clathrin-mediated endocytosis occurs (Fig. 2). This is facilitated through binding of the penton base to cellular integrins. Essential for the binding to cellular integrins are three amino acids (Arg-Gly-Asp) that are located within the penton base and together are called the RGD motif (185, 285, 356). Once in the endosome the adenovirus particle destabilizes due to a decrease in pH. This destabilization leads to dissociation of the fiber, penton base and minor capsid proteins IIIa, VI, VIII and IX from the adenovirus particle (114). The dissociation of these proteins liberates protein VI, which is normally located on the inner surface of the adenovirus particle where it connects the bases of the hexon proteins adjacent to the penton base (310). Once protein VI is liberated from the particle it exerts its membrane lytic activity, leading to endosomal membrane disruption (357). This process finally results in the release of the destabilized adenovirus particle into the cytosol. From here it is transported to the nuclear pore complex (NPC) through a dynein- and microtubule-mediated transportation (181). At the NPC the destabilized adenovirus particle docks to the NPC-filament protein CAN/Nup214, after which the viral DNA is released to enter the nucleus through a process that requires binding of the CAN/Nup214 docked capsids to nuclear histone H1 (333). Once the adenoviral DNA is located within the nucleus the processes of transcription, replication and assembly of new adenoviral particles will take place.

The route of cellular internalization described above is predominantly determined for Ad5 and Ad2. Through recent knowledge on other human adenovirus serotypes and fiber chimeric adenoviral vectors, some exceptions have become apparent. The RGD motif needed for the cellular integrin binding, is absent in serotypes Ad40 and Ad41 that belong to subgroup F indicating an alternative entry route (4, 66). The cellular internalization of Ad41 proves to be delayed in comparison to other human adenovirus serotypes, which is attributed to the lack of the RGD motif and the consequential inability to bind to the cellular integrins (4). In addition, Ad40 and Ad41 differ from other human adenoviruses in that their genome codes for two distinct fiber proteins that are both expressed and displayed on the viral particle, a short and a long fiber (161, 376). It has been convincingly shown that the long fiber of Ad41 is able to bind CAR, but the short fiber cannot (275). Although poorly
Chapter 1

understood at present, the role of the short fiber is considered to lie in alternative routes of
cellular internalization. The viral escape from the endosome upon cellular internalization, resulting in release of the
adenoviral particle into the cytosol and the subsequent transport to the NPC, is also not a
conserved process among human adenoviridae. For instance, Ad7 which is a member of
subgroup B does not escape the endosome but instead ends up in late endosomes and
lysosomes (219). Given the observation that an Ad5 vector containing only the fiber protein
of Ad7 (Ad5fib7) also ends up in late endosomes and lysosomes, it has been speculated that
the fiber protein is predominantly involved in determining the postinternalization trafficking
route (220). This finding was further supported by data demonstrating that an Ad5 vector
carrying only the fiber knob of a B-group adenovirus, Ad35, ended up in late endosomes
and lysosomes (297). Since the fiber knob interacts with a specific cellular receptor and both
Ad7 and Ad35 do not bind to CAR (275), it can be envisioned that specific receptor binding
of the adenoviral particle determines the trafficking pathway towards the nucleus.

![Figure 2: Adenovirus life-cycle.](image_url)

1) Receptor-mediated binding to a cell, 2) Upon integrin binding, endocytosis, 3) Adenovirus particle in early
endosome, 4) pH drop in endosome leads to particle destabilization and endosome membrane disruption through
protein VI, 5) Destabilized particle is transported to the nucleus through dynein- and microtubule-mediated
transport, 6) Adenoviral DNA enters the nucleus through the NPC.

**Adenoviral replication cycle**
The adenoviral transcription can be clearly distinguished in two phases, an early and a late
phase. In the early transcription phase, proteins derived from six different genomic regions
(E1A, E1B, E2A, E2B, E3 and E4; Fig. 3) are highly expressed. Most of these genomic
regions produce multiple messenger RNAs (mRNA) and protein products through
Replication-incompetent adenoviral vectors
differential mRNA processing. In the late transcription phase proteins derived from five
different genomic regions are expressed (L1 to L5; Fig. 3) and, like for the early regions,
multiple transcripts and proteins can be derived per region. The start of DNA replication
defines the transition between the early- and late transcription phase, sometimes also
designated as the intermediate phase. Given the complexity of the replication system and
vast amount of knowledge gathered on the diverse proteins and their role in replication, the
early and late phase as well the different proteins will be discussed in separate headings
below.

Figure 3: The adenoviral genome.
Schematic representation of the adenoviral genome, showing locations of the early (E) and late (L) transcription
regions and the proteins derived thereof. The packaging domain is indicated by Ψ.

Early phase of adenoviral replication
E1 proteins
Proteins derived from region E1A (289R and 243R) are known to trans-activate all other
early regions and activate the infected cell by pushing the cell into the active DNA
replication state (S-phase). By doing so, they create an environment that is optimal for
adenoviral replication (90). The cell activation by E1A-derived proteins occurs through
interference with cellular proteins from the retinoblastoma (Rb) family (pRB, p107 and
p130) resulting in free E2F (140, 149). In turn, E2F is a transcriptional activator that
promotes expression of genes necessary to push cells into the S phase (152). However, a
downside of this mechanism of E1A-derived proteins is that the cells are forced into
apoptosis via up-regulation of the tumor suppressor p53 levels (70). To counteract this
phenomenon, proteins of the E1B region are required. One of the products of the E1B
region, 55K, is able to block the p53-dependent apoptosis by direct binding to p53 (155,
205) and in cooperation with E4-orf6 (172, 262, 263). Alternatively, E1A-derived proteins
also induce a p53-independent cellular apoptosis pathway, mediated by the tumor necrosis
factor α (TNFα) (295). This pathway is effectively inhibited by another protein of the E1B
region called 19K. The 19K protein binds to the Bak protein thereby preventing the
interaction between the Bak and Bax proteins that would otherwise ensure triggering of the
caspase induced apoptosis pathway (318).

E2 proteins
The E2A region produces one transcript resulting in expression of the protein called
adenoviral DNA binding protein (DBP). This protein is involved in several processes
regarding adenovirus DNA replication such as initiation, elongation (reviewed in (69)), transcription control, DNA recombination, adenovirus particle assembly and mRNA stability (reviewed in (126, 340)). The two transcripts of the E2B region called precursor terminal protein (pTP) and DNA polymerase (pol), together with DBP and two cellular transcription factors (Nuclear Factor (NF) I and III) form a complex that is required for forming adenovirus progeny genomes (reviewed in (191)).

**E3 proteins**

From the E3 region seven transcripts are derived encoding for proteins called 12.5K, 6.7K, 19K, Adenovirus Death Protein (ADP), RIDα, RIDβ and 14.7K. Some of these proteins (RIDα and RIDβ) are involved in down-regulating cellular immunological response mechanisms in an attempt of the adenovirus to escape the host immune system (reviewed in (189)). For instance, the RIDα and RIDβ proteins (formerly known as E3-10.4K and E3-14.5K respectively) are both type I integral membrane proteins and together form the RID (receptor internalization and degradation) complex. The RID complex inhibits cellular apoptosis triggered by death ligands through down-regulation of their receptors (85, 188, 299, 327, 330). Well known death ligands include TNF-related apoptosis-inducing ligand (TRAIL), TNFα and Fas ligand and they are launched by the host in defence against virus-infected cells. In addition, the RID complex hampers the TNF-mediated apoptosis and inflammatory responses by blocking of arachidonic acid release and by down regulating NF-κB activation (94, 175).

Another protein of the E3 region (6.7K) is a type III integral membrane protein, localized in the endoplasmic reticulum (ER) (359), and known to suppress apoptosis by maintaining the cytosolic Ca²⁺ homeostasis (221). Protein 6.7K can also be found in the plasma membrane, where it forms a complex with the RIDα and RIDβ proteins to down-regulate the TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (31, 188).

Yet another protein of the E3 region (19K) is a type I integral membrane protein located mainly in the ER. In the ER 19K binds to major histocompatibility complex I molecules (MHC-I), resulting in inhibition of the transport of MHC-I molecules to the cell surface (9). Normally, MHC-I molecules are relocated to the cell surface to present foreing antigens to the immune system. To further elucidate the mechanism of action of the 19K protein, mutants of this protein have been constructed and studied. It has been shown using a 19K protein deleted for the ER-retention motif in the C-terminus of the protein that, even though the protein was unable to block the transport of MHC-I to the cell surface, only a portion of the MHC-I molecules reached the cell surface. The latter finding indicates an additional interference of the 19K protein on the MHC-I pathway, which by now is known to be caused by the inhibition of MHC-I/complex formation with TAP (32). This inhibition results in obstruction of normal MHC-I maturation, which also results in reduction of the CTL-mediated cellular killing upon adenoviral infection.

The last protein of the E3 region involved in immune escape is the 14.7K protein. In contrast to the other E3 derived proteins, 14.7K is not an integral membrane protein but is located in the cytoplasm and nucleus (108). Like the RID complex and the 6.7K protein, 14.7K inhibits TNF-mediated apoptosis. However, unlike the other proteins described, 14.7K exerts its action by inhibiting the internalization of TNF receptor 1 (290). Normally, the internalization of TNF receptor 1 leads to cellular apoptosis through so-called death inducing signaling complex (DISC) formation.

The former last protein of the E3 region called ADP and formerly known as E3-11.6K is not involved in immune escape mechanisms. This protein is essential for the release of newly assembled adenoviral particles from the infected cell since absence of ADP proved to significantly delay the release of adenoviral progeny from the cell (328). As a result of its
Replication-incompetent adenoviral vectors

function, ADP is predominantly expressed in the late phase of adenovirus replication in contrast to the other E3 proteins (329). The last protein derived from the E3 region is called 12.5K and despite several investigations no function could as yet be attributed to this protein.

Again, several exceptions exist to the number of proteins described from the E3 region derived from different adenoviridae. For instance the organization of the E3 region of the other adenoviral subgroups is not identical to those of subgroup C (reviewed in (361)). The only conserved E3-proteins throughout all subgroups are the proteins that form the RID complex and E3-14.7K indicating their importance. Some subgroups have E3 proteins that are unique to their own subgroup, like for example E3-20.3K and E3-20.6K for subgroup B (29) and E3-49K for subgroup D (360). Studying the function of these E3 proteins derived from alternative serotypes is in its infancy.

E4 proteins
The last early region (E4) codes for a number of proteins called open reading frame (orf) 1, 2, 3, 4, 6, and 6/7 that exert a variety of functions. The orf1 protein is related to dUTP pyrophosphatase (dUTPase) enzymes of viral and animal origin, however it does not appear to exert any enzymatic activity (355) and the precise function of the orf1 protein remains currently a mystery. Likewise, the function of orf2, has not yet been elucidated.

The orf3 protein shares several properties with orf6, including the interruption of the cellular double-stranded (ds) DNA repair mechanisms (39, 311) and they both mediate efficient transportation of adenoviral mRNA from the nucleus to the cytoplasm in order to increase late adenoviral protein synthesis (41, 142). Interference with ds DNA repair is based on the inhibition of adenoviral DNA concatamer formation, which otherwise would block adenoviral DNA replication and adenoviral packaging (311, 353). Both orf3 and orf6 bind to and inhibit the function of DNA damage-activated DNA-dependent protein kinase (DNA-PK) (39). Also, orf3 and orf6 both inactivate the Mre11 DNA repair complex (311) albeit through different mechanisms. For instance orf6 inactivates the Mre11 complex together with E1B-55K by proteasome-mediated degradation (311), while orf3 inactivates the Mre11 complex by directing the Mre11 complex from sites of DNA replication into large nuclear and cytoplasmic accumulations consisting of aggregates of misfolded proteins (12). The function of orf3 to relocate the Mre11 complex appeared not to be conserved between all human adenovirus serotypes, since it was found that the orf3 proteins of Ad4 and Ad12 are not able to relocalize the Mre11 complex (312). The second shared property of orf3 and orf6 i.e. the efficient transportation of adenoviral mRNA from the nucleus to the cytoplasm (41, 142) is poorly understood, although it is known that orf6, in contrast to orf3, forms a complex with E1B-55K to exert this function (40, 109, 242).

Besides the shared properties of orf3 and orf6, the proteins also display several unique functions. For example, orf3 has the ability to reorganize the promyelocytic leukemia protein (PML) nuclear body (also called PML oncopgenic domain (POD), PODs/ND10 or ND10) (75, 139), through interaction with PML isoform II (PMLII) (139). The PML nuclear body structures are thought to be involved in DNA replication, DNA repair, transcriptional regulation, post-translational modifications, apoptosis and IFN-induced antiviral state (37, 134, 268, 386). Likewise, recently it has been demonstrated that the orf3 protein interacts with transcriptional intermediary factor 1α (TIF1α) which, like PML, is a member of the tripartite motif (TRIM) protein family (377). Although the function of binding of orf3 to either PML or TIF1α is not completely understood, it has been postulated that the reorganization of the PML nuclear body by orf3 results in the inhibition of an IFN-induced antiviral response (337). In contrast to orf3, unique functions provided by orf6 are almost exclusively performed after complexing with E1B-55K. For instance, the E1B-55K/4-orf6
complex is involved in the shut-off of host cell protein synthesis, through inhibition of the transport of host cell mRNA to the cytoplasm. The latter finding contrasts with the finding that the E1B-55K/E4-orf6 complex mediates the efficient transport of the adenoviral mRNA to the cytoplasm. The mechanism by which the distinction between adenoviral mRNA and cellular mRNA is made has not yet been elucidated (91). In addition, the E1B-55K/orf6 complex is also involved in the inhibition of p53-mediated apoptosis, as has been described before in the E1-proteins section. The next E4-derived protein called orf4 is not essential for adenoviral growth (118), but is able to block transcription activation induced by E1A (36, 166, 227), auto-regulates its own transcription (36), regulates adenovirus alternative RNA splicing (154), induces hypophosphorylation of a variety of both viral and cellular proteins (154, 227), induces p53-independent apoptosis (203, 204), and induces cell cycle arrest (173). Protein orf4 exhibits all these functions by its binding to serine/threonine specific protein phosphatase 2A (PP2A) (166).

The last protein derived from the E4 region is called protein orf6/7 since it exists as a fusion protein obtained through alternative splicing. This protein is known to interact with the cellular transcription factor E2F (143). As mentioned before E2F is an important protein involved in the cell cycle (234). Through the interaction with E2F, orf6/7 activates the adenovirus E2 promoter and the E2F-1 promoter itself (269, 288) that is considered crucial in the adenoviral life-cycle.

Intermediate phase
The start of DNA replication initiates the expression of two intermediate phase proteins called IVa2 and IX. Both proteins activate the major late promoter (MLP) (197, 198), which is essential for expression of the late genes (see below). For IVa2 it has been demonstrated that activation of MLP is achieved only when IVa2 properly interacts with protein 33K derived from L4 (5, 197). Besides this primary role, protein IVa2 is part of a multi-protein complex that is involved in the packaging of adenoviral DNA (335, 383, 384) through binding to the packaging domain. The latter could be mediated by IVa2, since it has been demonstrated to interact with packaging sequences (244, 253, 382), possibly in conjunction with the proposed 22K protein derived from L4 (243). Also for protein IX multiple distinct functions have been proposed next to its role as MLP activator, such as involvement in nuclear reorganization induced by adenovirus (278), full length viral DNA packaging (105) and ensuring proper interaction between hexon proteins (95, 310).

Late phase of adenoviral replication
The late phase proteins are transcribed from one single promoter, the major late promoter (MLP) (Fig. 3). The proteins produced in the late transcription phase are the structural proteins that together form the adenovirus particle (minor and major capsid proteins) or are proteins that are essential for the encapsidation and maturation of the adenovirus particle. In the early phase of infection the MLP is already active albeit at low level, leading to transcription only up to L3 and a mRNA production of L1-52.55K (235). The MLP becomes fully activated upon adenovirus DNA replication, which is accomplished as described by protein IX (198) and protein IVa2 together with protein L4-33K (5, 197). In the late phase the MLP transcription proceeds up to L5, leading to a very large primary RNA transcript from which multiple mRNAs are produced through alternative 3’splice sites and five different poly(A) sites (296). All mRNAs contain the tripartite leader (TPL) sequence, which is important for nuclear export, and translation of the mRNAs (145, 195).
Replication-incompetent adenoviral vectors

**L1 proteins**
Late region-1 (L1) codes for 2 proteins called 52.55K and the minor capsid protein IIIa. Protein IIIa is a monomer that is in contact with four hexon proteins on the edge between facets (Fig. 1). In this way, IIIa stabilizes the interfaces between the facets in the adenoviral capsid (310, 342). The other L1 protein 52.55K plays an important role in adenoviral DNA packaging and virus particle assembly (116, 122). The precise mechanism of action is not determined yet. Nonetheless, it has been shown that 52.55K is able to bind to protein IVa2 (117) and that it is associated with the packaging domain (244). However, further studies illustrated that the binding of 52.55K to the packaging domain is not associated with protein IVa2 but dependent of another yet unknown protein (252, 253).

**L2 proteins**
The four proteins derived from the L2 region are called penton base, VII, V and X. The penton base has been described before and is an important structural protein of the capsid situated at the twelve vertices and anchors the fiber protein to the capsid (Fig. 1). Protein VII, one of the core proteins, associates with the adenovirus DNA inside the capsid together with protein V and protein X (54). Protein VII has been shown to interact with protein IVa2, protein 52.55K and unpackaged adenoviral DNA, suggesting a role for VII in an adenoviral DNA packaging complex (381). In addition, protein VII is involved in the nuclear entry of the adenoviral DNA upon infection (362). Protein V is one of the minor capsid proteins and forms a connection between protein VI and protein VII or the DNA (54, 211), thereby connecting the adenovirus DNA to the capsid. In adition, a recent study using an adenovirus 5 vector deleted for protein V suggested that this protein may be important for correct viral particle assembly and that it could play a regulatory role in adenovirus-infected cells (336). For protein X (mu), besides being one of the core proteins, not much is known. Although it has been hypothesized that it may have a role in the condensation of the adenovirus DNA (8).

**L3 proteins**
The L3 region expresses three proteins called protein VI, hexon and the protease (23K). Hexon is the major component of the adenovirus particle as described earlier. The hexon appears to be the major target of the immune system of the host, proven by the identification of epitopes for neutralizing antibodies (254, 317), CD8⁺ T lymphocytes (323) and CD4⁺ T lymphocytes (127) in the hexon protein. Minor capsid protein VI is, as most of the adenoviral proteins, a multi-functional protein. In the capsid it is positioned near the penton bases, where it connects the bases of the peripentonal hexons and connects the capsid to the DNA core through its connection with protein V (310). During cellular internalization upon infection, VI has a role in the endosomal escape of the viral particle by working as a membrane lytic factor (357). Near the end of the infection phase, protein VI plays a role in recruiting the hexon protein to the nucleus for assembly of the adenoviral particle (363). Finally, VI works as a stimulant for 23K protease activity (202). The last protein derived from L3 called 23K, is a protease (350) that is packaged within each viral particle released (53) and is essential for the adenoviral uncoating process that makes the cellular infection successful (113). The activity of the protease is upregulated by the adenovirus DNA and protein VI (202). In addition, the protease cleaves several minor capsid and core proteins during viral maturation, which is crucial for the stability of the adenovirus particle (212).

**L4 proteins**
The L4 region expresses three proteins, which are called 100K, 33K and protein VIII. The 100K protein is involved in hexon trimer formation (51), ribosome shunting, and preference of the translation of viral mRNA in the late phase of infection through specific binding to
adenoviral tripartite leader mRNAs (368). Finally protein 100K is involved in inhibition of granzyme B-mediated cell death (11). Protein 33K is derived from a spliced version of a L4 transcript (239). The 33K protein has been reported to be involved in the adenoviral particle’ assembly (86, 87). Additionally, 33K plays together with protein IVa2 a role in MLP activation (5) and is required to turn on full late gene expression (84), indicating that 33K is involved in both transcriptional and posttranscriptional mechanisms. Recently it has been found that at the start site of the 33K transcript, an alternative 22K transcript also initiates (243). The proposed 22K protein is found to bind the packaging domain together with IVa2 and truncation of 22K blocks the production of infectious virus (243). The last protein derived from the L4 region called protein VIII is one of the minor capsid proteins shown to interact with the inner surface of the hexon proteins in the particle (342). It is further hypothesized that VIII works as a “tape measure” determining the viral particle size (3) and that it is involved in the particle stability (190).

**L5 protein**

The only protein derived from L5 is the adenovirus fiber. The fiber protein is involved in binding to the cellular receptor as described earlier. The fiber protein determines the adenoviral tropism through fiber knob mediated binding to a specific cellular receptor. Additionally not only the fiber knob determines the transduction of cell, but also the fiber shaft domain plays a role (298). The fiber shaft is composed of several pseudorepeats of which the amount varies between serotypes. It was illustrated that by using different fiber shaft length the CAR-dependent transduction of cells could be altered; by using a long fiber shaft the transduction was more efficient in comparison to the use of a short fiber shaft (298). This indicates the role of the fiber shaft in adenoviral cell transduction.

**Virus-associated RNAs**

Besides all the proteins described above with clear time dependent expression, a number of viral RNAs are produced in the infected cells that exert important roles in the adenovirus replication cycle. These RNAs are called virus-associated (VA) RNA genes I and II and are transcribed first in the early phase and more abundantly in the late phase of the adenoviral infection. The genes encoding for the VA RNAs are the only genes in the adenoviral genome that are transcribed by RNA polymerase III (210). VA RNA I is required for the relocation of adenoviral mRNA from the nucleus to the cytoplasm (319), for efficient initiation of translation of the adenoviral mRNAs in the late phase (324), and in the defense against the host immune response evoked by dsRNA through inhibition of protein kinase R (PKR) (164) and the RNAi pathway (10). Little is known about the activity of VA RNA II. It has been demonstrated that VA RNA II is able to inhibit RNA helicase A (187) and that, like VA RNA I, VA RNA II interferes with the RNAi pathway (10). Not all human adenoviruses express two VA RNAs. Subgroup A, B2 and F adenoviruses express only one VA RNA (162). Whether this VA RNA gene has the same function as VA RNA I and II of the others is not known.

**Adenoviral vectors and the host immune response**

There are several features of adenoviruses that make them promising vectors for gene therapy and vaccination. First, adenoviruses are able to infect a wide range of tissues, and expression occurs regardless of the cell cycle status. Second, the genomes of Ad5, Ad2, and a variety of other serotypes are fully sequenced and well characterized, and these viruses have not been associated with severe disease. Third, initiation of the adenovirus replication cycle is tightly controlled by expression of E1 proteins. Therefore the generation of E1-deleted replication deficient vectors has easily been achieved using cell lines expressing the
Replication-incompetent adenoviral vectors

E1 protein in trans in order to complement the lack of E1 in the vector. Fourth, expression of foreign DNA under the control of heterologous promoters located in the former E1 region has been extremely successful, resulting in high levels of transcription and protein expression of foreign genes. Finally, mammalian cell lines have been constructed that can support large-scale generation of adenoviral vectors for clinical applications.

First generation adenoviral vectors are deleted for the E1 region, or the E1 plus E3 regions (Fig. 4). Subsequently, vectors have been constructed in which an additional early transcription region, E2A, was deleted, and these vectors are commonly known as second generation vectors (Fig. 4). Finally, vectors have been constructed for which essentially the entire adenovirus genome was deleted except for the ITRs and packaging domain (Fig. 4). These crippled or “gutless” vectors, also called third generation vectors, are completely dependent on helper viruses in order to replicate. The advantages and disadvantages of each generation of vectors are described below.

**Figure 4:** Schematic representation of the first, second and third generation of Ad5-based vectors.

The deletion of certain early transcription regions is indicated by a cross. The deletion of the E3 region is optional for the first and second generation vectors.

**First generation adenoviral vectors**

The first generation of Ad5 based vectors contain deletions in the E1 (E1A and E1B) region. This deletion renders them replication incompetent, since the activation of the E2 region required for DNA replication is strictly dependent on E1, as described above. The deletion of the E1 region, which encompasses approximately 5 kb of genomic viral DNA, can subsequently be used for the insertion of a foreign DNA expression cassette. This expression cassette in general consists of a heterologous promoter such as the cytomegalovirus promoter (CMV), a foreign DNA insert coding for a protein of interest (transgene) and a heterologous transcription termination signal (typically derived from SV40). For propagation and production of these E1-deleted vectors, E1 has to be provided in trans and therefore specific mammalian packaging cells expressing the Ad5 E1 proteins
have been generated. The first of such cell lines became known as HEK 293 cells (112). These cells contain approximately 4300 bp of the left end of Ad5 genome including the E1 coding domain as well as the left ITR, the packaging domain, and DNA coding for protein IX (129). This cell line allows efficient propagation of E1-deleted Ad5 vectors, however HEK 293 cells also allow the generation of replication-competent adenoviruses (RCA) (194). RCA can emerge from HEK 293 cells through a recombination event between overlapping sequences in the adenoviral vector and the E1 region sequences present in the cellular HEK 293 DNA. The presence of RCA in an adenoviral batch represents a potential safety risk. For this reason, a new E1-complementing cell line has been developed referred to as PER.C6® cells (82). PER.C6® cells do not result in RCA since no sequences other than the strict Ad5 E1 region are present. This has proven to avoid homologous recombination between the E1-deleted adenoviral vector and sequences within the cell line and thus the generation of RCA is prevented.

In addition to the E1 deletion in first generation adenoviral vectors, the E3 region can also be deleted. This has the advantage that it allows the insertion of larger expression cassettes (up to 8 kb). Since the E3 region is not essential for adenoviral growth, as described above, the loss of E3 does not have to be compensated. Therefore, the double E1/E3-deleted adenoviral vectors can also be propagated and produced on E1-complementing cell lines. In general, given the known functions of the E3-derived proteins, the E3 region is present in vectors used in gene therapy where long-term expression of the transgene is desired. In contrast, vectors are deleted for the E3 region when the vectors are used for vaccination where strong immune responses are desired. Since adenoviral vectors do not integrate into the cellular host genome, expression of a transgene is transient (157, 186, 307), which represents a serious limitation for gene therapy against inherited genetic disorders or other targets that require long-term transgene expression. The rapid loss of transgene expression, i.e. within two weeks after administration, is in part explained by the vigorous host immune response raised against the vector (63, 371, 372, 374). Confirmation of the importance of the immune system in eradicating transgene expression came from in vivo experiments using first generation adenoviral vectors in immune-compromised mice which demonstrated that transgene expression in these mice could be obtained for a long period of time (26, 218). This host immune response could theoretically be elicited simply by exposure of the administered adenoviral capsids to the immune system, or could be provoked by de novo synthesis of adenoviral proteins. The reason for considering both options was the observation that, even in the absence of the E1 region, low background expression of both early and late proteins could be readily detected in infected cells (372, 374). Since at the time both options seemed valid, the field moved into improving adenoviral vectors by deleting more adenoviral genes, which resulted in the second generation vectors.

Second and third generation adenoviral vectors
To eradicate expression of either early or late proteins in infected cells upon exposure to an adenoviral vector, an additional deletion of the E2A gene was performed. This resulted in second generation adenoviral vectors. Since the E2A region is essential for adenoviral replication, an additional cell line had to be generated that complemented the E2A gene function in trans (387). At first, the need for such a novel E1-E2A trans-complementing cell line was avoided by the use of a heat sensitive mutation (H5ts125) in the E2A gene (287). This mutation allows for growth and production of the adenoviral vector at the permissive temperature of 32°C, but not at 39°C. Initial results with this vector were encouraging, demonstrating less de novo production of adenoviral proteins in cell culture experiments and prolonged transgene expression in mice (79, 80, 107, 373). However, risk of virus reversion to the E2A wild type coupled to leaky E2A expression at 37°C of the H5ts125 mutation,
further stimulated the development of adenoviral vectors carrying a complete E2A deletion. 
Surprisingly, this second generation vector did not meet the expectation raised by the H5\textsubscript{ts}125 mutant vector (196) for reasons poorly understood. One plausible explanation was offered by finding that with the H5\textsubscript{ts}125 mutation low-level DBP was still produced in infected cells. The DBP down regulates expression of the E4 gene products 6 hours post infection (120, 236). Therefore, it was hypothesized that complete lack of DBP could result in prolonged expression of E4-derived proteins with possible negative impact on host immune responses launched against vector infected cells, resulting in diminished transgene expression (110). This hypothesis resulted in further attenuation by deleting the E4 region. Our own unpublished results using an Ad5 vector deleted for E1 and E2A as well as E4 failed to demonstrate prolonged transgene expression in mice. Furthermore, this vector failed to show any advantage in vector mediated toxicity as compared to a first generation vector in rats, as measured by identical dose dependent liver pathology, reduced platelet counts and liver enzyme levels (alanine transaminase) measured in blood that is indicative for liver damage (unpublished results).

**Figure 5:** Cre-lox system for third generation vector growth.

In this system loxP-sites are inserted around the packaging domain of the helper virus to prevent helper virus packaging through Cre-recombinase. CRE escape (1), inversion (2) and packaging domain recombination (3) can however still occur.

Third generation vectors (also called helper-dependent or “gutless” vectors) are deleted for almost the complete adenoviral genome, except for the ITRs and the packaging domain. Consequently, an expression cassette of up to 37 kb can be inserted. This in itself poses challenges since it is known that an adenoviral genome smaller than 27 kb poorly replicates (249). Thus in order to obtain a insert of the proper size, stuffer DNA must be used which does not influence transgene expression but also does not encode for any other protein (247). Another point of concern for third generation adenoviral vectors is the need for a helper virus during manufacture and the inability to eradicate this helper virus post production (88, 169). Potentially the helper virus may than induce an immune response. A strategy developed to reduce helper virus contamination is the use of the Cre-lox system. In this system loxP-sites are inserted around the packaging domain of the helper virus to prevent helper virus packaging through Cre-recombinase (248). However, complete loss of contamination with helper virus is not yet achieved, since CRE escape, inversion and packaging domain recombination can all occur (Fig. 5). Several in vivo studies have demonstrated encouraging results in that both expression and detection of the transgene DNA was prolonged in pre-clinical models (72, 77, 217). However, other reports
demonstrated that third generation adenoviral vectors still induce adenoviral-specific T lymphocyte responses (230, 280).

The lack of convincing data demonstrating the advantage of further attenuated second and third generation vectors in escaping the immune response turned the focus of the field in investigating the antigenicity of the adenoviral particle itself. A convincing study administering biologically inactivated adenoviral vectors in mice (153), together with the anti-vector responses observed for the third generation vectors (230, 280), demonstrated that the adenoviral particle itself plays an important role in the elicited immune response.

**Figure 6:** Uptake of adenoviral particles by DC.

The uptake of adenoviral particles by DC and subsequent antigen presentation in the lymphnode results in activation of CD8+ CTL. The CTL than directly kills adenovirus infected cells upon antigen presentation.

**Immune responses against adenovirus vectors and the transgene**

Further detailed investigations of the antigenicity of adenoviral vectors revealed that the adenoviral particle induced anti-vector cellular immune response through mediation of the innate immune response (228, 229, 291). The importance of the adenoviral capsid for induction of an innate immune response was exemplified by a study which showed that the hexon protein alone has adjuvant-like qualities since it was capable of inducing a cell mediated immunity (CMI) (222). Cells exposed to adenovirus react by releasing pro-inflammatory cytokines and chemokines including TNF-α, IL-6, IL-8, IL-12, IFNγ, MIP-2, MIP-1β, IP-10 and RANTES (229, 245, 291, 385). These cytokines and chemokines ensure that the infection remains local by inhibiting viral protein synthesis and ensure attraction and activation of effector cells (e.g. granulocytes, natural killer (NK) cells, dendritic cells (DC) and macrophages) (115). The effector cells do not only execute cytolytic functions at the site of infection but also secrete additional cytokines and chemokines that aid the adaptive
Replication-incompetent adenoviral vectors

immune response. In addition, uptake of adenoviral particles by DC and macrophages and subsequent antigen presentation results in activation of CD8\(^+\) CTL and CD4\(^+\) T-lymphocytes (Fig. 6) (385).

The first evidence that an immune response can be obtained against an inserted antigen was demonstrated by showing prolonged expression in mice of a mouse antigen as compare to the same vector carrying the human equivalent (332). Another elegant study demonstrating the immunogenicity of the transgene utilized adenoviral vectors carrying human factor IX (FIX) that were administrated to mouse strains with different genetic backgrounds. This study proved that expression of FIX depended on the type of immune response elicited (humoral versus cellular) and on the tolerance of the mouse strain to the transgene (218). The induction of an innate immune response and the capability of inducing a transgene-specific immune response are clearly serious limitations for the use of these vectors for gene therapy purposes. However, these studies triggered interest in the adenovirus vector system for vaccination, since the induction of vigorous immune responses are desirable features of a vaccine carrier. The potential of adenoviral vectors as vaccines has raised enormous interest in a variety of disease models. Most of these studies employ Ad5 since this vector system was readily available. The remainder of this chapter will describe the scientific findings thus far on the use of adenoviral vectors for vaccination purposes, in particular in the field of infectious diseases.

Adenoviral vectors for vaccination

The rationale for using adenoviral vectors as vaccine carriers is based on the many observations demonstrating the vigorous host immune response upon adenoviral vector administration. This section will review the advantages and disadvantages of Ad-based vectors as vaccine carriers.

Table II: Infectious agents to which Ad5-based vaccine vectors are developed.

<table>
<thead>
<tr>
<th>Infectious agent</th>
<th>Gene of interest</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human/simian immunodeficiency virus</td>
<td>Gag, Env, Pol</td>
<td>(48, 49, 184, 207, 286, 302)</td>
</tr>
<tr>
<td>Malaria</td>
<td>CS</td>
<td>(42, 106, 272, 273)</td>
</tr>
<tr>
<td>Ebola</td>
<td>GP, NP</td>
<td>(313, 314, 347)</td>
</tr>
<tr>
<td>Marburg virus</td>
<td>GP</td>
<td>(346)</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>E, prM</td>
<td>(135, 150, 159)</td>
</tr>
<tr>
<td>Influenza</td>
<td>HA</td>
<td>(102)</td>
</tr>
<tr>
<td>SARS</td>
<td>spike, membrane, nucleocapsid protein</td>
<td>(103, 193, 380)</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>glycoprotein</td>
<td>(258, 370)</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>NS3, core, E2</td>
<td>(15, 246, 277)</td>
</tr>
<tr>
<td>Herpes simplex 2 virus</td>
<td>gB</td>
<td>(99, 100)</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Ag85A</td>
<td>(348)</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>PA</td>
<td>(213, 214, 322)</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>V antigen</td>
<td>(55)</td>
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</table>

Pre-clinical and clinical studies using Ad5 based vaccines

As mentioned above, many vaccine studies have been performed using adenovirus 5 (Ad5)-based vaccine vectors. An overview of such studies is provided in Table II. A common denominator of these studies is that they target human pathogens that require induction of cell-mediated immunity (CMI) as well as antibodies. Ad5-based vaccines are potent
inducers of transgene-specific CD8+ T lymphocytes. In addition to transgene-specific CD8+ T lymphocytes, Ad5-based vaccines also induce transgene-specific antibodies and CD4+ T lymphocytes. Several landmark studies have been reported demonstrating the potency of Ad5-based vaccines. For instance, a single administration with Ad5 vectors has proven to protect either mice and monkeys upon challenge against various infectious pathogens (55, 99, 100, 102, 258, 273, 313, 322, 370). As candidate AIDS vaccines, Ad5 vectors have achieved partial protection against SHIV and SIV in the non-human primate model (302). Based on these promising pre-clinical studies, a number of Ad5-based vaccines have entered human clinical trials (a partial list is shown in Table III). Recently, the first two phase I clinical trials for Ad5 based vectors have been finalized. The first included an Ad5-based vector expressing HIV clade B Gag or a combination of Ad5-based vector expressing clade B Gag, Pol and Nef (76). The Ad5-based vaccines proved well-tolerated, safe and transgene-specific T cell responses were readily detected in approximately 70% of the participants. Following this phase I clinical study a phase IIb proof-of-concept study was launched with the Ad5-based vector expressing clade B Gag, Pol and Nef. This study aims to answer the pivotal question regarding whether cell-mediated immunity will prevent or control HIV in humans (59). The other phase I clinical trial completed utilized a vaccine consisting of a 3:1:1:1 ratio of Ad5-based vectors expressing HIV clade B Gag-Pol fusion protein, HIV clade A Env, HIV clade B Env and HIV clade C Env (50). This study demonstrated induction of transgene-specific CD4+ and CD8+ T lymphocytes with 93.3% and 60% response rates respectively. Additional studies are evaluating whether priming with a DNA vaccine can further potentiate immune responses obtained with Ad5-based vectors. This DNA prime, Ad5 boost regimen is expected to enter phase IIb testing later this year.

Table III: Ad5-based vaccine vectors currently in clinical trials.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Regimen</th>
<th>Phase</th>
<th>Sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 clade B Gag, Pol and Nef, and clades A, B and C Env</td>
<td>p: DNA b: Ad5 vector</td>
<td>I</td>
<td>NIH VRC</td>
</tr>
<tr>
<td>Ebola Zaire and Sudan-Gulu GP</td>
<td>p: Ad5 vector b: Ad5 vector</td>
<td>I</td>
<td>NIH VRC</td>
</tr>
<tr>
<td>P. falciparum CSP and AMA1</td>
<td>p: Ad5 vector b: Ad5 vector</td>
<td>IIa</td>
<td>USAID, CDMRP, MIDRP</td>
</tr>
<tr>
<td>HIV-1 clade B Gag, Pol and Nef and clades A, B and C Env</td>
<td>p: DNA b: Ad5 vector</td>
<td>IIb</td>
<td>Merck</td>
</tr>
<tr>
<td>HIV-1 clade B Gag, Pol and Nef and clades A, B and C Env</td>
<td>p: Ad5 vector b: Ad5 vector</td>
<td>I/IIa</td>
<td>NIH VRC</td>
</tr>
</tbody>
</table>

Prime (p), boost (b), National Institutes of Health Vaccine Research Center (NIH VRC), United States Agency for International Development (USAID), Congressionally Directed Medical Research Programs (CDMRP), Military Infectious Diseases Research Program (MIDRP).

The use of prime-boost regimens is a strategy to increase the immunogenicity of Ad5-based vectors. In pre-clinical studies it was shown that heterologous prime-boost regimens, either Ad5-vector in combination with DNA vaccines or poxvirus vectors, were substantially more potent than homologous prime-boost regimens (Ad5-vector prime and Ad5-vector boost) (48, 106, 207, 213, 246, 286, 302, 348) and achieved and/or improved protection against a variety of pathogens (42, 106, 184, 213, 302, 314). Although these studies with prime-boost regimens have shown improvement on immune induction, from a product perspective the combined use of two different vaccine components poses challenges to the vaccine industry. However, results obtained with homologous Ad5 prime-boost regimens have thus far been disappointing. Reason for the latter are the very high and potent neutralizing antibody response induced against the Ad5 vector after the initial priming vaccination that largely prevent effective homologous boosting with the same vector. These findings have propelled investigation into the worldwide seroprevalence of Ad5 in human populations.
The problem of Ad5 pre-existing immunity and possible solutions

Pre-clinical studies using both mice and non-human primates have shown that pre-existing anti-Ad5 immunity blunts the immunogenicity (Fig. 7) of Ad5-based vaccine vectors (18, 48, 375). These findings have been confirmed by results obtained from the completed phase I human trials as described earlier (50, 76). In mice, it has been demonstrated that both Ad5-specific neutralizing antibodies (NAbs) and T lymphocytes play a role in the anti-Ad5 immunity, with the Ad5-specific antibodies being the most important component (215, 316). In humans, Ad5-specific NAbs are highly prevalent and in certain regions (e.g. sub-Saharan Africa) NAb titers are very high (136, 174, 237, 344). Ad-specific CD4+ and CD8+ T lymphocytes have been found in humans (128, 323), although the importance of Ad-specific T lymphocyte responses remains unclear. The high prevalence of anti-Ad5 immunity in the human population is a potential major limitation to the utility of Ad5-based vaccine vectors. Pre-existing anti-Ad5 immunity could reduce the immunogenicity and protective efficacy of Ad5-based vaccines as well as prevent accurate dose control.

Currently several research strategies are being pursued to tackle the problem of Ad5 pre-existing immunity. One strategy is exploring the possibility that Ad5-based vaccines may still be effective as a boosting agent following an efficient alternative priming agent. Therefore, a combination of a DNA vaccine prime and Ad5 vector boost is being investigated (18, 49, 375). A second strategy is based on the generation of adenoviral vectors that are derived from alternative human adenoviruses. To identify human adenoviruses with low seroprevalence among human populations, seroprevalence surveys using sera derived from healthy adults are conducted (237, 344). These surveys resulted in a panel of human adenoviruses that have a low seroprevalence and low NAb titers in individuals who are seropositive (174, 237, 344). Vectors derived from one of these rare human adenoviruses, adenovirus 35 (Ad35), have been studied as vaccine carriers for HIV/SIV (20, 27), malaria (241) and tuberculosis (123, 264). Pre-clinical studies in mice (20) and non-human primates (27, 233) have demonstrated that an Ad35-based vaccine is capable of inducing potent antigen-specific CD4+ and CD8+ T lymphocyte responses against SIV Gag and HIV Env. Furthermore, an Ad35-based vector expressing the Plasmodium yoelii circumsporozoite (PyCS) protein resulted in a 96% inhibition of liver infection upon high Plasmodium yoelii sporozoite challenge (241). In addition, a tuberculosis challenge model demonstrated the potency of an Ad35-based vector expressing the tuberculosis
antigens Ag85A, Ag85B and TB10.4 as a fusion protein (Ad35.TBS) and protective efficacy against challenge with *Mycobacterium tuberculosis* (264). These studies also demonstrated that in contrast to Ad5, an Ad35-based vaccine retained its full potency in presence of experimentally induced high levels of anti-Ad5 immunity (see Fig. 7). Given these promising results in pre-clinical models, Ad35-based vectors are now the first vectors derived from a rare-serotype adenovirus to enter phase I clinical trials. A third strategy aims to the construction of chimeric Ad5 vectors capable to escape anti-Ad5 immunity. However, the exact mechanism behind the anti-Ad5 immunity is not well understood. Conflicting data are published in respect to which component(s) of the adenoviral capsid (hexon, penton or fiber) is/are the main target of the NAb (97, 98, 137, 283, 378). At least one study has demonstrated that an Ad5-based fiber chimeric vector, expressing the fiber of Ad35 (Ad5f35), was not able to escape anti-Ad5 immunity (240), suggesting that antibodies directed towards the fiber do not play an important role in the anti-vector immunity. Therefore, additional studies to better understand which adenoviral capsid components are the main targets for NAb are essential to construct rAd5-based vectors that are no longer hampered by anti-Ad5 immunity. Finally, another strategy to circumvent anti-Ad5 immunity exploits vector systems that are derived from non-human adenoviruses, such as ovine (133), porcine (266), bovine (267), and chimpanzee (89, 270, 284). In particular, the chimpanzee rAd vaccine vectors are pursued for human vaccine development. They have been shown to induce both potent transgene-specific CD8⁺ T lymphocytes and antibodies in mice (89, 168, 255, 284, 369) and to afford protection upon otherwise fatal Ebola challenge (168, 284). Most importantly, chimpanzee rAd vaccine vectors are only marginally affected by anti-Ad5 immunity (89, 255). Together these studies demonstrate that the use of non-human adenoviral vectors for vaccination is feasible. However, understanding the potency of these non-human vectors as well as their safety profile awaits the initiation of human clinical trials. Regulatory issues associated with the clinical development of chimpanzee adenovirus vectors will likely prove more complex than for human serotype adenoviral vectors.
HIV/SIV-specific CD8⁺ T lymphocytes are convincingly shown to be important for the control of HIV/SIV viremia in humans and monkeys respectively (38, 289). Both preclinical and clinical studies have illustrated the potency of rAd5-based vaccine vectors to elicit HIV/SIV-specific CD8⁺ T lymphocytes. Unfortunately, the high prevalence of anti-Ad5 immunity in the human population poses a threat to the usage of rAd5-based vectors as vaccine vehicles. Alternative strategies, such as chimeric rAd5-based vectors capable of escaping anti-Ad5 immunity may be a solution. However, to be able to construct such chimeric vectors, a better understanding of anti-Ad5 immunity is pivotal. Therefore, we aimed to determine the main target of the Ad5-specific NAb in chapter 2.

During the first vaccine potency studies with the rAd35 vector in comparison to the rAd5 vector it became apparent that the Ad5-based vector was superior in naïve mice but, as expected, not in the mice with anti-Ad5 immunity (20). The reason for the higher immunogenicity of Ad5-based vectors in naïve settings is currently not well understood. A possible explanation may be the usage of different cellular receptors by rAd5- and rAd35-vectors, resulting in different intracellular trafficking pathways (see section 1.3: Tropism and internalization). To investigate the role of the Ad5 fiber knob on the immunogenicity of the vaccine vector, a capsid chimeric rAd35 containing the fiber knob of Ad5 (rAd35k5) was constructed in chapter 3 and its immunogenicity in comparison to rAd5 and rAd35 was assessed.

Even though the rAd35 vector is less immunogenic in naïve settings it still warrants a solution to the limitations for the Ad5-based vaccine vector caused by the high prevalence of anti-Ad5 immunity in the human populations. As shown for the Ad5-based vectors, rAd35 homologous prime-boost regimens elicited far less potent transgene-specific immune responses than a heterologous rAd35 vaccine regimen (20). However, any heterologous prime-boost regimen that includes the rAd5 vector may still substantially be limited by the presence of anti-Ad5 immunity. Therefore, alternative Ad-based vectors with low seroprevalence worldwide are still needed to expand the possibilities for prime-boost vaccine regimens. The remainder of the chapters in this thesis focuses on the construction and HIV/SIV-vaccine potency of several newly developed rare human Ad-based vectors: rAd11 in chapter 4, rAd49 in chapter 5 and 6, and rAd26, rAd48 and rAd50 in chapter 7.