HIV IMMUNOTHERAPY

host immunity and virus evolution

Anna de Goede
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HIV IMMUUNANTHERAPIE
gastheer-immuniteit en virus evolutie

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GENERAL INTRODUCTION
HUMAN IMMUNODEFIENCY VIRUSES

HIV is a lentivirus belonging to the family of Retroviridae, causing acquired immunodeficiency syndrome (AIDS). Retroviruses are enveloped viruses using reverse transcription of viral RNA into DNA during replication [1]. Two closely related but distinct types of HIV have been characterized: HIV-1 and HIV-2. Both HIVs originate from multiple cross-species transmissions of simian immunodeficiency viruses (SIV) that naturally infect African primates. Zoonotic transfers gave rise to viruses that can spread in humans. The HIV-2 lentivirus originates from the transmission of SIV from sooty mangabey to humans [2]. The majority of HIV-2 infections have been found in West Africa and, with lower prevalence rates in countries with historical and socio-economic ties such as Portugal, France, Brasil, India and the United States (2, 3). The considerably more virulent HIV-1 originates from the transfer of SIV from the chimpanzee to humans [4] and shows marked genetic variability. The pandemic form of HIV-1, also called the main (M) group is the major cause of AIDS. The other three HIV-1 lineages, termed group N, O and P are considerably less prevalent than group M. Each lineage results from an independent cross-species transmission event [5]. Group M is genetically further diversified into subtypes or clades designated by letters A to K. In different parts of the world, different subtypes of HIV-1 predominate [6]. Subtype B is the most common form in West and Central Europe, Australia, North and South America, and several Southeast Asian countries, northern Africa and the Middle East. The epidemic global spread of HIV-1 began in the late 1970s. By the end of 1981, a total of 270 cases of severe immunodeficiency among homosexual men were reported and a year later the term AIDS was formally established [7].

VIRAL REPLICATION CYCLE

The HIV replication cycle begins with adhesion of the virus to the host cell and ends with fusion of the cell and viral membranes with subsequent delivery of the viral core into the cytoplasm (Figure 1). The first entry step is the binding of the gp120 portion of the envelope protein (Env) to its primary cell-surface receptor, CD4. Interaction of gp120 with CD4 results in rearrangement of the variable loops of gp120 enabling binding to a co-receptor, either chemokine receptor CCR5 or CXCR4 or an alternative, minor co-receptor such as CCR2, CCR3 or CXCR6 [8]. Upon binding of gp120 to CD4 and the co-receptor, the virus moves to the site where productive membrane fusion occurs (Figure 2). In the final step of HIV entry, the gp41 portion of Env inserts into the host membrane and causes a fusion pore of the viral envelope with the plasma membrane of the cell [9].
Upon fusion, the virion is uncoated and its contents are released into the cytoplasm. In the cytoplasm, the viral RNA genome is reverse transcribed into complementary DNA (cDNA) by the viral enzyme reverse transcriptase (RT). RT is a multifunctional protein and serves as a polymerase to copy the RNA and cDNA template and also has an RNase H function to degrade template RNA in the RNA-DNA duplex. The reverse transcription process yields the so-called HIV pre-integration complex (PIC) that is transported into the nucleus [10]. The PIC can cross the intact nuclear membranes of nondividing cells as a result of lentiviral Vpr function [11]. In the nucleus, the double stranded cDNA is integrated into the host-cell genome by the viral enzyme integrase. Once integrated, the proviral DNA is copied as part of cellular DNA during cycles of cell division. At this stage, the provirus may become latent and (re-)activation of infected cells is required for the production of new viral transcripts [12]. Viral RNA serves as a template for protein production as well as genomic RNA in progeny virions (Figure 1).

After (re-)activation of the HIV-infected cell, viral transcription is initiated and host cellular transcription factors NF-κB and NFAT that are expressed in activated T cells enhance viral replication [15, 16]. Control of viral gene transcription is exerted mainly by Tat and Rev, which act directly on viral RNA structures. Tat binds to the transcriptional activation region TAR in the nascent HIV-RNA transcripts and enhances elongation of viral RNA by the cellular RNA polymerase II complex [17]. In this early stage, all HIV transcripts are spliced by the host spliceosome. The multiple spliced mRNAs that are exported from the nucleus and are subsequently translated to encode the early expressed proteins Tat, Rev and Nef [18-20]. In a positive feedback, Tat further enhances viral RNA transcription. Rev binds the viral RNA Rev response element (RRE) through multimerization, enabling translocation of RNA from the nucleus before being spliced, thereby preventing splicing [21, 22]. This results in export of singly spliced mRNAs encoding Env and the accessory proteins and unspliced mRNAs encoding Gag and Pol and the viral genomic RNA for inclusion into virions [23]. All virion components need to traffic from their site of synthesis in the cytoplasm to the site of virion assembly at the plasma membrane. This process of trafficking and subsequent packaging of the virion components Gag, Gag-Pol, Vpr and genomic RNA is coordinated by Gag and Gag-Pol polyproteins. The assembled virion buds at the plasma membrane which provides the lipid envelope [24]. The budding process is arrested by the host antiviral protein tetherin that retains the newly budded viral particles to the cell surface. Tetherin is antagonized by viral Vpu, thereby allowing release of viral particles [25]. As the immature virion buds, viral maturation starts. PR gets activated and cleaves Gag and Gag-Pol polyproteins into fully processed proteins (MA, CA, NC, p6, PR, RT, IN) whereby the virion is converted into its mature state and gets ready to infect a new host cell and replicate therein [26-28].

VIRAL STRUCTURE AND PROTEIN FUNCTION

Each HIV particle, or virion, consists of 3 major elements: an outer coat of a lipid bilayered envelope (Env) containing viral glycoproteins, an associated matrix (MA) surrounding the capsid (CA) that ensures the integrity of the virus particle and an inner core which encloses two identical copies of the single-stranded RNA genome of 9600 nucleotides, polymerase and additional viral proteins, packaged by the nucleocapsid (Figure 3). The virus particle is about 100 nm in diameter [1].
The genome of the integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length and the ends are flanked by long terminal repeat (LTR) sequences [30]. The genome encodes at least nine proteins divided into three classes: major, structural proteins Gag, Pol and Env; regulatory proteins Tat and Rev; and accessory proteins Vif, Vpr, Vpu and Nef. The HIV-1 genome has a complex organization to enable the formation of 15 proteins from only 9800 nucleotides. From the nine open reading frames, primary translation products are produced and three of the primary polyproteins are subsequently cleaved to final proteins. Several genes overlap but are located in different reading frames and the regulatory proteins Tat and Rev are encoded in two exons per protein located in different reading frames (Figure 4).

The env gene encodes the glycoproteins of the viral envelope and are responsible for receptor engagement and virus-cell membrane fusion; its product gp160 has to be cleaved by host-cell proteases into a surface (SU) glycoprotein gp120 and transmembrane (TM) glycoprotein gp41 which form a trimeric complex at the viral membrane [32, 33]. The gag gene encodes the immature Gag polyprotein p55 from which several structural proteins for the viral core are derived by viral protease cleavage. The matrix protein (MA or p17) is associated with the inner viral membrane, whereas nucleocapsid (NC or p7) coats the viral RNA genome and the capsid (CA or p24) surrounds the nucleocapsid. A smaller peptide cleaved from Gag is p6 that coordinates membrane binding, Gag interactions in immature virions and virus budding [34]. The pol gene overlaps with the reading frame of gag and is synthesized by ribosomal frameshifting as part of a Gag-Pol fusion protein. Proteolytic cleavage by the viral protease, releases the three enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) that are essential to the viral infectious lifecycle: PR catalyses the proteolytic processing of the Gag and Gag-Pol precursor proteins, RT mediates...
the conversion of single stranded RNA into double stranded viral DNA and IN regulates the integration of the linear double stranded viral DNA into the host cell chromosome. The RT protein part of Pol is a heterodimer that consists of a large (p66) and a small (p51) subunit. By removal of the ribonuclease hybrid (RNase H) domain from p66, the subunit p51 is formed. Only by dimerization of the two subunits, RT is enzymatically active [35].

The tat gene consists of two separately located exons encoding the Tat (transactivator of transcription) protein that plays an essential role in virus replication [17]. Tat promotes the elongation phase of transcription by stimulating viral RNA polymerase II resulting in the production of full-length transcripts [36]. The arginine-rich functional domain of Tat binds to the transactivation responsive element (TAR) on the 5' end of all viral transcripts and the activation functional domain recruits cellular proteins to relieve the repression on the LTR of integrated provirus, both contributing to the synthesis of full length viral mRNAs [17]. A multitude of additional Tat functions have been proposed that contribute to HIV pathogenesis. Tat alters expression levels of genes involved in immune suppression and apoptosis: it downregulates the expression of human leukocyte antigen (HLA) class I molecules and upregulates several immune suppressive cytokines such as IL-10 and TGF-β, the HIV-1 CCR5 co-receptor and the IL-2 receptor. It also upregulates apoptosis inducing factors such as TRAIL and caspase 3 and 8 [17]. On the contrary, Tat can also inhibit apoptosis by upregulating the anti-apoptotic protein BCL-2, thereby promoting the establishment of HIV latency [17]. Microtubuli can be destabilized by Tat, resulting in the progressive loss of T cells. Acetylation of lysine residues in Tat may modulate the interaction of Tat with host cellular elements and contribute to the diverse and sometimes opposite effects of Tat [37]. Extracellular Tat can enter uninfected bystander cells and have its effects there [17]. The rev gene consists of two separately located exons encoding the Rev (regulator of expression of virion proteins) protein regulating late steps of the viral replication cycle required for the expression of the structural proteins Gag, Pol and Env. Rev binds to the Rev-responsive element (RRE) on nuclear viral RNAs, thereby enabling export of the RNA to the cytoplasm where the encoded proteins are expressed [18]. The RRE is an RNA structure present on unspliced RNA encoding Gag and Gag-Pol and on singly spliced RNA encoding Env [18]. The activity of the Rev-RRE complex fluctuates during the virus replication cycle, indicating that Rev plays a role in the control of viral replication [38]. During the early phase of replication, Rev was found to suppress viral integration, probably preventing superinfection with other viruses [18]. The nef gene encodes the accessory protein Nef (negative factor) and although its name suggests differently, Nef accelerates progression to immunodeficiency and viral replication. Nef decreases the expression of viral (co-)receptors at the cell surface, such as CD4, CCR5, and to a lesser extent CXCR4, to prevent superinfection [39, 40]. A decrease of CD4 also promotes Env incorporation and virion budding and thus increases virus production [40].

Other functions of Nef, such as the stimulation of Gag protein levels in infected cells and the transport of Gag to the cell membrane [41], further contribute to the enhancement of viral infectivity. Nef mediated downregulation of HLA class I decreases the efficiency of cytotoxic T cell (CTL) mediated killing of infected cells suggesting that it plays a central role in immune evasion [42-44]. Nef increases viral spread by several mechanisms: it counteracts, the antiviral host factor tetherin that normally prevents viros from budding off the host-cell membrane, and it favors cell-cell transmission of viros [39]. Moreover, it induces the formation of long-distance intercellular conduits targeting B-cells and evading systemic and mucosal IgG2 and IgA immune responses [45]. Nef affects T-cell activation by a mechanism that is not fully understood [39]. Moreover, apoptosis is modulated by Nef by both pro-apoptotic signals such as overexpression of apoptosis-inducing Fas Ligand and downregulation of Bcl-2, as well as cell survival increasing signals such as protection from apoptotic signals [39]. The multiple functions of Nef are maintained during chronic infection [46].

The vif gene encodes the accessory protein Vif (virus infectivity) factor which supports viral replication by overcoming the natural cellular defense against retroviruses. Vif promotes the proteasomal degradation of APOBEC3 deaminases produced by the host-cell to destroy the genetic information in viros by converting cytosine to uracil (dC into dU) in the negative strand of HIV cDNA [47]. The vpr gene encodes the accessory protein Vpr (viral protein R) promoting viral replication and pathogenesis by a wide range of functions. Vpr mediates nuclear import of the viral pre-integration complex, contributing to the permissiveness of non-dividing cells for HIV. Furthermore, it causes G2 cell cycle arrest and has inhibitory effects on the adaptive immune system [11]. Vpr induces T-cell apoptosis, regulates NF-kB activity and promotes the expression of a variety of viral and host genes involved in antigen presentation and cytokine production [11]. The vpu gene encodes the accessory protein Vpu (viral protein U) which is required for efficient release of viros from the surface of infected cells. The antiviral host factor tetherin, a membrane-associated protein that captures viros at the cell surface, is counteracted by Vpu [47]. A second function of Vpu is the degradation of newly synthesized CD4 [48].
Biology and Epidemiology of Transmission

Transmission of HIV typically occurs after the transfer of body fluids from an infected to an uninfected person, most commonly across mucosal surfaces by sexual intercourse and alternatively by contaminated needles for intravenous use, infected blood products or by vertical transmission from mother to child in utero, intrapartum or during breast feeding [49]. Dominant sites of primary infection are the gastrointestinal and genital mucosa. The probability of HIV transmission per exposure event depends on a wide range of risk factors such as transmission route, HIV disease state, plasma viral load, exposure route, male circumcision, genital ulcer disease, presence of other sexually transmitted infections (STI) and socio-economic factors [49]. Transmission of HIV depends on a variety of factors but is relatively inefficient with in general the lowest transmission risk for sexual contact (ranging from 1 in 20 to 1 in 2000 [49]).

Target cells

The main cells targeted by HIV are T cells, macrophages, monocytes and dendritic cells (DCs) [50]. This narrow tropism is predominantly determined by the cell surface receptors required for entry. The major receptor for HIV is CD4 and is expressed predominately on T-helper cells but also on monocytes, macrophages and DCs. Besides CD4, a co-receptor is required for fusion of HIV to the target cell [51]. Virus isolates are classified on their ability to use co-receptor CCR5 (R5 variants), co-receptor CXCR4 (X4 variants) or both (R5X4 variants).

In acute infection, replication of R5 variants is favored and X4 variants are not transmitted [52]. CCR5 is expressed on memory CD4+ T cells in multiple tissues, especially gut-associated lymphoid tissue (GALT) and these initial targets are massively infected and lost in primary HIV infection [53, 54]. CD4-independent infection is mediated through attachment to a variety of cell-surface receptors or pattern recognition factors such as the C-type lectin receptors langerin (CD207), mannose receptor (CD206) and DC-SIGN [55]. However, CD4-independent interactions with the host cell are generally inefficient [51]. HIV entry into the central nervous system that contributes to HIV neuropathogenesis is largely mediated through infected monocytes that differentiate into macrophages and lymphocytes that enter perivascular spaces and harbor viruses that replicate in macrophages [56].

Table 1 HIV proteins and their function

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus protein</th>
<th># copies per virion</th>
<th>Interactions with other viral factors</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gag</td>
<td>MA, matrix (p17)</td>
<td>~5000 TM</td>
<td>-</td>
<td>Plasma membrane targeting for virion assembly; Env incorporation; post-entry events</td>
</tr>
<tr>
<td>gag</td>
<td>CA, capsid (p24)</td>
<td>~5000 TM</td>
<td>-</td>
<td>Virion core structure and assembly</td>
</tr>
<tr>
<td>gag</td>
<td>NC, nucleocapsid (p7)</td>
<td>~5000 RNA genome of virus</td>
<td>-</td>
<td>Virion packaging of viral RNA; virion assembly</td>
</tr>
<tr>
<td>gag</td>
<td>p6</td>
<td>~5000 Vpr</td>
<td>Promotes virion budding</td>
<td></td>
</tr>
<tr>
<td>pol</td>
<td>PR, protease</td>
<td>~250 Gag, Pol</td>
<td>-</td>
<td>Proteolytic processing of Gag and Gag-Pol polyproteins</td>
</tr>
<tr>
<td>pol</td>
<td>RT, reverse transcriptase (p51-p66)</td>
<td>~250 RNA genome of virus, IN</td>
<td>cDNA synthesis; RNase H domain degrades RNA</td>
<td></td>
</tr>
<tr>
<td>pol</td>
<td>IN, integrase</td>
<td>~250 viral cDNA, RT</td>
<td>Insertion of virus cDNA into cellular DNA</td>
<td></td>
</tr>
<tr>
<td>env</td>
<td>SU, surface glycoprotein (gp120)</td>
<td>4-35 trimers TM</td>
<td>Virus binding to cell-surface receptors; mediates virus attachment and entry</td>
<td></td>
</tr>
<tr>
<td>env</td>
<td>TM, transmembrane glycoprotein (gp41)</td>
<td>4-35 trimers SU, MA</td>
<td>Fusion peptide; mediates membrane fusion and virus entry</td>
<td></td>
</tr>
<tr>
<td>tat</td>
<td>Tat, trans-activator of transcription</td>
<td>none viral RNA, via transacting response element (TAR)</td>
<td>Potent activator of viral transcription elongation</td>
<td></td>
</tr>
<tr>
<td>rev</td>
<td>Rev, regulator of expression of virion proteins</td>
<td>none viral RNA with introns, via Rev response element (RRE)</td>
<td>Induces nuclear export of intron-containing viral RNAs</td>
<td></td>
</tr>
<tr>
<td>nef</td>
<td>Nef, negative factor</td>
<td>cleaved by PR</td>
<td>CD4 and HLA down-regulation; T-cell activation; enhances viral infectivity; blocks apoptosis; pathogenicity determinant</td>
<td></td>
</tr>
<tr>
<td>vif</td>
<td>Vif, virion infectivity factor</td>
<td>1-150</td>
<td>Suppresses host infection restriction factors (APOBEC)</td>
<td></td>
</tr>
<tr>
<td>vpr</td>
<td>Vpr, viral protein R</td>
<td>~700 p6</td>
<td>Enhancer of post-entry infectivity; cell-cycle arrest</td>
<td></td>
</tr>
<tr>
<td>vpu</td>
<td>Vpu, viral protein U</td>
<td>none</td>
<td>CD4 and HLA down-regulation; induces virion release from host cell surface</td>
<td></td>
</tr>
</tbody>
</table>

CA, capsid; IN, integrase; MA, matrix; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; SU, surface glycoprotein; TM, transmembrane. Adapted from [27].
Viral quasispecies
Infection is in most cases established by transfer of only one or a few virus strains (called transmitted-founder viruses) and HIV is relatively monoclonal during the acute phase of infection [49]. Thereafter many variants are generated because of the rapid replication of HIV and the high mutation rate due to the error-prone retroviral replication. As a result, within a single infected human, the HIV population becomes heterogeneic and the genetically distinct but closely related variants are referred to as ‘quasispecies’. Within the quasispecies, wild type is determined as the most fit and most common variant [49, 57].

Epidemiology
HIV attacks the body’s immune system and without effective treatment the immune system is weakened to a stage where opportunistic infections and other illnesses occur, causing acquired immunodeficiency syndrome (AIDS). The number of individuals living with HIV or AIDS continues to grow but the incidence of new HIV infection is nowadays increasing more slowly in many regions of the world. In Eastern Europe and in Asia, the incidence of HIV infections is rising faster than in the rest of the world. In 2012, 35.3 million individuals were infected with HIV worldwide, including 2.3 million new cases, and more than 1.6 million deaths were related to AIDS [58]. Hence HIV/AIDS is the leading infectious cause of adult death in the world today and also fuels other epidemics of global concern, most notably opportunistic infectious diseases such as tuberculosis [58]. In the Netherlands, over 21,000 people are HIV-infected and 1,100 new diagnoses were made in 2012.

CLINICAL COURSE OF INFECTION

Following transmission, early HIV infection starts with the eclipse phase during which the virus replicates in the mucosa. The eclipse phase ends when the virus becomes first detectable in blood, ~ 1-2 weeks after infection (Figure 5). Thereafter, the virus spreads to the mucosa-draining lymph nodes where further replication occurs [60]. During this period, the virus disseminates via the bloodstream to secondary lymphoid tissue with a preference for the GALT where the highest numbers of effector memory CD4+ T cells in the body reside, mainly expressing co-receptor CCR5 [60]. The large number of replicating viruses in mucosa and circulating in the peripheral blood (viremia) causes extensive death of CD4+ T cells and up to 80% of the CD4+ T cells in the GALT can be depleted in the first 3 weeks of infection [61]. The clinical stages of acute infection, Fiebig stages, are based on the development of detectable concentrations of HIV-markers. When HIV-specific antibodies become detectable in blood by ELISA, ~ 3-4 weeks after infection, this is known as seroconversion, typically during Fiebig stage III when plasma viral load peaks [61]. At the time of peak viremia, up to 80% of the patients may develop symptoms such as influenza-like illness [62]. Death of infected CD4+ T cells occurs either through apoptosis or pyroptosis. Of the infected CD4+ T cells, 5% is activated and productively infected when circulating in peripheral blood. These cells die after 1-2 days of viral replication through apoptosis. In contrast, the other 95% of the CD4+ T cells that die during HIV infection are non-activated ‘bystander’ cells, mainly present in lymphoid tissues refractory to reproductive infection. It was recently proposed that these cells die through pyroptosis resulting in cell-lysis and the release of highly inflammatory cytokines [63, 64]. During the acute phase, CD8+ T cells that kill HIV-infected cells (cytotoxic T cells, CTL) become activated and control virus production, but do not eradicate the viral pool. Subsequently, the peak plasma viral load declines to reach a stable viral set point and CD4+ T-cell counts rebound to subnormal levels.
CD4+ T cells have a half-life of around 44 months [67]. The long-term survival of memory cells enables the immune system to respond rapidly upon re-exposure to an antigen. When latently infected cells become (re-)activated by cytokines or antigens, they produce virus that can spread to and infect other CD4+ T cells [66].

After the acute viremic phase, a chronic, asymptomatic phase of infection is initiated that lasts for about 10 years (range: 1-20 years) on average [71] (Figure 6). Although the name of the asymptomatic phase suggests differently, the virus still replicates and both the number and function of CD4+ T cells gradually decline. As a result of this gradual loss of immune competence, the asymptomatic phase turns into a more symptomatic phase that eventually, in the absence of treatment, will develop into full-blown AIDS [71]. The severity of HIV disease can be assessed based on the number of CD4+ T cells and the presence of HIV-related conditions. HIV-related conditions include recurrent bacterial pneumonia, candidiasis, cytomegalovirus disease, chronic herpes simplex ulcers, Kaposi sarcoma, B-cell lymphoma (especially Burkitt lymphoma), Mycobacterium infections, Pneumocystis carinii pneumonia, HIV-related encephalopathy and HIV-related wasting syndrome. AIDS is diagnosed when the immune system of an HIV-infected person becomes severely compromised with CD4+ T-cell counts <200 cells/µl and/or when the person becomes ill with an opportunistic infection or illness [72].

**IMMUNE RESPONSE AGAINST HIV**

Infection with HIV triggers the host immune system to eliminate the invading pathogen from the infected tissue. The two branches of the immune system, innate and adaptive, both play a critical role in the host’s defense against viruses and bacteria.

**Innate immune system**

The first line of defense against pathogens is formed by the non-specific innate immune system. The cells of this inborn system detect invading HIV rapidly after infection and try to control or eliminate it. Constituent cells of the innate immune system are granulocytes, DCs and macrophages. These are constantly patrolling mucosal tissues and other sites by a variety of pattern recognition receptors (PRR) on the cell membrane to detect invading viruses and recognize proteins and nucleic acids shared among viruses [73]. Examples of pattern recognition receptors are surface receptors such as Toll-like receptors (TLRs), DC-SIGN and mannose receptor as well as RIG-I and MDA-5 in the cytoplasm [55, 74]. During HIV infection, ligands derived from HIV itself or from opportunistic infections or translocated bacteria bind TLR 7/8 and TLR3 [75]. Once DCs and macrophages detect these ligands, they phagocytose...
the virus and digest it into small peptide fragments called epitopes that are displayed on the cell's surface; hence these cells are called antigen-presenting cells (APC). Subsequently, signal transduction pathways involving NF-kB, MAPK and interferon (IFN) regulatory factors (IRF) 3/7 are activated resulting in the activation of resting immune cells. The activated innate immune system produces cytokines, tries to eliminate the virus or limit its dissemination and initiates activation and shaping of the adaptive branch of the immune system [75]. The innate immune system cannot respond to or remember specific antigens, in contrast to the adaptive immune system. Based on this premise, natural killer (NK) cells are not strictly cells of the innate immune system. On the one hand they are innate effector cells that mediate antibody dependent cellmediated cytotoxicity and viral inhibition and the secretion of antiviral cytokines and chemokines, thereby providing the first wave of the antiviral attack [76]. On the other hand, NK cells have capacities characteristic of the adaptive immune system: they are capable of recognizing, targeting and remembering specific antigens suggesting that antigen-specific NK-cell memory exists [77]. As such, NK cells could shape the immune system by killing infected cells but dampen the adaptive immune response by killing activated CD4+ T cells [76]. Not only NK cells but also DCs can dampen the antiviral innate immune response. DCs can be subdivided in myeloid DCs (myDCs) as APCs initiating T-cell responses and plasmacytoid DCs (pDCs) as major producers of type I IFN and IDO. IDO is an immunoregulatory enzyme that leads to apoptosis of effector T cells and induction of regulatory T cells (Tregs) thus restraining the immune response [78]. But pDCs were demonstrated capable to (cross-)present antigens as well [79]. The innate immune system partly controls viral replication in the earliest phase of infection and becomes deregulated during chronic infection [61].

As part of, or even preceding innate immunity, host-encoded cellular restriction factors provide a defense mechanism against viruses. Four anti-HIV host factors are: the apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like 3 (APOBEC3) family, tetherin, tripartite-motif-containing 5α (TRIM5α) and SAMHD1 [47, 80]. HIV-1 generally evades the viral capsid thereby disrupting the architecture of the reverse transcriptase complex and blocking reverse transcription [47]. SAMHD1 has recently been discovered to limit viral replication by decreasing the amount of intracellular nucleotides that are key substrates for viral DNA synthesis. SAMHD1 can be neutralized by lentiviral Vpx but in general not by HIV-1 Vpr in infection of humans [47]. In HIV-2 infected individuals, the efficiency of Vpx-mediated SAMHD1 antagonism is not correlated with the potency of viral control [81]. Besides anti-HIV activity, host factors may also enhance HIV infectivity, as becomes clear from semen-derived enhancer of virus infection (SEVI) fibrils that promote attachment of virus to target cells [82].

Adaptive immune system
When the innate immune response is not capable of eliminating the virus or bacteria, or if these responses are evaded by the pathogen, the adaptive branch of the immune system kicks in. The adaptive immune system is mediated by T lymphocytes and B lymphocytes that are pathogen-specific, establish immunological memory and take longer to become activated – typically several days. The specificity of T and B cells is determined by their membrane-exposed receptor (T cells: TCR or B cells: BCR) generated by random rearrangement of genes. The receptors can detect antigens that are foreign to the body and therefore are a potential threat of infection or disease. Upon antigen detection, the adaptive immune cell becomes activated and proliferates, mounting an immune response against the antigen carrier. Few progeny cells of the activated cells become memory cells, which have a longer lifespan and are rapidly reactivated if the specific antigen is encountered again. This antigen-specific memory is the hallmark of the adaptive immune response [83].

B cells are activated by APC that are present in the lymph nodes draining infected sites. This results in the production of antibodies, which are proteins that work primarily by latchting onto viruses and preventing them from infecting their target cells. The induction of antibodies depends on T follicular helper cells [84]. TThe only target for antibodies that is expressed on HIV is the envelope protein. Most antibodies directed against envelope proteins gp120 and gp41 are unable to clear infection because they bind poorly to intact enveloped virions or infected cells. Antibodies can be either neutralizing when they block HIV entry into its target cell or they can be non-neutralizing when directing other components of the immune response to destroy infected cells. An example of the latter are IgG antibodies that bind NK cells leading to antibody-dependent cellular cytotoxicity. In the context of natural infection, neutralizing antibodies play a limited role in durable control of infection given that their diversity is mainly driven by increased viral loads [85].

T-cell responses involve CD4+ T helper cells that orchestrate the functioning of activated CD8+ T cells, known as CTL which can kill virus infected cells. Strong and effective HIV-1 specific CD4+ and CD8+ T-cell responses are generally regarded as the backbone of antiviral immune activity. T cells use their TCR to recognize tiny parts of ‘nonself’ or ‘foreign’ pathogens, called peptides, bound to HLA molecules expressed on the APC surface. Differences in the exact sequence of these HLA molecules matter, because they determine which parts of an antigen are presented to T cells. HLA class I molecules present peptides that are cleaved from tagged intracellular proteins by the proteasome, to CD8+ T cells. HLA class II molecules present antigens of extracellular origin phagocytosed into intracellular vesicles, to CD4+ T cells. Phagocytosed antigens can also be presented in the HLA in class I pathway to CD8+ T cells, a phenomenon known as cross-presentation [86]. The heavy chain of the HLA class I molecule
is encoded by HLA-A, -B and -C alleles. In general, HLA molecules bind peptides of 8 to 10 amino acids in length that can be recognized by CD8+ T cells. Requirements for peptide length and sequence for HLA class I binding are strict and computer algorithms can be used to predict the binding potential of peptides to HLA molecules [87]. Interaction of the TCR with a peptide bound to HLA on the target cell or APC activates signaling pathways causing T-cell proliferation, differentiation and effector functions such as cytotoxicity and production of antiviral cytokines IFN-γ and tumor necrosis factor alpha (TNF-α). Antiviral efficacy of T cells is co-determined by the TCR clonotype, suggesting that a structural interaction between the TCR and the corresponding peptide-HLA class I complex is important for viral control [88].

CD8+ T cells that encounter viral antigens for the first time, i.e. naïve T cells, are expanded to a pool of effector CD8+ T cells. Effector T cells are in general short-lived, have a high expression of cytotoxic granule components (granzyme and perforin) and migrate out of the lymphoid tissue to kill virus-infected cells in the periphery. CD8+ T cells can also induce apoptosis by direct cell surface molecule interactions of the T-cell expressed Fas ligand with Fas binding molecules on the target cells inducing apoptosis of the target cell. For long-lasting immunity mediated by CD8+ T cells, a pool of antigen-specific memory cells should be established that can proliferate rapidly when the antigen is re-encountered. The memory pool is divided in central memory CD8+ T cells (Tcm) that remain in lymphoid tissue and effector memory CD8+ T cells (Tem) found in spleen, liver and mucosa and can perform immediate effector functions [89]. CD8+ T cells have been found repeatedly to be one of the major contributors in containing viral load and in natural control of HIV-1. In humans, expansion of CD8+ T cells during acute infection and associations between HIV-specific cytotoxic CD8+ T-cell activity and control of viremia have been demonstrated [90-93]. In non-human primates it was shown that SIV load declines in the presence of CD8+ T cells [94, 95]. Experimental depletion of CD8+ T cells in SIV-infected macaques leads to loss of control of SIV replication [94]. In addition, evidence for the major role played by CD8+ T cells in viral control results from the association between certain HLA class I alleles and viral load set-point. Certain class I HLA types are associated with a more benign disease course, such as HLA-B*57, HLA-B*5801, HLA-B*27 or HLA-B*35 [96, 97]. Whether HIV-specific CD8+ T cells mediate their role through direct killing of productively infected cells before release of viral progeny [90, 94], or mechanisms other than cytotoxic clearance [98, 99] is yet unknown.

CD4+ T cells not only are a major target for HIV infection [54] but also play a role in the host response to HIV-infected cells. Proliferation of CD4+ T cells has been associated with reduced viral load and CD4+ T cells support the antiviral effects of CD8+ T cells by secretion of cytokines IL-2, IL-7 and IL-21 [100] and upregulation of costimulatory membrane markers such as CD40L, CD80 and CD86. Regulatory CD4+ T cells have an immunosuppressive effect whereas CD4+ T-helper-17 (Th17) cells producing IL-17 are pro-inflammatory and form a crucial component of the mucosal antimicrobial defense [101]. During HIV infection, Th17 cells are significantly depleted by direct infection and by type I IFN. The balance between Th17 cells and Tregs that modulate immune responses is lost during chronic infection as the number of Tregs gradually decline [102]. A distinct subset of CD4+ T cells contributing to immune functions in HIV infection is constituted by follicular T-helper cells that play a role in B-cell expansion and maturation [84].

Antiviral immunity is also mediated by a range of soluble proteins secreted by CD4+, CD8+ and NK cells that inhibit virus spreading. These are chemokines such as CCL3, CCL4 and CCL5 that compete with HIV for binding to the CCR5 co-receptor and cytokines such as IFN-α, IFN-γ, TNF-α and interleukins.

** IMMUNE MODULATION **

Infection with HIV generates innate and adaptive immune responses resulting in a good initial recovery, but they appear to be insufficient or occur too late to completely eradicate the virus. In most patients, viral replication cannot be controlled to undetectable levels. Direct and indirect immune activation, virus mediated cytopathic effects, and insufficient T-cell regeneration establish the chronic state, during which the immune system is progressively destructed and results in AIDS [71]. Functional deficits of T-cell responses such as impaired CD4+ T-cell proliferation [103] and defective cytolytic function of CD8+ T cells [104, 105] may result from diminished antigen presenting capacities of DCs in infection, reduced CD4+ T cell help to CD8+ T cells or loss of CD4+ T cells due to infection [54, 106].

** Immune activation during HIV infection **

The same cells and responses aimed at eliminating the virus seem to play deleterious roles by driving ongoing immune activation and progressive immunodeficiency. Paradoxically, HIV-induced immunodeficiency is dominated by chronic immune activation and high cell turnover, apoptosis, and activation induced cell death instead of inactivity of the immune system. This persistent immune activation is the driving force for progressive immunodeficiency and plays a central role in the immunopathogenesis of HIV [107, 108]. The state of immune activation is characterized by upregulation of activation markers such as CD38, HLA-DR and K67 [109] and receptors for immune suppressors, such as PD-1 and CTLA4 on CD4+ T cells [110]. A major factor contributing to the persistence of immune activation is the translocation of bacterial lipopolysaccharide (LPS) from the gastrointestinal tract to the systemic circulation during HIV infection [111]. Immune activation and inflammation is induced by secretion of IL-1β and IL-18 during the recently discovered process of pyroptosis.
cell death. Pyroptosis is caspase-1-mediated and occurs in non-activated CD4+ T cells [63, 64]. Other factors being involved in the paradox of immune activation are proinflammatory cytokines such as type I interferons (IFN-α and IFN-β) that are overexpressed in the presence of viral proteins and RNA [50, 61]. Type I IFNs cause undesirable immune activation and killing of uninfected T cells by upregulation of co-receptor CCR5, suppression of the Th17 population, and transformation of pDCs into killer DCs [80]. In addition, type I IFNs can upregulate expression of PD-L1, the ligand to the PD-1 receptor that is associated with T-cell exhaustion and HIV progression [112]. Low levels of regulatory T cells are associated with higher levels of immune activation but also with viral control [113]. During acute infection the antiviral and anti-inflammatory effects of the immune system are beneficial but come at the cost of general immune activation that may be harmful during the chronic phase of infection [80].

Exhaustion

HIV-specific immunity is diminished in chronic HIV infection by direct loss of CD4+ T-cells but also by the interlinked processes of immune activation, exhaustion and senescence [109]. Aspecific activation of the innate immune system drives the overactivation of the adaptive immune response. This chronic immune activation plays a key role in HIV pathogenesis, for example by an increased susceptibility of CD4+ T-cells to infection and a decreased responsiveness to antigenic stimulation [108]. Persistent antigenic stimulation but also high viremia and a limited TCR clonotype repertoire lead to exhausted T-cells that are functionally unresponsive to further antigen stimulation and subsequently physically deleted [114, 115]. Moreover, the immune system deteriorates over time due to the intrinsically limited replicative capacity of T-cells, referred to as immunosenescence. HIV-infected persons who maintain suppression of viremia and stable CD4+ T-cell counts without cART medication, also referred to as ‘elite controllers (EC), express lower levels of transcripts involved in lymphocyte exhaustion and senescence than persons with progressive viremia. Myeloid DCs from ECs can generate potent antiviral effector responses without enhancing overall immune activation through selective upregulation of leukocyte immunoglobulin-like receptor (LILR) immunoregulatory receptors [110].

AIDS and non-AIDS related pathology

Persistent inflammation as a result of chronic immune activation may cause AIDS as well as non-AIDS related pathology. Important insights into the role of immune activation in the pathogenicity of HIV are derived from SIV infection in different monkey species. SIV infection in its natural host sooty mangabeys (SM) and African green monkeys (AGMs) does not lead to AIDS. In contrast, in rhesus macaques (RMs), SIV infection is pathogenic. Interestingly, in both pathogenic and non-pathogenic SIV infection, strong cellular and humoral immune responses are mounted and high viral loads are detected. However, immune responses in RM fail to clear the virus resulting in high levels of immune activation and excessive inflammation associated with progression to AIDS whereas in SM and AGM, inflammation rapidly declines by downregulation of IFN and T-cell responses [108, 116, 117]. Non-AIDS related comorbidities such as premature aging, accelerated cardiovascular disease, cancer and neurological events are related to chronic immune activation [108, 118]. The virus-induced inflammatory state is thought to contribute to the aforementioned long-term complications, evidenced by the observation that EC have more cardiovascular comorbidities than antiretroviral treated patients, who in general have lower levels of immune activation than ECs [119]. On the other hand, treated patients have upregulated markers of inflammation compared to uninfected controls and are diagnosed also more often with non-AIDS comorbidities (after correction for risk factors including drug toxicity) [120].

Immune evasion

Host immune selection pressure by HIV specific CD8+ T cells and antibodies causes evolutionary pressure, leading to the selection of escape variants. These viral variants are less well recognized by the immune system. Selective escape from CD8+ T cells and other immune cells by viral mutations has been suggested to be the major driving force of HIV-1 sequence diversity within individual patients as well as at the population level [121, 122]. Mutations within immunodominant regions may not only allow the mutant virus to evade host immune responses but may also antagonize T cells responsive to the wild-type epitope [123]. The rate of escape is influenced by mutational pathways required for virus escape, the ‘strength’ of the CD8+ T-cell response, the cost on replicative fitness of the infecting HIV strain and stochastic processes [57, 122]. The balance between these factors determines whether an escape mutation will persist or decline during infection [124]. Escape mutations occur in CD8+ T-cell targeted epitopes of the virus and abrogate recognition by CD8+ T cells by the reduction of peptide binding to the restricting HLA class I allele, interference with recognition by TCR contact residues or inhibition of intracellular epitope processing [125]. Mutations in conserved regions of the virus genome often result in substantial loss of replicative fitness of the virus for which the virus tries to compensate by additional mutations outside the epitope sequence [122, 126-131]. Vaccine-induced immune responses should target vulnerable regions of the viral genome within which mutations impose a high fitness cost [132, 133].

NATURAL CONTROL OF HIV

A small percentage (less than 5%) of infected people who seroconvert, spontaneously control HIV replication. They have normal CD4+ T-cell counts and immune function, undetectable or low viral loads and do not progress to AIDS, even without the aid of antiretroviral therapy [134]. These long-term nonprogressors (LTNP), subdivided in elite controllers (EC) who
have undetectable viral loads and viremic controllers who maintain a low-level viremia, are being studied intensively to discover their mechanism of viral control and resistance to AIDS progression [135]. What is known is that the uncommon, natural control of HIV is influenced by the genetic makeup of the host. HLA class I alleles involved in viral control are HLA-B*57, HLA-B*5801, HLA-B*27 and HLA-B*51 [96, 97]. Other host genetic factors involved in control are polymorphisms in killer cell immunoglobulin-like receptors (KIR) on NK cells, expression profiles of members of the ILLR on DCs, epigenetic control mechanisms, the Δ32 mutation in the CCR5 receptor and mutations that affect the production of antiviral cytokines IL-10 and IFN-γ [136]. HIV-1 specific CD8+ T cells from EC can be distinguished from progressor T cells for their cytotoxic and proliferative capacities and cytokine seetion, together referred to as ‘polyfunctionality’, instead of the magnitude and breadth of HIV-specific CD8+ T cells [136, 137]. The expression of cytotoxic granule components of the CTL from controllers is maintained by rapid expansion and upregulation of transcription factor T-bet [138, 139]. CD8+ T cells from EC tend to recognize more virus variants and mutants (they are more cross-reactive) [140] and seem to target more conserved functionally constrained antigens [138] and vulnerable parts of the virus where the virus pays a higher fitness cost in order to escape from these CTL-mediated immune pressure [141]. The CD8+ T cells are also less susceptible to the inhibitory effects of Tregs, possibly as a result of the decreased expression of the TIM3 protein [142]. In EC, HIV-1 specific CD4+ T cells have higher functional avidities.

**PREVENTION AND TREATMENT MODALITIES FOR HIV**

**Modalities to reduce infection rates and transmission**

In the era of a persisting HIV/AIDS epidemic and the absence of an effective HIV vaccine, there is a high need for effective prevention strategies to constrain the spread of HIV. In order to achieve effective prevention, behavioral, biomedical and structural interventions must be combined and tailored to the target population [143, 144]. Behavioral strategies include condom usage, sexual abstinence, HIV counseling and testing and monogamy for sexual transmission and the use of safe injection equipment for intravenous drug use. These strategies have been shown necessary, but not sufficient to reduce HIV transmission or acquisition rates, and are hard to maintain [144]. For an effective prevention approach, behavioral strategies should be combined with structural interventions for the social, political, economic and environmental context in which behavior occurs, and with biomedical interventions. Regarding the latter, male circumcision was one of the first tools proposed for prevention of HIV acquisition [144]. Later on, pre-exposure prophylaxis (PrEP) with antiretroviral drugs was added to the prevention toolkit. These drugs can either be administered orally, or topically as vaginal microbicides providing a female-initiated method for PrEP. Antiretroviral drugs can also be applied after either occupational or nonoccupational exposure to HIV as post-exposure prophylaxis (PEP). The reduction in viral load as a result of antiretroviral therapy in individuals, who tested positive for HIV, reduces infectiousness and thus helps to reduce transmission as secondary prevention [145]. As discussed above, STIs increase the risk of HIV transmission and therefore, STI treatment-interventions are included in HIV prevention approaches [144].

**Antiretroviral treatment, benefits and limitations**

Antiretroviral drugs form the cornerstone of medical management of HIV-1 infection. Administration of a cocktail of at least three HIV-1 specific antiviral drugs, also known as highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (cART), has dramatically reduced AIDS-related mortality and morbidity. The use of cART suppresses viral replication to undetectable levels in plasma and a steady recovery of CD4+ T cells is achieved [146]. Antiretroviral drugs can be divided into six classes based on their mechanism of action: NRTIs, NNRTIs, PI, INIs, I镊IS and fusion inhibitors. These classes target different steps of the viral life cycle, and each class has a different mechanism of action. The main groups of antiretroviral drugs and the generic drug names are indicated and the different steps of the viral replication that are targeted by antiretroviral drugs are depicted. NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors. Adapted from [13].
of action on the HIV-1 replication cycle (Figure 7). The viral entry step of the replication cycle, is targeted by fusion inhibitors and by antagonists of CCR5. The next replication step targeted by antiretroviral drugs is the reverse transcription of viral RNA into proviral cDNA by the viral RT enzyme. Nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) block the generation of full-length cDNA by RT. Following transport of the viral pre-integration complex into the nucleus of the host cell, the integration of the viral genome in the host DNA is the target of integrase inhibitors. The sixth class of antiretroviral drugs is comprised of the protease inhibitors (PI) that target the viral protease enzyme. PIs prevent the formation of infectious viral particles by inhibiting proteolytic processing of the Gag and Gag-Pol precursor proteins in the newly formed virions [147]. In general, three antiretroviral drugs with at least two different mechanisms of action are used because when only one or two antiretroviral drugs are used, therapy resistance develops relatively fast as the threshold for selecting resistant virus clones is too low.

Despite the dramatic positive effects of cART on the life expectancy of HIV-infected individuals, it does not provide a cure: latent proviruses residing in long-lived cellular reservoirs are not eradicated and certain compartments of the immune system remain compromised [146]. Because cessation or interruption of cART leads to a rapid rebound of virus replication from the latently infected cells in the vast majority of patients, lifelong therapy adherence is required [148]. However, one remarkable case has been described in which the use of antiretroviral therapy has resulted in the cure of HIV infection. In this 'Mississippi case', an in utero HIV-infected neonate was treated immediately after birth with cART for 18 months, and after treatment interruption no evidence of active HIV infection could be found. The early initiation of treatment combined with the particularities of the fetal immune system may have contributed to the functional cure in this case [149]. Purging HIV from the latent reservoir by inducing the re-expression of latent genomes within resting CD4+ T cells is the primary strategy to deplete this reservoir. This can be achieved by the administration of cytokine IL-7, histone deacetylase inhibitors such as the chemotherapy drug Vorinostat, NF-κB activating agents and others [150]. All of these strategies carry the risk of global T-cell activation. Other disadvantages of therapy are side-effects, unaffordable costs for the vast majority of HIV-infected patients and the risk of development of resistance to the drugs as a result of the high mutation rate of the virus. Hence, there is a clear need for therapeutic alternatives to life-long cART treatment in HIV-infected patients.

**Therapeutic alternatives to antiretroviral therapy**

Therapeutic alternatives to life-long cART treatment should either provide full virus eradication or a functional cure [151]. Eradication, also referred to as sterilizing cure, has been reported in one case of a chronically HIV-infected man who was transplanted with allogeneic stem cells from a CCR5 Δ32/Δ32 donor, stopped cART afterwards and in whom viral replication remained absent since then [152, 153]. Other approaches to achieve virus eradication are the intensification of cART, stem cell therapy, gene therapy and elimination of the viral reservoir of latently infected cells [154]. When HIV is not eradicated but its replication is controlled below detection levels in the absence of antiretroviral therapy, a functional cure is achieved [154]. Since EC spontaneously control HIV replication, they are an example of a functional cure. Characteristics of their immune responses may provide clues for the development of therapeutic vaccines for HIV.

Therapeutic vaccines are currently developed to harness the immune response to treat diseases ranging from cancer to Alzheimer’s disease [155, 156]. Therapeutic vaccination for HIV is intended to increase existing immune responses against HIV or induce de novo beneficial HIV immune responses by vaccination with a suitable immunogen [157]. Ideally, induced responses control viral replication while avoiding increased susceptibility of target cells to HIV. A partially effective therapeutic vaccine carries the risk of generating more targets for HIV (i.e. T-cell proliferation and/or activation) thereby allowing ongoing HIV replication. Furthermore, vaccine induced immune activation can lead to inflammation and non-AIDS related events and to immune system exhaustion [151]. Nevertheless, partial viral control observed during interruption of cART after immunotherapy would provide a proof of concept for a therapeutic vaccine candidate [151].

**HIV immunotherapy trials**

The numerous prototype HIV-1 vaccine candidates that have been evaluated in human clinical trials cover a wide range of approaches to induce antibody and/or T-cell responses in terms of vector systems (viral, non-viral), adjuvant formulations, antigens, and administration regimens. So far, prophylactic vaccines have failed to protect against subsequent HIV infection and have not been shown to significantly reduce viral load following infection. Only the RV144 trial, evaluating a recombinant canarypox-HIV vector prime and recombinant HIV-1 envelope subunit plus aluminium hydroxide (alum) adjuvant boost, showed modest efficacy in reducing HIV-1 infection rates by 31% [158]. Different therapeutic vaccine candidates have been tested in human clinical trials. To date, results from over 80 trials haven been reported for multiple vaccine strategies including inactivated whole virus, virus-like particles, peptides, naked DNA, viral vectors and modified DCs. In general, these immunotherapy trials are limited by the low number of included patients and the lack of a control group. Most of the therapeutic vaccination trials failed to demonstrate improvement of CD4+ T-cell count, control of HIV RNA load or delay of clinical progression.

Specific challenges for therapeutic vaccines are the presence of mutant viruses that escape antibodies and CTLs that have evolved during the course of infection. Second, the vaccine
induced immune responses cannot target the latency persisting transcriptionally silent provirus. Moreover, HIV vaccine development is hampered by the lack of insight into which immunological functions correlate with protection. Since a large variety of immune cells and functions are involved in controlling infections, it is difficult to define correlates of protection. Moreover, a clear correlate of protection for HIV does not exist as no one has successfully cleared HIV-1 infection by an immune-mediated mechanism. For the definition of a correlate of protection, it is essential to define what is meant with ‘protection’. Is it protection against HIV acquisition, mucosal infection, systemic infection, disease progression or other clinical parameters? In addition, the timing of the immune assay as well as pathogen and host factors must be taken into consideration. Efficacy of a future HIV vaccine may depend on several correlates of protection, including HIV-specific cellular immunity [159].

Vaccines based on antibodies
A significant part of the development of prophylactic HIV vaccines aims at the induction of antibodies that target the envelope protein in a way that disables (neutralizes) most circulating varieties of HIV-1 in laboratory studies. These so-called broadly neutralizing antibodies (bnAbs) are correlates of protective immunity for most of the highly effective vaccines for viral infections other than HIV. In non-human primates, the passive transfer of bnAbs can provide sterilizing protection from infection [160, 161] by binding to HIV Env thereby preventing the virus to enter target cells. To date, human vaccination studies failed to actively induce such bnAbs, systemically and/or mucosally. The RV144 trial which showed moderate reduction in HIV infection rates did not induce bnAbs, suggesting that other mechanisms such as antibody-dependent cellular cytotoxicity and antibody-dependent cellular virus inhibition may play a role [162, 163].

Recently, a number of particularly broad and potent monoclonal bnAbs have been isolated [33, 164, 165]. The question is still how to present the epitopes of such bnAbs in a particular antigenic context such that the same type of antibody response is elicited after immunization [166]. Env is the only viral-encoded antigen accessible to antibodies on the surface of virions and infected cells and thus antibody vaccine strategies aim at eliciting protective antibodies based on the Env glycoprotein subunits. HIV has multiple tricks to escape from neutralizing antibodies targeting Env: it is highly mutable, has a low density on the virion surface and its conserved epitopes are shielded and often glycosylated. Taken together it is hard to construct immunogenic epitopes not subjected to tolerance mechanisms and without rapid escape [167] but recently proposed germline-targeting strategies enable eliciting bnAbs by vaccination [168]. Neutralizing antibodies can also be transferred directly by passive immunization to persons at risk of infection although they cannot enter the central nervous system. Ideally they are provided in a way allowing sustained expression.

Vaccines aimed at inducing cellular immunity
Virus-specific CD8+ T-cell responses are a dominant feature of durable immune control and CD8+ T cells play a pivotal role in controlling viremia in acute infection and contribute to delay of disease progression. However, in most HIV-1 infected patients, CD8+ T cells are generally unable to adequately control acute or chronic HIV-1 infection [90, 92, 169-171].

A common approach in the field of HIV vaccine design is to induce HIV-specific T cells. In non-human primate models, T-cell inducing vaccines have been shown to attenuate acute replication of SIV and lower viral load although not being able to protect against SIV infection [172-174]. For proper CD8+ T cell effector function and strong immunological memory it is essential that help is provided by CD4+ T cells [159]. CD4+ T cells are also needed to induce and maintain B cell responses, thus CD4+ T-cell activation by a vaccine seems warranted. On the other hand, CD4+ T-cell activation may result in increased HIV-1 acquisition or viral load after infection because more target cells are available to the virus. Non-persistent antigen expression mainly elicits TCM whereas evidence is accumulating that T reg are essential for maintaining memory and for suppressing viral replication [175]. Since CTL responses are triggered by recognition of virus-infected host cells, T-cell vaccines are more likely to attenuate early viral replication and maintain control of viremia so that disease progression is delayed rather than to provide sterilizing immunity.

Vaccine design
Various vaccine platforms are studied in an attempt to develop vaccines against HIV. Two basic questions in HIV vaccine design are: how to make potentially better antigens and how to deliver them more effectively? The choice of the immunogen as well as strategies to deliver them is discussed in this paragraph.

Choice of the immunogen
A crucial step in vaccine design is the choice which parts of the HIV genome should be used as vaccine antigens to induce immune responses. In general, the viral protein used as an immunogen should be associated with control of HIV or SIV replication. Given the fast kinetics of HIV replication, early recognition of infected cells by CTL may increase the chance of target cell elimination before the release of progeny virus [176, 177]. The regulatory proteins Tat, Rev and Nef are expressed early in the lifecycle of HIV [178], rendering these antigens interesting candidates for HIV vaccine development and early HIV inhibition. Indeed, several pre-clinical studies that used early expressed genes as immunogens showed encouraging results in terms of resistance to acquisition of infection and induction of cellular immunity [179-183].
Tat-specific immune responses correlate with a slower progression to disease [130] and the added value of CTL responses directed against Tat, Rev and Nef compared to Gag alone was demonstrated in rhesus macaques [184]. In humans, an inverse correlation between CTL precursors against Tat and Rev progression to AIDS was found [177]. In contrast to these findings, studies in humans controlling HIV-1 infection in the absence of therapy did not show an advantage of CTL directed against early rather than against late expressed proteins [185] and the breadth of T-cell responses directed against the accessory or regulatory proteins appeared to be unrelated or even negatively correlated with viremia [171]. Moreover, CTL from these patients preferentially targeted Gag over other proteins [185]. Gag-specific T-cell responses have been associated with a reduced viral load [171, 186, 187] and conserved regions in Gag are critical for viral fitness [122]. In most EC, inhibitory activity on viral replication is mediated by Gag-specific CD8+ T cells and protective HLA class I alleles that present immunodominant Gag epitopes. Gag is expressed late in the replication cycle but is nevertheless possibly recognized early in infection because incoming virions contain considerably more Gag protein than other HIV proteins [188]. As a result, Gag or Gag epitopes are commonly used as immunogens for T-cell based vaccines. However, many HIV progressors express Gag-specific T cells and viral control can be achieved in the absence of Gag-specific T cells in nonhuman primates [93]. Env is inferior as a vaccine antigen as the virus easily escapes from CD8+ T-cell pressure by mutations [122]. Probably a combination of proteins is needed as immunogen in an HIV vaccine, but the optimal combination is not known yet.

Most of the immunogens used in vaccine candidates contain HIV gene sequences from a single virus or a consensus sequence composed of different viruses circulating in a certain geographical region. For therapeutic vaccines, a sequence from a single virus matching the autologous virus from the patient increases its immunogenicity compared to consensus sequences [189]. Because of the high mutation rate of HIV, there is an enormous variation among viruses circulating in the global population but even within a single infected individual. Attempts to cover this high sequence diversity have been made by the design of conserved or mosaic sequences. Conserved sequences are epitopes within which mutational escape from CD8+ T-cell responses is constrained or disadvantageous due to higher viral fitness cost [141, 190] and these regions can be identified from computationally derived viral fitness landscapes [133]. The mosaic approach uses in silico based algorithms to systematically screen recombined virus sequences from multiple strains for optimal coverage of epitopes in a broad range of circulating viruses [191]. Pathogen and mammalian genomes differ considerably allowing codon-optimization to increase the expression of the immunogen [192].

**Antigen delivery strategies**

To elicit HIV immunity, the antigen must be delivered to the patient’s immune system and this can be done by a wide variety of methods. For therapeutic vaccines, popular antigen delivery strategies include inactivated or attenuated virus, viral vectors, non-viral vectors, DNA, viral proteins or peptides, virus-like particles, and dendritic cells (Figure 8). Although each strategy has its own advantages and disadvantages, a major differentiating feature is the possibility of endogenous antigen production. When the vaccine antigen is produced endogenously, induction of HLA class I restricted CD8+ T cell responses is accomplished more efficiently than via cross-presentation of exogenous proteins.

**Inactivated HIV and virus-like particles**

Many successful viral vaccines are composed of killed virus (inactivated whole virus) or weakened virus known as live attenuated virus, the latter which does not cause disease but does raise a protective immune response. However, for an HIV vaccine, this approach is
hampered by safety issues. There is concern that a whole inactivated virus preparation may not be completely inactivated, and for attenuated virus there is the risk of recombination of weakened vaccine strains and wild-type virus or the occurrence of mutations resulting in the acquisition of full virulence [193]. As an alternative to inactivated HIV, virus-like particles (VLP) have gained attention. VLPs are similar in size and conformation to intact virions but lack crucial genetic information which render them non-infectious and a safer alternative to live-attenuated viruses. They contain many copies of antigen mimicking viral surface structure and can be internalized and processed by antigen presenting cells. Therefore VLPs have the potential to efficiently induce antibodies as well as CTLs [194].

Viral vectors

Many of the currently tested vaccine candidates utilize viral vectors to shuttle HIV antigens into the body to elicit immune responses. Genetically engineered recombinant viral vectors induce endogenous synthesis of antigenic peptides that can be presented on HLA class I molecules to CD8+ T cells. As live viruses carry considerable safety risks, they are attenuated to gain a better safety profile although this comes at the cost of decreased immunogenicity [195]. Major challenges in viral vector design are the sometimes complicated manufacturing process, the vector's capacity for antigen uptake and presentation, vector stability and host immune responses directed against the vector. In the field of HIV vaccine research, viruses used for the development of vectors include poxviruses, adenovirus and cytomegalovirus (CMV). Poxvirus-based vectors such as canarypox (ALVAC), NYVAC and modified vaccinia Ankara (MVA) have been extensively evaluated. They have the capacity to carry large amounts of foreign (viral) genetic material but the disadvantage of low immunogenicity [196]. Adenoviruses have also been widely used as vaccine vectors as they are easy to manipulate and manufacture and are highly immunogenic, even after attenuation. Adenovirus- and poxvirus-based vectors for HIV-vaccines have been tested in large clinical trials. The STEP study used a replication-incompetent adenoviral vector (Ad5) encoding Gag, Pol and Nef (MRKAd5) that induced CD8+ T cells in vaccine recipients but did not protect from HIV acquisition and did not decrease post infection viral load [197]. The HIVTN 505 trial tested three prime immunizations with DNA expressing Gag, Pol and Nef (MRKAd5) that induced CD8+ T cells in vaccine recipients but did not protect from HIV acquisition and did not decrease post infection viral load [197]. The HV/HIV 505 trial tested three prime immunizations with DNA expressing Gag, Pol, Nef and Env and one Ad5 boost in Ad5-seronegative persons. The Ad5 vector was live-attenuated and encoded the Gag-Pol fusion protein and Env. Vaccinees had a non-statistical higher acquisition rate of HIV than placebo controls with no effect on post infection viral load [198]. In contrast, the RV144 trial that used a canarypox vector (ALVAC-HIV) encoding Gag, Pol and Env did show a modest effect in protecting from HIV acquisition with a 31% reduction in infection rates compared with placebo [158]. Attempts are made to improve these vectors and circumvent adenoviral vector neutralization by pre-existing antibodies or antibodies induced during multiple vaccination inoculations. For adenovirus, rare human serotypes such as Ad26 and Ad35 and non-human adenoviruses are used in vector development but so far seem to be less potent than Ad5. Replication defective, non-persistent vectors have the disadvantage that the transgene is only expressed transiently which can lead to rapid waning of the immune response. Replicating competent vectors such as adeno-associated virus and cytomegalovirus could overcome these problems and allow long-term antigenic exposure. CMV-based vectors offer the potential to induce and maintain differentiated effector memory T cells by continuous antigen expression, as has been demonstrated in rhesus macaques vaccinated with a recombinant CMV vector encoding SIV Gag, Pol, Env, Tat, Rev and Nef and subsequent challenge with SIV [172, 175, 179]. The use of CMV vectors in humans is considered but impeded by the risk of primary infection and resultant damage of fetuses by maternal acquisition of CMV as well as CMV disease in transplant patients. Apart from poxvirus, adenovirus and CMV, various other viruses and bacteria have been tried as vectors for HIV antigens [195].

Non-viral vectors

An alternative approach to the use of viral vectors is the use of DNA, RNA or proteins. Although in general less immunogenic, they have the advantage of the absence of immunity against the vector, they are stable, do not carry infectious risks and they can be combined with recombinant viral vectors. Epitope presentation via HLA class I molecules to generate CD8+ T-cell responses can be achieved via low-efficiency cross-presentation of the exogenous protein. In contrast, proteins are endogenously produced when the antigen is administrated via HIV DNA engineered into a plasmid. DNA vaccines have additional advantages: they are inexpensive to manufacture, can be administered in several tissues and are well-tolerated and neutralization of the vector by antibodies present pre-vaccination is not a concern for DNA vaccines [192]. However, the transfer of DNA to APCs is inefficient and therefore DNA vaccines generally have low immunogenicity. Multiple efforts have been made to further improve the immunogenicity of plasmid DNA. For example, antigen uptake can be improved by electroporation, a vaccine delivery technique that pulses naked DNA into muscle or skin cells, the latter targets skin DCs in vivo [199]. Targeting DCs by plasmid DNA was shown for the DermaVir vaccine that uses DNA to express 15 HIV antigens in a nanomedicine formulation to deliver antigens to lymph node DCs [199]. Another approach to improve antigen uptake and immunogenicity is the incorporation of DNA into lipid formulations. A novel format of lipid formulations is represented by antigen-expressing immunostimulatory liposomes (AnExILs). The AnExIL platform is based on the cell-free production of protein antigens from DNA templates both within and outside liposomes, mediated by an in vitro transcription and translation mix. It offers the potential of combining antigen production, adjuvanticity and antigen delivery in one system. AnExILs have the advantage over DNA plasmids and conventional liposomal vaccine formulations that antigens are directly transcribed and translated in vitro from the expression vector in and outside the liposomes and can be taken up by DCs in vivo [150].
DNA vaccines probably show their greatest promise in combinatorial approaches of heterologous prime-boost systems in which the immune system is primed with one agent and the provoked response is boosted with a different agent. Generally, DNA acts better as a prime than a boost vaccination because it tends to stimulate a CD4+ T-cell biased response, providing necessary T-cell help to boost a response. Even in a DNA prime, heterologous boost platform, multiple administrations of the DNA vaccine are required because of its low immunogenicity. Prime-boost combinations of a vector prime and a vector or protein boost require less vaccinations to achieve adequate priming [195].

**Dendritic cell-based immunotherapy**

*Ex vivo* cell based therapeutic approaches use autologous patient cells loaded *in vitro* with various preparations and loading strategies of HIV antigens. Especially DCs have been evaluated in clinical trials of therapeutic vaccination for HIV as they have ideal antigenic presentation properties: proteins are processed through both HLA class I and II pathways for stimulation of CD8+ or CD4+ T cells, respectively. Thus DCs can directly activate CD8+ T cells as well as induce CD4+ T cell help to CD8+ T cells [200]. Enhanced CD8+ T cell responses were observed in trials with DCs loaded with whole inactivated HIV or HIV particles [201-204], peptides [205], or autologous HIV mRNA [206, 207]. In a number of these trials, a decrease in plasma viremia was observed [201, 202, 204]. Promising results have recently been obtained in a randomized, placebo-controlled trial with DCs pulsed with autologous heat-inactivated whole HIV. Placebo treatment consisted of non-pulsed DCs. After the therapeutic immunization, cART was interrupted. Plasma viral load was significantly decreased 3 and 6 months after cART interruption in DC-HIV versus DC-placebo and was associated with a consistent increase in HIV-specific T cells [204].

The potential risk of HIV infection of DCs administered as a vaccine is low because HIV replication is inefficient in mature DCs and myeloid DCs are relatively resistant to HIV infection [208]. Moreover, in the context of immunotherapy trials, DCs are administered to cART treated patients with undetectable plasma viral load, even further reducing the risk of infection of vaccine DCs. For vaccine preparation DCs must be matured or activated in order to be capable to stimulate T cells. Different cocktails of maturation stimuli can lead to different DC effector functions. Most clinical protocols have used a mixture of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, to which prostaglandin E2 is often added to increase expression of the lymph node homing receptor CCR7 [157]. Antigens can be loaded to DCs as proteins, DNA or mRNA. Cross-presentation of large proteins is ineffective in DCs whereas transfection of DNA results in increased cell death, lower antigen expression levels and possibly more non-specific immune responses. Electroporation of mRNA overcomes these problems. Although mRNA is biological instable, it offers the advantages that it can include a large number of antigens and variants, does not carry infection or insertion risk or risk of vector-related adverse immune reactions [120]. Furthermore, mRNA allows for further optimization by electroporation of maturation stimuli and addition of HLA class II targeting sequences for optimal presentation to CD4+ and CD8+ T cells [209].

**Adjuvants**

Adjuvants in vaccine formulations are used to enhance quality and strength of immunogenicity, often by mimicking pathogen danger signals activating innate immune signaling pathways. A wide range of adjuvants is used in HIV vaccine trials, including traditional chemical adjuvants such as alum and MF59 as well as plasmid encoded co-stimulatory molecules, cytokines such as IL-12, IL-15, chemokines and TLR ligands [210]. The T cell stimulatory capacities of DC-based vaccines can be enhanced by electroporation of immature DCs with mRNA encoding co-stimulatory molecules such as CD40 ligand (CD40L) which plays a crucial role in priming immune cells. Autologous DCs electroporated with CD40L mRNA have been evaluated *in vivo* [211]. The combination of mRNA encoding CD40 ligand with 4-1BBL [212] or with CD70 and TLR4 as the TriMix adjuvant [213] was shown *in vitro* to enhance T-cell responses.

**AIMS AND OUTLINE OF THIS THESIS**

Untreated HIV infection leads to a gradual loss of immune competence and progresses to AIDS. Treatment with combined antiretroviral drugs (cART) can decrease viral replication below detectable levels but is not sufficient to eradicate the virus. The success of antiretroviral therapy to control HIV infection is limited by the requirement of life-long adherence, side effects, development of drug resistance, high cost and incomplete access. There is an urgent need for a highly effective prophylactic HIV vaccine to prevent infection but also for therapeutic strategies as an alternative to cART for life. Immunotherapy, or therapeutic vaccination, is intended to increase existing immune responses against HIV or induce *de novo* immune responses with a suitable immunogen. These immune responses must provide a functional cure by controlling viral replication and preventing disease progression in the absence of cART. The key difficulty in the development of an HIV vaccine is the uncertainty about the type of immune responses that are required for control of viral replication, how these responses can be elicited and how they can be measured in assays.

The research described in this thesis aims to evaluate dendritic cell (DC)-based HIV immunotherapy, by host immunity and viral evolution and to explore both viral and non-viral HIV antigen delivery strategies. In Chapter 2 we evaluated the feasibility, safety and
immunogenicity of a DC-based therapeutic vaccine in a non-randomized phase IIa clinical trial. We generated mature autologous DCs and electroporated them with mRNA encoding the early expressed HIV proteins Tat, Rev and Nef. The vaccine, referred to as DC-TRN, was administered to 17 HIV-1 subtype B infected patients treated with cART. We tested if the vaccine could induce or enhance T cells specific for Tat, Rev or Nef. These HIV-specific T cells were characterized for their capacity to produce cytokines or to proliferate. In order to identify the effects of DC-TRN immunotherapy on clinical parameters, cART was interrupted after the last vaccination. Effects on viral replication, CD4+ T-cell counts and time off-cART were studied. Chapter 3 describes the evolution of HIV in patients who received DC-TRN immunotherapy. It is known that the high mutational rate of HIV can result in escape from vaccine-induced immune pressure. The potential effects of vaccination on viral evolution can be identified at the level of the whole genome and at the level of specific HIV-1 epitopes. For both approaches, viral sequences obtained before and after vaccination and within and outside vaccine genes tat, rev and nef were compared. Chapter 4 describes the identification of a novel HIV-1 Rev epitope. We characterized the sequence evolution and immunogenicity of this epitope in one of the DC-TRN study participants. Sequence similarity between viral and human epitopes, known as molecular mimicry, could impair T-cell immunogenicity or cause cross-recognition. The research described in Chapter 5 further expands on the host immune responses to DC-TRN immunotherapy and reports the blood transcriptome profile upon vaccination. Monitoring only a few parameters of an immune response after immunotherapy may not be sufficient to provide a comprehensive analysis on the complexity of the interactions that shape the immune response in immune cells depending on the interaction of innate and adaptive immune system, different cell types and receptors. Transcriptome analysis may help in a detailed understanding of vaccine responses. Chapter 6 and 7 address antigen delivery strategies for HIV vaccination as an alternative to the DC-based cell-therapy platform. In Chapter 6 we evaluated the immunogenic potential of recombinant influenza virus as a vector for HIV antigens. Viral vectors in general are highly immunogenic and influenza virus offers the potential of needle-free administration to the respiratory tract by inhalation. Chapter 7 describes the humoral and cellular immunogenicity of a non-viral liposomal vector system from which antigens are transcribed and translated in vitro in combination with antigen delivery in vivo to antigen presenting target cells.

Overall, the research described in this thesis evaluated HIV immunotherapy and its effects on host immunity and viral evolution. It addressed key difficulties in the development of HIV immunotherapy: what type of immune responses are necessary for control of viral replication, how can these responses be elicited and how can they be monitored?
PART I
DENDRITIC CELL-BASED IMMUNOTHERAPY IN HIV INFECTION

Chapter 2, 3, 4 and 5
A PHASE I/IIA IMMUNOTHERAPY TRIAL

of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption

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HIV immunotherapy: approach and immunogenicity


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ABSTRACT

In a phase I/IIa clinical trial, 17 HIV-1 infected patients, stable on cART, received 4 vaccinations with autologous dendritic cells electroporated with mRNA encoding Tat, Rev and Nef, after which cART was interrupted. Vaccination was safe and feasible. During the analytical treatment interruption (ATI), no serious adverse events were observed. Ninety-six weeks following ATI, 6/17 patients remained off therapy. Although induced and/or enhanced CD4+ and CD8+ T-cell responses specific for the immunogens were observed in most of the patients, we found no correlation with the number of weeks off cART. Moreover, CD4+ T-cell counts, plasma viral load and the time remaining off cART following ATI did not differ from historical control data. To conclude, the vaccine was safe, well tolerated and resulted in vaccine-specific immune responses. Since no correlation with clinical parameters could be found, these results warrant further research in order to optimize the efficacy of vaccine-induced T-cell responses.

INTRODUCTION

The number of people living with HIV has reached an estimated 33.3 million in 2009 [214]. Combination antiretroviral therapy (cART) is currently the only means to halt progression to AIDS [215]. However, chronic suppression of HIV replication is required in order to contain persistent infection in reservoirs such as latently infected CD4+ T lymphocytes and cells of the macrophage–monocyte lineage [216]. Despite its success, chronic suppressive therapy is limited by its significant cost, the requirement of lifelong therapy adherence and the occurrence of potentially severe side effects [216, 217]. Moreover, by suppressing HIV replication, cART limits the production of HIV antigens below a threshold needed for HIV-specific T cell stimulation, resulting in low residual immune defenses and a rapid viral rebound following discontinuation of cART [103, 218, 219].

Given the limitations of cART, immune intervention strategies by means of therapeutic vaccination are being explored [217,220-222]. The development of these immunotherapeutic approaches is based on the central tenet that HIV replication in chronic human infection can be constrained by the induction and expansion of potent virus-specific cellular immune responses [223, 224]. Indeed, several lines of evidence support the assumption that there is a link between virus-specific CD4+ and CD8+ T cells and disease outcome in HIV infection, as reviewed elsewhere [225-227]. Unfortunately, the T-cell response against HIV is often insufficient to maintain durable control of viral replication and disease progression occurs in most patients. Therapeutic immunization targeting both the CD4+ and CD8+ T-cell compartments would therefore be a valuable method to boost and/or redirect endogenous immune responses in patients with a chronic HIV infection.

To achieve an optimal effect of immune therapy, the choice of the antigen and of its vector may be of critical importance. As professional antigen presenting cells, dendritic cells (DCs) are known to play a critical role in the generation of highly specific immune responses against a variety of pathogens and tumor antigens. Although they have already been used in human vaccination strategies for a variety of diseases [228], so far, only a limited number of phase I autologous DC-based therapeutic HIV vaccination trials have been published [157, 200-203, 205, 206, 229-232]. In the majority of these trials, DCs were pulsed with peptides derived from the antigen(s) of choice [205, 229, 230, 232]. However, in some trials, DCs have been pulsed with inactivated autologous HIV-1 virus [221, 222, 228] or with a recombinant canarypox vector (ALVAC vCP1452) [220]. While these trials used different products and doses, the collective experience suggests that DC-based vaccines can be safely used in HIV-infected patients. Moreover, in some of these trials, encouraging results were obtained with respect to the observed immune responses and reduction in pVL [233].
The advantage of DCs pulsed with heat or aldrithiol-2 inactivated HIV-1 virus over the use of peptide-pulsed DCs is that no prior knowledge of the HLA haplotype of the patient is needed and that multiple and as yet undefined epitopes from all structural viral antigens may contribute to T-cell activation. However, the methods used to inactivate HIV are difficult to standardize and require the implementation of a meticulously standardized inactivation procedure in order to avoid any biological risk of infection [157].

In our study, HIV-infected patients were vaccinated with mRNA electroporated DCs, a non-viral gene delivery system. In addition to the advantages of inactivated-virus pulsed DCs, several other benefits can be envisaged for this approach. First, it is less labor-intensive since the same mRNA constructs can be used for a whole cohort of patients. Moreover, mRNA constructs can be human codon-optimized to increase expression levels [234] and further modified to abrogate immune suppressive effects of certain HIV proteins (e.g. the Nef-mediated MHC class I downregulation) [235]. This approach also offers the possibility to select the antigens to be presented by the vaccine. Furthermore, by linking the viral antigens to a MHC class II-targeting sequence, presentation to both CD4+ and CD8+ T cells can be achieved [209]. Finally, this method of antigen delivery is safe as mRNA is not immunogenic, has a relatively short half-life, and lacks the potential to integrate into the host genome [236, 237].

Virtually all HIV proteins have been considered as immunogens to be presented by DCs. Since strong anti-Gag immune responses have been detected in long-term nonprogressors (LTNP) and elite controllers, a lot of attention is currently directed toward Gag as an immunogen [140, 185, 186, 238-240]. Although the early expressed proteins Tat, Rev and Nef have not been extensively studied as vaccine antigens, they also represent interesting immunogens [176, 181, 241]. Early recognition of infected cells by Tat-, Rev- and/or Nef-specific cytotoxic T lymphocytes (CTLs) increases the chance of target cell elimination before the release of progeny virus has occurred, possibly resulting in a more effective control of HIV replication [223]. Moreover, prophylactic vaccination experiments in non-human primates using Tat and Rev expressing vectors resulted in reduced plasma simian immunodeficiency virus (SIV) loads after challenge, compared with control or vaccination with Pol/Gag expressing vectors [176, 181, 182, 241]. Similar or even better results were obtained using different vaccination schedules [179, 180, 183, 242]. Wilson et al. described the added value of CTL responses directed against Tat, Rev and Nef compared to Gag alone in rhesus macaques [184]. Finally, Hansen et al. showed encouraging results using rhesus cytomegalovirus vectors expressing SIV Gag, Rev/Nef/Tat and Env in persistently infected rhesus macaques [179].

We conducted a phase I/IIa immunotherapy trial in HIV-1 infected patients, stable on cART. Patients were administered autologous DCs electroporated with mRNA encoding Tat, Rev or Nef before being submitted to an analytical treatment interruption (ATI). We describe the clinical and laboratory data as well as the immunological responses obtained during the first 96 weeks following ATI.

Our results indicate that the vaccine was safe and well tolerated. Vaccination had no measurable impact on clinical parameters following treatment interruption, despite the induction and/or enhancement, in most patients, of CD4+ and CD8+ T-cell responses specific for the vaccine immunogens.

**METHODS**

**Study design and endpoints**

This study was a non-randomized, non-blinded phase I/IIa trial, registered under number NTR2198 at the Netherlands Trial Register (www.trialregister.nl), and conducted in two clinical centers. Between October 2006 and October 2007 HIV-1 infected patients underwent an eligibility screening that had to be completed within 30 days and at least 24 h prior to study entry. The leukapheresis procedure was scheduled 3 to 4 weeks before the administration of the first vaccine (Figure 1). Patients were vaccinated on 4 occasions with an interval of 4 weeks between each vaccine. All patients were vaccinated at the UZ Brussel using autologous mature DCs (mDCs) electroporated either with sig-Tat-DC-LAMP, sig-Rev-DC-LAMP or sig-Nef-DC-LAMP mRNA. On each vaccination day, the 3 autologous DC-based vaccines were administered subcutaneously (50% of the volume) and intradermally (50% of the volume) via 2 separate needle tracts at the anteromedian side of an arm or a thigh. The same injection site was used for each of the antigens during subsequent vaccinations. Patients were treated as outpatients and were observed for 1 h following injection. Local site reactions, systemic events and vital signs were registered systematically prior to immunization as well as 30 and 60 min after each immunization. Patients were asked to record their axillary temperature, all medications taken, as well as any symptom they experienced at the time of vaccination and throughout the following 6 days. During this immunization phase, patients attended the clinic on the day of vaccination and one week thereafter.

Two weeks after administration of the last vaccine, all patients were submitted to an ATI, during which clinical and laboratory data were monitored at regular time intervals. cART was resumed either when the CD4+ T-cell count decreased below 50% of the baseline value (i.e. at time of study entry) or when re-treatment was considered necessary, according to the then current guidelines for the use of anti-retroviral agents in HIV-1 infected adults and adolescents, developed by the Panel on Clinical Practices for Treatment of HIV Infection...
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(www.AIDSinfo.nih.gov). Patients who needed to resume cART within 96 week post ATI will be further referred to as ‘resumers’, whereas those patients remaining off cART at 96 weeks following ATI are referred to as ‘non-resumers’.

All adverse events were graded for severity according to the National Cancer Institute CTC Scale (Version 2.0, published April 30, 1999). The possible association between an adverse event and the investigational agents was determined by the investigators based on their clinical judgment. Immunological assays were performed at different time points (Figure 1).

The primary endpoints were the safety and tolerability of the administered cellular vaccine, of the ATI and of cART resumption. The secondary endpoints were the evolution of the clinical and laboratory parameters (kinetics of HIV viral load rebound and of CD4+ and CD8+ T-cell counts) as well as the duration of the period off cART during the ATI phase. Furthermore, the ability of the cellular vaccine to enhance HIV-specific T-cell responses against Tat, Rev and Nef in vaccine recipients was evaluated.

FIGURE 1  Study overview

The leukapheresis procedure was scheduled 3 to 4 weeks before the administration of the first vaccine. Each vaccination cycle, indicated by a syringe cartoon, consisted of autologous mDCs electroporated either with sig-Tat-DC-LAMP, sig-Rev-DC-LAMP or sig-Nef-DC-LAMP mRNA. At week 14, all patients were submitted to ATI. cART was resumed when considered necessary (see Methods). Immunological assays were performed at different time points during follow-up, as indicated below the timeline. The peptide-based ELISpot assay was performed using PBMCs derived at the time of leukapheresis (‘PreVac’) and at 1 week after the administration of the last vaccine (‘Vac#4+1wk’). Alternatively, the DC-based ELISpot and CFSE dilution assays were performed using PBMCs derived at 4 different time points: at ‘PreVac’, at 1 week following the administration of the second vaccine (‘Vac#2+1wk’), at Vac#4+1wk, and at 4 weeks following the interruption of cART (ATI+4wk).

This trial was conducted in full conformity with the principles expressed in the Declaration of Helsinki. Patients were recruited within the Infectious Diseases Department of the UZ Brussel and the Erasmus MC and were given oral and written information about the trial. The clinical trial protocol and related documents were reviewed and approved by the respective institutional review boards from the Vrije Universiteit Brussel and Erasmus MC (clinical protocol numbers VUB-05-001 and MEC-2005-227, respectively). All patients provided written informed consent before any trial-specific procedure was performed.

Study population

Patients eligible for enrollment were men and women ≥ 18 years of age with HIV-1 infection, as documented by two licensed ELISA test kits and by Western blotting or immunoblotting at any time before study entry. Since the immunogens used in the cellular vaccine were of the subtype B, the HIV subtype of the patients had to be of the same genetic subtype. Patients were required to be on a cART regimen, defined as a combination of at least 3 antiretroviral drugs, with a stable virological and immunological response (i.e. HIV-1 RNA levels ≤ 50 copies/ml and CD4+ T-cell counts ≥ 500 cells/μl for a period of at least 3 months prior to study entry) and to have a documented nadir CD4+ T-cell count of ≥ 300 cells/μl. Patients were excluded if any of the following conditions had been fulfilled: (1) occurrence of any acute infection or serious medical illness within 14 days prior to study; (2) history of lymph node irradiation, allergy to neomycin or history of other serious acute allergic reaction; (3) prior use of any HIV vaccine or non-established experimental therapy; (4) use of any immune modulator or suppressor within 30 days prior to study entry; (5) HIV-1 seroconversion within one year prior to study entry or (6) presence of chronic hepatitis B or C infection.

Plasmids

All antigen-encoding mRNA constructs contained both the transmembrane and cytoplasmic but not the lumenal region of the DC-LAMP (lysosome associated membrane protein) targeting sequence [209]. The construction and in vitro transcription of the plasmids pGEM-sig-Tat-DC-LAMP, pGEM-sig-Rev-DC-LAMP and pGEM-sig-Nef-DC-LAMP mRNA. At week 14, all patients were submitted to ATI. cART was resumed when considered necessary (see Methods). Immunological assays were performed at different time points during follow-up, as indicated below the timeline. The peptide-based ELISpot assay was performed using PBMCs derived at the time of leukapheresis (‘PreVac’) and at 1 week after the administration of the last vaccine (‘Vac#4+1wk’). Alternatively, the DC-based ELISpot and CFSE dilution assays were performed using PBMCs derived at 4 different time points: at ‘PreVac’, at 1 week following the administration of the second vaccine (‘Vac#2+1wk’), at Vac#4+1wk, and at 4 weeks following the interruption of cART (ATI+4wk).

Vaccine production

All patients underwent a leukapheresis, which was performed at the Red Cross Blood Transfusion Center of the Vrije Universiteit Brussel using a COBE® Spectra Apheresis System, as described previously [245]. The cellular vaccines were manufactured on an individual basis at the Laboratory of Molecular and Cellular Therapy, according to Good Manufacturing Practice.
standards, as previously described [243, 245]. In brief, following cell-washing, peripheral blood mononuclear cells (PBMCs) were seeded in Cell Factories* using X-vivo 15 medium (BioWhittaker, USA) supplemented with 1% heat-inactivated autologous plasma, allowing plastic adherence of monocytes. After removal of the non-adherent fraction, monocytes were cultured in X-vivo 15 medium supplemented with 1% heat-inactivated autologous plasma in the presence of GM-CSF and IL-4 for 6 days. On day 6, maturation of immature monocyte-derived DCs was induced by the addition of a cytokine cocktail consisting of GM-CSF, IL-4, IL-6, IL-18, TNF-α and PGE2 for 24 h. Mature DCs were harvested and electroporated with mRNA encoding HIV-1 antigens, before being stored in liquid nitrogen [243, 245]. For all subjects, DCs were assayed for viability, sterility and purity. Upon thawing, approximately 10⁷ viable electroporated mDCs were seeded in 250 µl phosphate buffered saline (PBS, BioWhittaker) containing 5% human serum albumin and drawn in a 1 ml syringe for vaccination.

Clinical laboratory evaluations
Blood chemistry determination and complete blood counts were performed at the clinical laboratories of the respective study sites. Laboratory evaluations concerning autoimmunity were performed at the day of screening and 1 week after ATI. CD4⁺ and CD8⁺ T-cell counts were measured using standard flow cytometry protocols (Tri-Test, BD Pharmingen). Plasma HIV viral loads (pVL) were determined using the Ultrasensitive Roche Amplicor HIV-1 RNA Monitor test (version 1.5), following the manufacturer’s instructions. This assay detects pVL values between 50 and 100,000 copies/ml (1.7 and 5.0 log₁₀ copies/ml, respectively). In the Monitor test (version 1.5), following the manufacturer's instructions. This assay detects pVL values between 50 and 100,000 copies/ml (1.7 and 5.0 log₁₀ copies/ml, respectively). In the

Cell culture and reagents
PBMCs were routinely collected through leukapheresis or buffy coat and stored in liquid nitrogen. After thawing, PBMCs were left overnight at 37 °C in Iscove’s modified Dulbecco’s medium (IMDM, Gibco) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.24 mM L-asparagine and 0.55 mM L-arginine (all from Cambrex), further referred to as I10H, and of any stimulus served as positive and negative controls, respectively. For the DC-based ELISpot assays, 200,000 PBMCs were co-cultured, in triplicate, with 20,000 mDCs electroporated with either Tat-, Rev-, Nef-, Gag- or Flu-NP-encoding mRNA. mDCs cultured in the presence of PMA and ionomycin, in the presence of mock electroporated DCs or in the absence of any stimulus served as positive and negative controls, respectively. Following overnight incubation, plates were washed and sequentially incubated with biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate (both from Dianova) before being developed with BCIP/NBT buffer (Dianiode). The number of IFN-γ spot forming units (SFU) was determined using an AID ELISpot reader and software (Autoimmun Diagnostica GmbH). The peptide based ELISpot was repeated if background values (IFN-γ spot forming in the absence of any stimulus) exceeded 200 SFU/million cells.

CFSE proliferation assays
CFSE proliferation assays were performed in parallel with the DC-based ELISpot assays. PBMCs were incubated for 5 min at 37 °C in a 0.3 µM carboxyfluorescein succinimidyl ester (CFSE) solution (Molecular Probes) at a final concentration of 1 million PBMCs/ml. One million CFSE-labeled PBMCs were then co-cultured for 6 days with 100,000 electroporated mDCs in a final volume of 1 ml I10H. As positive and negative controls, PBMCs were cultured in the presence of 0.5 µg/ml PHA, in the presence of mock electroporated DCs or in the absence of any stimulus. No IL-2 was added at any time. On day 6, PBMCs were stained with CD3-PE (DAKO), CD8-PerCP (BD Biosciences) and CD4-APC (Pharmingen) monoclonal antibodies in PBS/0.5%BSA for 30 min at 4 °C and analyzed on a FACSCalibur (BD Biosciences).

Synthetic peptides
HIV-1 clade B consensus Tat, Rev, Nef and Gag 15-mer peptides overlapping by 11 amino acids (aa) were obtained from the NIH AIDS Research & Reference Reagent Program. The Tat, Rev, Nef and Gag proteins were covered by respectively 2, 3, 5 and 12 peptide pools. A set of peptides most frequently recognized in the context of CMV, EBV and influenza infection (CEF pool, NIH) was used as an additional positive control.

ELISpot plates (Millipore) were coated overnight with human IFN-γ capture antibody (Dianova). The following day, plates were blocked with I10H. For the peptide-based ELISpot assays, 200,000 PBMCs were cultured, in quadruplicate, in the presence of Tat, Rev, Nef or Gag peptide pools at a final concentration of 2 µg/ml. PBMCs cultured in the presence of 10 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) and 10 µg/ml ionomycin (Sigma-Aldrich), in the presence of the CEF peptide pool or in the absence of any stimulus served as positive and negative controls, respectively. For the DC-based ELISpot assays, 200,000 PBMCs were co-cultured, in triplicate, with 20,000 mDCs electroporated with either Tat-, Rev-, Nef-, Gag- or Flu-NP-encoding mRNA. mDCs cultured in the presence of PMA and ionomycin, in the presence of mock electroporated DCs or in the absence of any stimulus served as positive and negative controls, respectively. Following overnight incubation, plates were washed and sequentially incubated with biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate (both from Dianova) before being developed with BCIP/NBT buffer (Dianova). The number of IFN-γ spot forming units (SFU) was determined using an AID ELISpot reader and software (Autoimmun Diagnostica GmbH). The peptide based ELISpot was repeated if background values (IFN-γ spot forming in the absence of any stimulus) exceeded 200 SFU/million cells.
Data analysis

Data processing and statistical analysis were performed using SPSS for Windows. Data from ‘resuers’ and ‘non-resuers’ were compared using the Mann–Whitney U test (asymptotic two-tailed). P values < 0.05 were considered significant. A ‘historical control group’ was composed using data from published treatment interruption studies similar to our study trial. From an electronic literature search on Scopus™ (www.scopus.com, accessed June 2008, Elsevier BV, the Netherlands) with the search terms HIV + treatment interruption + humans + (viral load or CD4), 121 titles were retrieved. Of these, 15 publications described guided (not scheduled) treatment interruptions in study populations similar to ours and were available for data extraction (246-260). CD4+ T-cell counts were standardized, pVL were transformed to decimal logarithms and time points to resume treatment were estimated from Kaplan–Meier curves. Pooling was performed by calculating averages weighted for number of studies and number of persons. The resulting curves were smoothed.

The immune assays were first evaluated using arbitrarily defined cut-off values, which are commonly used in the literature. T-cell responses, as measured by ELISpot assays, were considered positive whenever the number of SFU/million PBMCs were above the cut-off value of 50 and exceeded 2 times the standard deviation above the mean of replicate negative controls, referred to as ‘threshold for positivity’. Responses below background values (unstimulated PBMCs and mock DCs for peptide and DC-based ELISpot, respectively) were set at an arbitrary value of 1 SFU/million cells. Postvaccination T-cell responses were considered to be de novo (induced) whenever their value before vaccination did not exceed the threshold for positivity. Alternatively, postvaccination T-cell responses were considered to be enhanced whenever the number of SFU was at least 3 times higher than before vaccination. Proliferative responses, as measured by DC-based CFSE proliferation assays, were considered positive whenever the antigen loaded DCs increased the percentage of CFSE+ T cells by at least 1.5x compared to the mock DCs.

In addition, data derived from DC-based ELISpot and CFSE proliferation assays were analyzed by statistical modeling and linear regression (Figure 5, Figure S2 and S3). Pre-processing of the raw data (smoothing and baseline correction) was first applied. Next, based on the data obtained from the different time points analyzed, regression models were constructed for each patient and each vaccine antigen-specific response. The regression lines were only computed where the difference between the maximum and the minimum of the independent variable (responses to Tat-, Rev or Nef electroporated DCs) was larger than 0.3 log10 SFU/million cells or % CFSE+ events. The Tat-, Rev- and Nef-specific T-cell responses were plotted against the corresponding Gag- or Flu-NP-specific T-cell responses. The slopes from these regression curves were analyzed. Slope values of 1 indicate that, over the 4 different time points analyzed, the change in the vaccine-specific T-cell response occurs in parallel with the change observed for the Gag- or Flu-NP-specific T-cell response. Slope values of 0 were found whenever T-cell responses against vaccine antigens changed over the 4 different time points analyzed while those against the control antigens did not. Slope values below 0.5 were considered to indicate relative changes of the vaccine-specific T-cell responses, compared to the T-cell responses directed against the control antigens. Custom programs, written in R (R Development Core Team 2011, www.R-project.org), were used to perform statistical analyses on the data from the immune assays.

RESULTS

Characteristics of the study population

A total of 17 cART-treated HIV-1 infected patients were enrolled. All patients were males infected through unprotected homosexual contact, staged CDC A at diagnosis (CDC A1: 11/16 (65%) patients and CDC A2: 6/17 (35%) patients). All but one met the inclusion criteria regarding the CD4+ T-cell counts. The pre-treatment HIV-1 viral load varied between 4.5 log10 copies/ml and >4.7 log10 copies/ml (data missing for 1 out of 17 patients). The characteristics of the patients are shown in Table 1.

Primary endpoint

For the majority of the patients (13/17), a single leukapheresis product allowed the generation of DC numbers sufficient for the preparation of multiple vaccine doses. However, 4 patients required a second leukapheresis due to low DC recovery. For one patient, the leukapheresis procedure had to be performed by central venous access, because of an inability to gain peripheral venous access. Although no unwanted clinical events were recorded during the majority of the leukapheresis procedures, 4 out of 21 had to be interrupted prematurely due to a complication of short duration, including one case of generalized itching with facial redness, one case of a sustained cramp at the access arm and 2 cases of vasovagal reaction with syncope of short duration.

All 17 patients received the full vaccination schedule of 3 injections on 4 occasions. The vaccine injections were generally well tolerated. Mild redness and/or induration at the injection sites were the most common adverse events (n = 16/17 patients), resolving spontaneously within 8 days. No general reactions or serious adverse events were recorded during the vaccination period. Injections of the DC-based vaccine were not associated with any clinical or serological evidence of auto-immunity (data not shown).
All patients underwent interruption of cART. Following ATI, one patient experienced worsening of his psoriasis. Another patient reported an HIV-seroconversion-like syndrome 8 weeks after ATI. The pVL of this patient had increased to 5.6 log\(_10\) copies/ml with a decrease of CD4\(^+\) T cells to 428/\(\mu l\) (14% of the CD3\(^+\) T cells), compared to 639/\(\mu l\) (37% of the CD3\(^+\) T cells) 2 weeks prior to ATI. The patient’s symptoms resolved spontaneously after 14 days. One patient developed tonsillitis 6 weeks following the interruption of cART. The CD4\(^+\) T-cell count of this patient had decreased from 749/\(\mu l\) (37% of the CD3\(^+\) T cells), 2 weeks prior to ATI, to 127/\(\mu l\) (33% of the CD3\(^+\) T cells) 2 weeks before the onset of the clinical event with an increase in pVL to >5 log\(_10\) copies/ml. Tonsillitis was treated with co-trimoxazole for 7 days. CD4\(^+\) T-cell count was treated with co-trimoxazole for 7 days. CD4\(^+\) T-cell count then spontaneously increased to 616/\(\mu l\) (18% of the CD3\(^+\) T cells) and pVL decreased to 4.59 log\(_10\) copies/ml. None of these adverse events was an indication to resume cART. In patients having to resume cART (see below), no cART-related adverse events were recorded.

### TABLE 1  Patient’s characteristics

<table>
<thead>
<tr>
<th>Characteristics of the study population</th>
<th>ALL ((n = 17))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45 (36-72)</td>
</tr>
<tr>
<td>Time between HIV diagnosis and cART initiation (weeks)</td>
<td>32 (2-442)</td>
</tr>
<tr>
<td>Time on cART before enrollment (months)</td>
<td>91 (36-142)</td>
</tr>
<tr>
<td>Nadir CD4(^+) T cells (cells/(\mu l))</td>
<td>350 (290-660)</td>
</tr>
<tr>
<td>CD4(^+) T cells before cART initiation (cells/(\mu l))</td>
<td>390 (290-682)</td>
</tr>
<tr>
<td>CD8(^+) T cells before cART initiation (cells/(\mu l))</td>
<td>1450 (620-2890)</td>
</tr>
<tr>
<td>CD4(^+) / CD8(^+) ratio before cART initiation</td>
<td>0.27 (0.12-0.90)</td>
</tr>
<tr>
<td>Baseline CD4(^+) T cells (cells/(\mu l))</td>
<td>610 (500-960)</td>
</tr>
<tr>
<td>Baseline CD8(^+) T cells (cells/(\mu l))</td>
<td>706 (400-1338)</td>
</tr>
<tr>
<td>Baseline CD4(^+) / CD8(^+) ratio</td>
<td>1.14 (0.47-1.50)</td>
</tr>
<tr>
<td>Time until HIV-RNA &gt; 1.7 log(_{10}) copies/ml following ATI (weeks)</td>
<td>3.1 (2.0-6.3)</td>
</tr>
<tr>
<td>Time until HIV-RNA &gt; 3 log(_{10}) copies/ml following ATI (weeks)</td>
<td>4.7 (2.0-8.3)</td>
</tr>
<tr>
<td>Lowest CD4(^+) T cells following ATI (cells/(\mu l))</td>
<td>280 (127-583)</td>
</tr>
<tr>
<td>Lowest CD8(^+) T cells following ATI (cells/(\mu l))</td>
<td>1400 (670-4400)</td>
</tr>
</tbody>
</table>

Results are shown as median (range). ATI, analytical treatment interruption; cART, combined antiretroviral therapy.

### Evolution of clinical and laboratory parameters

The CD4\(^+\) and CD8\(^+\) T-cell counts as well as the pVL remained stable during the vaccination course. Following cART interruption, the median time to pVL rebound (i.e. first detection of a pVL value), was 3.1 weeks (range 2.0–6.3) (Table 1). Plasma HIV-RNA increased to a level greater than 3 log\(_{10}\) copies/ml after a median of 4.7 weeks (range, 2.0–8.3). During ATI, the lowest CD4\(^+\) T-cell count ranged between 127 and 583 cells/\(\mu l\) (median of 280 cells/\(\mu l\)). The median peak CD8\(^+\) T-cell count during this same period (1400 cells/\(\mu l\); range 670–4400) was comparable with the median number of CD8\(^+\) T cells prior initiation of cART (1450 cells/\(\mu l\); range 620–2890).

Ninety-six weeks following interruption of cART, 6/17 patients (35%) remained off therapy. These ‘non-resumers’ had a median pVL and CD4\(^+\) T-cell count at 96 week post treatment interruption of 4.58 log\(_{10}\) copies/ml (\(n = 5\), pVL at 96 weeks missing for one patient; range 4.05–5.33 log\(_{10}\) copies/ml) and 463 cells/\(\mu l\) (\(n = 5\), CD4\(^+\) T-cell count at 96 weeks missing for one patient; range 270–645 cells/\(\mu l\)), respectively. Based on repeated CD4\(^+\) T-cell counts below 300 cells/\(\mu l\), resumption of cART was considered necessary in 11/17 patients (65%). The median time to CART resumption was 50 weeks (range 27–93). In all the ‘resumers’ the pVL evolved to a value below the lower limit of detection.

### Analysis of the HIV-specific immune responses

The antigen-specific T-cell responses were evaluated at different time points using 3 different immunological assays (Figure 1). The peptide-based ELISpot assay was performed on PBMCs drawn at 2 different time points (Figure 1). Overnight IFN-γ production from whole and CD4\(^+\)-depleted PBMCs was comparable to the results obtained with whole PBMCs (data not shown).

The DC-based ELISpot assay was performed on PBMCs drawn at 4 different time points (Figure 1). Patient D could not be included in the subsequent analyses, due to a lack of DCs. Based on the arbitrarily defined cut-off values, Tat-, Rev and Nef-specific T-cell responses were considered to be induced in respectively 7, 6 and 4 patients following vaccination (Figure 3). In addition, in respectively 2, 4 and 3 patients enhanced Tat-, Rev- or Nef-specific T-cell responses were observed postvaccination (Figure 3).
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Figure 2 Immune responses by a peptide-based IFN-γ ELISpot assay

(A) Whole PBMCs were stimulated overnight using overlapping pools of Tat, Rev, Nef and Gag peptides. Data from each individual patient are shown and each antigen is depicted by a different color. The IFN-γ production in the absence of any peptide was considered as background. Results are shown as the mean number of SFU/million PBMCs, based on 4 replicate wells. The error bars represent the SEM (standard error of the mean). The response from patient G to Gag pools 7–12 was not analyzed for the ‘PreVac’ time point due to a lack of DCs (*). Based on the arbitrarily defined cut-off values, Tat-, Rev- and Nef-specific T-cell responses were considered to be induced postvaccination in respectively 2, 4 and 9 patients, namely patients M (pool 1) and Q (pool 2) for Tat, patients C (pools 1 and 2), F (pool 1), M (pool 2) and O (pools 1 and 2) for Rev and patients C (pool 4), D (pools 1, 3, and 4), F (pools 1 till 4), H (pool 2), L (pools 2 and 4), M (pool 4), O (pools 2 and 3), P (pool 5) and Q (pool 5) for Nef. In addition, 5 patients showed enhanced Nef-specific T-cell responses postvaccination, namely patients C (pools 3 and 5), D (pool 5), G (pool 2), H (pools 3 till 5) and L (pool 3). (B) Box-and-whisker plots showing the overall evolution of the antigen-specific responses during the vaccination course. The number of SFU observed following stimulation with the individual peptide pools was arbitrarily set at 0 for conditions with numbers of SFU/million cells ≤50 or lower than background value. For conditions >50 SFU/million PBMCs, background values were subtracted from the number of SFU obtained after stimulation with the peptide pools. The numbers of SFU/million PBMCs obtained with the different peptide pools were cumulated for each antigen. Values ≤1 were arbitrarily set at 1. SEM, standard error of the mean; SFU, spot forming units.
The data derived from peptide and DC-based ELISpot assays were also submitted to a web tool designed by Moodie et al. to determine positivity of ELISpot responses ([www.scharp.org/zoe/runDFR/](http://www.scharp.org/zoe/runDFR/)) [261, 262]. The majority (75.6%) of the peptide-based ELISpot responses considered positive using the pre-defined cut-off values were also found to be positive with at least one of the 2 methods described by Moodie et al. For the DC-based ELISpot, the concordance between both methods was even higher: 97% of the values calculated with the pre-defined cut-off evaluation corresponded with at least one of the 2 methods described by Moodie et al. (data not shown).

The T-cell proliferative capacity was analyzed by CFSE proliferation assay (Figure 4). Data obtained in parallel with the DC-based ELISpot and CFSE proliferation assays are shown for one representative patient in Figure S1. Spontaneous proliferation was absent or low with a median (25%–75% interval) percentage of CFSE<sup>low</sup> events of 0.13 (0.06–0.28) and 0.09 (0.04–0.17) for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, and generally lower than the percentage CFSE<sup>low</sup> events in response to mock electroporated DCs (0.6 (0.35–1.96) and 0.52 (0.27–1.8) for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively). Antigen-specific proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was already observed before vaccination in most patients (Flu-NP: 8 and 14/14 patients; Gag 8 and 13/14 patients; Tat: 5 and 7/15 patients; Rev 5 and 13/15 patients; Nef 10 and 13/15 patients, respectively). As shown in Figure 4, following vaccinations, we observed an increase of the proliferative responses against mock electroporated DCs in approximately 50% of the patients, which was generally paralleled by an increase in proliferative responses against Gag and Flu-NP electroporated DCs. One week following completion of the vaccination procedure, a Tat-, Rev- and Nef- specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation was observed in respectively 6 and 10/14, 4 and 10/15 and 7 and 12/14 patients. Four weeks following treatment interruption, these proliferative T-cell responses remained relatively stable.

**FIGURE 3** Immune responses by a DC-based IFN-γ ELISpot assay

Whole PBMCs were stimulated overnight with electroporated mDCs. Data from each individual patient are shown and each antigen is depicted by a different color. The IFN-γ production in response to mock DC was considered as background. Results are shown as the mean number of SFU/million PBMCs, based on 3 replicate wells. The error bars represent the SEM. Based on the arbitrarily defined cut-off values, Tat-, Rev and Nef-specific T-cell responses were considered to be induced postvaccination in respectively 7, 6 and 4 patients, namely patients B, F, G, J, N and Q for Tat, patients B, C, F, M, N and Q for Rev and patients B, I, K and M for Nef. In addition, in respectively 2, 4 and 3 patients enhanced Tat-, Rev and Nef-specific T-cell responses were observed postvaccination, namely patients G and M for Tat, patient G, H, J and L for Rev and patients C, F and N for Nef. SEM, standard error of the mean; SFU, spot forming units.
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CD4

CD8

Percentage CD4\(^+\) cells

Percentage CD8\(^+\) cells

- Tat
- Rev
- Nef
- Gag
- Flu
- Mock
In order to more accurately differentiate between changes in T-cell responses directed against vaccine antigens and changes in responses directed against mock and control antigens (Gag and Flu-NP), the data derived from both the DC-based ELISpot and CFSE proliferation assays were further analyzed by regression analyses (Figure 5 and Figure S3). The differences between the Tat-, Rev, and Nef-specific T-cell responses, as measured by DC-based ELISpot assay pre and postvaccination, were considered significant (>0.3 log_{10} SFU/million cells) in respectively 10/15 (67%), 13/15 (87%) and 10/15 (67%) of patients. Relative to Flu-NP, changes in Tat-, Rev- and Nef-vaccine-specific T-cell responses were found in respectively 4/10 (40%), 6/13 (46%) and 4/10 (40%) patients (Figure 5A). Relative to Gag, changes in responses were found in respectively 4/10 (40%), 8/13 (62%) and 4/10 (40%) patients (Figure 5A). The differences between the Tat-, Rev- and Nef-specific CD4\(^+\) and CD8\(^+\) T-cell proliferative responses, as measured by DC-based CFSE proliferation assay pre and postvaccination, were considered significant in respectively 14/15 (93%), 13/15 (87%) and 12/15 (80%) and 14/15 (93%), 12/15 (80%) and 13/15 (80%) patients. Relative to Flu-NP, changes in Tat-, Rev- and Nef-specific CD4\(^+\) T-cell proliferation were found in respectively 9/14 (64%), 8/13 (62%) and 6/12 (50%) patients (Figure 5B). Relative to Gag, changes in responses were found in respectively 11/14 (79%), 10/13 (77%) and 7/12 (58%) patients (Figure 5B). For the CD8\(^+\) T-cell proliferative responses, the changes relative to Flu-NP and Gag were, respectively, 7/14 (50%) and 9/14 (64%) for Tat, 7/12 (58%) and 7/13 (54%) for Rev and 7/13 (54%) and 7/13 (54%) for Nef (Figure 5B). The large majority of changes, relative to Flu or Gag, in the measured Tat-, Rev- and Nef-specific T-cell responses were due to an enhancement of these responses during the course of vaccination.

Using the data obtained with the DC-based ELISpot assay, we compared the T-cell responses as evaluated by the use of arbitrarily defined cut-off values, on the one hand, and after regression analysis, relative to Gag, on the other hand. Of the 33 measured T-cell responses, 17 were considered induced or enhanced by both methods of analysis. The remaining 16 T-cell responses showed discordant results since T-cell responses were considered induced or enhanced with only one of the two methods of analysis (12 and 4 T-cell responses considered induced or enhanced based on respectively the arbitrarily defined cut-off values and the regression analysis).

Correlations
In order to relate the clinical data to the immunological data, we analyzed whether there was a correlation between the duration of the period off cART and the induced or enhanced immune responses against Tat, Rev and Nef. Moreover, we compared the clinical characteristics and laboratory parameters of ‘resumers’ and ‘non-resumers’. Finally, in order to obtain an overall impression of a potential efficacy of the vaccine, the characteristics of the study patients were compared to a historical control group.

Association between vaccine-specific T-cell responses and the duration of the period off cART

As we observed increased immune responses against vaccination antigens, a regression analysis was performed to evaluate whether a correlation between the T-cell responses, as measured by DC-based ELISpot assay, and clinical outcome, defined as the number of weeks off cART, was present. No correlation between any of the T-cell responses and the time remaining off cART was found (data not shown).

Comparison of clinical characteristics and laboratory parameters of resumers and non-resumers

In order to identify possible predictive factors in the resumption of cART following treatment interruption, the clinical characteristics and laboratory parameters of the ‘resumers’ were compared with those of the ‘nonresumers’. Lower CD4\(^+\) T-cell count before first cART initiation correlated with a higher probability of having to resume cART within 96 week post ATI (\(p = 0.021\)). However, the CD4\(^+\) T-cell count at baseline (i.e. at study inclusion) did not correlate with cART resumption (\(p = 0.227\)). The median time until HIV-RNA level rises above 3 log_{10} copies/ml was positively correlated with the time remaining off cART (\(p = 0.039\)). As expected, during interruption of treatment, significantly lower CD4\(^+\) T-cell counts were observed in the ‘resumers’ compared to the ‘non-resumers’ (\(p = 0.004\)).

Comparison with historical controls

Since there was no control group included in our trial, the evolution of the relative CD4\(^+\) T-cell counts and of the pVL following cART interruption as well as the proportion of patients remaining off cART was compared with historical controls. This analysis was performed in order to obtain an overall impression of a potential efficacy of the vaccine (Figure 6 and 7). Based on these data, no indication of therapeutical efficacy of the vaccine was observed.
The evolution over time of the Tat-, Rev- and Nef-specific T-cell responses, as measured by DC-based ELISpot (A) and CFSE proliferation assay (B and C), was plotted against the corresponding evolution of the Flu-NP and Gag-specific T-cell responses. Following preprocessing of the raw data obtained at the different time points analyzed, regression models were constructed for each patient and each vaccine antigen-specific response. The Tat-, Rev- and Nef-specific T-cell responses were then plotted against the corresponding Gag- or Flu-NP-specific T-cell responses. The slopes from these regression curves were analyzed. Each antigen is depicted by a different color. The upper part of each panel shows the vaccine-specific T-cell responses relative to the Flu-NP-specific T-cell responses whereas the lower part shows vaccine-specific T-cell responses relative to the Gag-specific T-cell responses. SE, standard error.
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**FIGURE 6** CD4+ T-cell count and plasma viral load following ATI

The dynamics of the CD4+ T-cell counts (A) and pVL (B) of the study population following cART interruption were compared with historical controls. Gray line: pooled data from 651 subjects in 13 studies (A) and from 445 subjects in 7 studies (B). Black dots: means (with 95% CI bars) of 17 patients.

**FIGURE 7** Kaplan–Meier curve of time to resumption of cART

The data on time to resumption of cART following ATI of the study population was compared with that of historical controls. Gray line: pooled data from 304 subjects in 3 studies. Gray line: data from 17 patients with 95% CI.

**DISCUSSION**

Here, we present the results of a non-randomized, nonblinded phase I/IIa immunotherapy trial of chronically HIV-1-infected patients stable on cART, by means of Tat, Rev and Nef expressing DCs. The administration of the vaccine proved to be safe, well tolerated and feasible. Although the data on the vaccine-specific T-cell responses varied depending on the immunological assay used (peptide-based ELISpot, DC-based ELISpot or DC-based CFSE proliferation assay) and the method applied to evaluate these data (arbitrarily defined cut-off values or regression analysis), we conclude that the administration of the vaccine resulted in induced and/or enhanced CD4+ and CD8+ T-cell responses specific for the vaccine antigens in most of the patients.

The primary endpoint of our study was safety and tolerability of the administered cellular vaccine, of the analytical treatment interruption and of cART resumption. Four of the 21 leukapheresis procedures performed to prepare the vaccine had to be interrupted prematurely due to a complication of short duration. This frequency corresponds to the
number of complications observed with leukapheresis procedures performed at our center in the setting of cancer vaccination studies. The administration of the candidate vaccine proved to be safe. Prior trials of DC-based vaccines in HIV-1 infected individuals have, although different in their study set-up, also demonstrated favorable safety profiles [201-203, 205, 206, 229-232, 263], with no grade 3 or higher adverse events.

In conformity with the study protocol, all patients underwent interruption of cART. During ATI, none of the observed clinical adverse events was an indication to resume cART. Although the safety of ATI in the context of immunotherapy remains a matter of debate, it has proven to be safe in several clinical trials [157]. So far, 4 other publications have reported ATI to be safe following the administration of a DC-based vaccine [203, 205, 231, 263].

Following ATI, 11 out of the 17 patients had to resume cART within 96 weeks. None of these patients experienced drug-related adverse events and for all of them pVL evolved to a value below the lower limit of detection, concomitant with an increase in CD4+ T-cell count. Angel et al. used criteria different to ours to determine whether restarting cART was indicated at 24 week post treatment interruption [264]. If we apply the same criteria to our study population at week 24 post-ATI, 18% of the patients had not met the criteria to restart cART. This is comparable with the 20% of patients in the study group vaccinated with ALVAC+/-- Remune [264].

One of the secondary endpoints of our study was enhancement of HIV-specific T-cell responses against Tat, Rev and Nef in vaccine recipients. Although the peptide-based ELISPOT assay is currently the golden standard to screen for vaccine-induced immune responses [265, 266], it cannot be excluded that certain antigen-specific CD4+ T-cell responses are not detected when using 15-mer peptides as antigens since the length of MHC class II epitopes can vary considerably [267]. Therefore, we extended our analyses and performed ELISPOT assays using DCs electroporated with whole antigen encoding mRNA. We show that the DC-based ELISPOT assay is more sensitive than the peptide-based ELISPOT assay. Apart from the possibility that some CD4+ T-cell responses were not detected with the peptide-based ELISPOT assay, the presence of certain co-stimulatory molecules on the surface of the DCs, which could boost antiviral memory T-cell responses, may also explain the higher sensitivity observed [268].

As a third assay to monitor immune responses upon vaccination, CFSE proliferation experiments were performed. Since this is a flow cytometry based assay, it has the advantage that several phenotypic markers can be assessed together with the functional read-out. Furthermore, an important defect in the immune response against HIV is an impairment of the proliferative capacity of CD8+ and CD4+ T cells [269, 270] and strong CD8+ T-cell proliferative capacity correlates with a better prognosis during HIV infection and after therapeutic vaccination [171, 270]. Therefore, we hypothesized that vaccine-specific T-cell proliferative responses may be indicative for the clinical outcome of DC-based vaccination.

Based on the results of these 3 immunological assays, we conclude that the administration of the vaccine resulted in induced and/or enhanced CD4+ and CD8+ T-cell responses specific for the vaccine antigens. T-cell responses remained stable during the first weeks following ATI. In some patients, we observed an increase in Gag- and/or Flu-NP-specific T-cell responses following vaccination. Possible explanations for this observation are a general immune activation induced by the DC-based vaccine or an undefined bystander effect. Although the regression analysis showed that the increase of the Tat-, Rev- and Nef-specific T-cell responses during vaccination was generally more pronounced than the increase of the Flu-NP- or Gag-specific T-cell responses, this was not the case for all patients. Indeed, based on cut-off values that were arbitrarily defined but widely used in the literature, a number of patients were considered to have enhanced vaccine-specific T-cell responses, whereas a regression analysis showed that the increase of these responses was comparable with the increase of the responses observed for the control antigens Flu-NP and Gag. Thus, monitoring of responses against control antigens may provide valuable information since it offers the possibility to determine whether vaccine-induced enhancement of T-cell responses is due to an overall T-cell activation or an antigen-specific activation. This is an important issue, since overall levels of immune activation are directly associated with HIV-1 disease progression [109]. Most DC-based vaccination studies published to date did not directly compare T-cell responses specific for vaccine antigens with T-cell responses specific for control antigens e.g. [201, 206, 230]. Ide et al. observed an increased T-cell response against a Gag region not included in the vaccine in 1/4 patients [205]. Based on the regression analysis relative to Gag, 8/15 (53%) patients showed vaccine-induced increases in Tat-, Rev- and/or Nef-specific T-cell responses, as measured by the DC-based ELISPOT assay. Furthermore, respectively 13/15 (87%) and 9/15 (60%) patients showed vaccine-induced increases in Tat-, Rev- and/or Nef-specific CD4+ and CD8+ T-cell proliferative responses. The majority of these T-cell responses were directed against more than one of the immunogens. The first report on immunotherapy using autologous DCs electroporated with RNA was published by Routy et al. (AGS-004 study) [206]. The authors reported full or partial HIV-specific proliferative immune responses in 7 of the 9 evaluable subjects. Recently, Routy et al. reported data on the phase 2 AGS-004 study in which patients received 4 doses of AGS-004 while on cART and then interrupted cART while continuing to receive study drug [263]. HIV-specific CD8+ T-cell proliferative responses were shown to be generated in response to the vaccine. Other therapeutic DC-based vaccination trials reported increased HIV-specific T-cell responses in 50–100% of the study patients after vaccination [201-203, 205, 229, 231, 232, 271]. Although our results cannot directly be compared with these trials, these observations suggest that DC-based vaccines are indeed immunogenic.
Neither the immunogen-specific immune responses detected prevaccination or postvaccination, nor their increase during the vaccination course correlated with clinical outcome of the patients, defined as the number of weeks off cART. This is in contrast with the data from Routy et al., showing a correlation between the CD8+ T-cell proliferative responses to the individual antigens present in AGS-004 and the level of viral control during STI [263]. Moreover, in this study, a significant difference in the percent of subjects with pVL rebound ≥ 50 copies/ml at day 14 was seen when compared to historical controls. This study differed from our experimental set-up in several aspects, i.e. choice of the antigens, use of autologous viral genes, co-electroporation of CD40L encoding mRNA, criteria to restart cART and the use of the Swiss cohort as historical control group. Moreover, patients received 4 doses of AGS-004 while on ART and then interrupted antiretroviral drug treatment while still receiving study drug. We have indeed shown that co-electroporation of DCs with the co-stimulatory molecules CD40L and 4-1BBL results in more immunogenic DCs compared to cytokine matured DCs [212]. These highly immune stimulatory DCs are promising for testing in future vaccination trials. Finally, based on data of previously published clinical trials, vaccination based on the autologous viral sequence might give better results than vaccination based on a consensus viral sequence [233].

The spontaneous immune activation (defined as the IFN-γ production measured in the absence of an antigen stimulus) increased in some patients following vaccination and these patients were more likely to resume cART early (data not shown). This may indicate that immune activation is advantageous for the virus and thus deleterious for the HIV-infected patient, as described previously [109].

Following withdrawal of cART, the kinetics of HIV viral load rebound and CD4+ T-cell counts were extensively monitored. A significant correlation was found between the CD4+ T-cell count observed before initiation of the first cART and the probability of resuming cART within 96 week post ATI. However, the probability of cART resumption was not associated with lower nadir CD4+ T-cell counts. This is in contrast with other studies [272-275] and is most likely explained by the lack of power of our study. However, a more rapid rise of the HIV-RNA level above the threshold of 3 log10 copies/ml was significantly associated with a higher probability of cART resumption. Finally, the CD4+ T-cell count at baseline (i.e. at study inclusion) did not correlate with the probability of cART resumption.

Despite a clear enhancement of HIV-specific immune responses by the vaccine, no indications of clinical success could be found, as the kinetics of CD4+ T-cell counts and pVL and the time remaining off cART following ATI did not differ from historical control data (Figure 6 and 7). The decision to resume cART is however not only based on immunological and virological parameters and is therefore not a robust parameter. Lack of viral control despite high frequencies of HIV-1 specific IFN-γ secreting CD8+ T cells and recognition of multiple viral epitopes, has been observed [276, 277]. It has been hypothesized that the immune system is damaged to such an extent that even powerful vaccination approaches cannot elicit effective immune responses. Moreover, the activation of CD4+ T cells by the vaccine could have provided more target cells to the virus. Routy et al. sought to avoid CD4+ T-cell activation in the AGS-004 study, by omitting HLA class II targeting signals [206]. The lack of a better disease prognosis after vaccination may also be explained by the fact that the antigen-specific T-cell responses elicited by the vaccine are still too low to result in clinical benefit.

A lot of attention is currently directed toward Gag as an immunogen. This is in part due to the fact that strong anti-Gag immune responses have been detected in long-term nonprogressors (LTNP) and elite controllers [140, 185, 186, 238-240]. Moreover, Gag-specific CD8+ T cells have been shown to recognize infected cells before SIV proviral DNA integration and viral protein synthesis [187, 278, 279]. Since these data were not yet available at the time of our study design, Gag was not studied as vaccine antigen in our trial. Therefore, in collaboration with Vanham G. (Institute of Tropical Medicine), a second phase I immunotherapy trial comparing the immunological data using DCs electroporated with both TaReNef, a chimeric mRNA construct encoding Tat, Rev and Nef [243], and Gag versus TaReNef encoding mRNA alone [280] was recently initiated.

In conclusion, we report that therapeutic vaccination of HIV-infected patients stable on cART, with DCs electroporated with either Tat, Rev or Nef is safe, well tolerated and feasible. Induction and enhancement of vaccine-specific CD4+ and CD8+ T-cell responses were observed. Therefore, dendritic cell-based vaccines show substantial promise as immunotherapeutic strategy for the treatment of chronic HIV-1 infection. Our results warrant further research in AIDS-free chronically infected patients in order to identify vaccine-induced immune responses that are effective against HIV and to discriminate these from counter-effective vaccination effects, due to general immune activation.
ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

FIGURE S1: Raw data from DC-ELISpot and DC-CFSE assays

PBMCs from patient F were co-cultured with the indicated DCs and assayed for IFN-γ production (DC-based ELISpot assay, left panel) proliferation (DC-based CFSE proliferation assay, right panel). Raw data images are shown for one of the 3 replicate ELISpot wells and for the CD3+CD4+ and CD3+CD8+ T-cell proliferation.
Statistical modeling and linear regression, referred to as regression analysis, were performed on the data derived from the DC-based ELISpot (A) and CFSE proliferation (B and C) assays. Pre-processing of the raw data (smoothing and baseline correction) was first applied. Smoothing was based on a generalized linear model with a Poisson response. A discrete roughness penalty [281] was applied on all raw data (upper left part of each panel). The amount of smoothing was chosen subjectively after comparison of graphs by some of the authors (AdG, CvB and PE). Baseline correction for the mock stimulus was applied by subtraction (upper right part of each panel), unless the T-cell response observed with mock DCs was stronger than with antigen-expressing DCs. In the latter case, no subtraction was applied. The corrected numbers were transformed to decimal logarithms. Based on the data obtained from the different time points analyzed, linear regression models were constructed for each patient and each antigen-specific response. In these analyses, the Flu-NP- (lower left part of each panel) and Gag- (lower right part of each panel) specific T-cell responses were used as the dependent variables whereas the Tat-, Rev- and Nef-specific T-cell responses were used as the independent variables. The regression lines were only computed when the difference between the maximum and the minimum of the 1201 independent variable was larger than 0.3 log10 SFU/million cells or % CFSElow, in order to avoid unreliable slope estimates. Each character stands for the prevaccination data of a particular patient whereas the numbers represent the different time points following vaccination.

The data on the vaccine-specific T-cell responses obtained with the regression models were plotted against the corresponding Flu-NP- (upper part of each panel) or Gag- (lower part of each panel) specific T-cell responses, as measured by DC-based ELISpot (panel A) and CFSE proliferation (panels B and C) assays. The slopes from these regression curves were analyzed. Slope values of 1 indicate that, over the 4 different time points analyzed, the change in the vaccine-specific T-cell response occurs in parallel with the change observed for the Gag- or Flu-NP-specific T-cell response. Slope values of 0 were found whenever T-cell responses against vaccine antigens changed over the 4 different time points analyzed while those against the control antigens did not. Slope values below 0.5 were considered to indicate relative changes of the vaccine-specific T-cell responses, compared to the T-cell responses directed against the control antigens. Each character stands for the prevaccination data of a particular patient whereas the numbers represent the different time points following vaccination. A standard error of the estimated slope was obtained. The slopes and the standard errors were collected in Figure 5.
Chapter IV: Immunotherapy: Approach and Immunogenicity

B

CD4 regression lines

log10(Tat) vs. log10(Nef)

log10(Rev)

C

CD8 regression lines

log10(Tat) vs. log10(Nef)

log10(Rev)
HIV-1 EVOLUTION

in patients undergoing immunotherapy with Tat, Rev, and Nef expressing dendritic cells followed by treatment interruption

HIV immunotherapy: virus sequence evolution

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ABSTRACT

Objectives
This study aimed to evaluate HIV sequence evolution in whole genes and in CD8+ T-cell epitope regions following immunotherapy and subsequent analytical treatment interruption (ATI). A second objective of this study was to analyze associations between vaccine-specific immune responses and epitope mutation rates.

Design
HIV-1-infected patients on combined antiretroviral therapy (cART) were subjected to immunotherapy by the administration of an autologous dendritic cell-based therapeutic vaccine expressing Tat, Rev, and Nef and subsequent ATI.

Methods
HIV-1 genes were amplified and sequenced from plasma RNA obtained before initiation of cART as well as during ATI. Control sequences for virus evolution in untreated HIV-1-infected individuals were obtained from the HIV Sequence Database (Los Alamos). CD8+ T-cell epitope regions were defined based on literature data and prediction models. HIV-1-specific immune responses were evaluated to analyze their impact on sequence evolution.

Results
Viral sequence evolution in the tat, rev, and nef genes of vaccinated patients was similar to that of controls. The number of mutations observed inside and outside CD8+ T-cell epitopes was comparable for vaccine-targeted and nontargeted proteins. We found no evidence for an impact of vaccine-induced or enhanced immune responses on the number of mutations inside or outside epitopes.

Conclusion
Therapeutic vaccination of HIV-1-infected patients with a dendritic cell-based vaccine targeting Tat, Rev, and Nef did not affect virus evolution at the whole gene level nor at the CD8+ T-cell epitope level.

INTRODUCTION

Because host cellular immunity plays a central role in containing HIV replication, therapeutic immunization could be a valuable method to boost or re-direct HIV-specific immune responses in HIV-infected patients. The induction and expansion of potent virus-specific cellular immune responses upon acute infection can constrain HIV replication in chronic HIV infection [90, 282]. However, these T-cell responses are often insufficient to maintain durable control of HIV replication and most patients eventually progress toward disease.

Previous immunotherapy studies have shown varying results with respect to viral sequence evolution. Despite strong T-cell immune responses associated with a longer time off therapy, sequence variation was shown to be minimal in patients receiving consensus sequence Gag p24 peptide, providing no evidence for immune response-induced viral evolution in p24 [283]. Similarly, dendritic cell immunotherapy with autologous Gag p17 and p24 did not influence sequence variability in immunodominant epitopes, whereas CD8+ T-cell responses weakened after immunization [284]. In MVA-nef immunotherapy, a lack of CD8+ T-cell escape mutations in immunodominant epitopes was observed, notwithstanding high Nef-specific T-cell frequencies [285]. In contrast, others reported that immunotherapy with MVA-nef was associated with a decrease in nef variability compared with control genes and patients [286]. In contrast, in a rAd5-gag immunotherapy trial, viral diversity in gag was greater during treatment interruption than pretreatment [287].

We conducted a phase I/IIa immunotherapy trial in 17 HIV-1-infected patients, stable on antiretroviral treatment (combined antiretroviral therapy, cART). The immunogens tat, rev, and nef were chosen on the basis of encouraging preclinical studies, showing that cytotoxic T-cell (CTL) recognition of the early expressed antigens increased the chance of target cell elimination before progeny virus was released [19, 241]. Patients were administered four vaccinations of autologous dendritic cells electroporated with mRNA encoding Tat, Rev, and Nef (DC-TRN) before analytical treatment interruption (ATI). Previously, we reported that this immunotherapy resulted in induced and/or enhanced CD4+ and CD8+ T-cell responses specific for the vaccine antigens in most of the patients, whereas plasma viral load and time remaining off cART did not differ from historical control data [288].

Here we evaluate the effect of dendritic cell immunotherapy and subsequent ATI on HIV-1 sequence evolution. Pre and postvaccination sequences of whole genes and CD8+ T-cell epitopes were analyzed in both the vaccine and control genes in order to compare the sequence evolution in vaccine and control genes; compare sequence evolution in vaccinated and unvaccinated patients; assess mutation rates within and outside epitopes;
and explore possible associations between epitope mutations and immune responses measured before and after vaccination.

**METHODS**

Seventeen HIV-1 subtype B-infected individuals, stable on cART, participated in the DC-TRN immunotherapy trial [288]. Institutional review boards approved the study (VUB 05-001 and METC 2005-227). Patients provided written informed consent. The trial was conducted in full conformity with the principles expressed in the Declaration of Helsinki and registered at the Netherlands Trial Register (www.trialregister.nl, NTR2198). On four occasions with an interval of 4 weeks, study participants were vaccinated with autologous mature dendritic cells electroporated either with sig-Tat-DC-LAMP, sig-Rev-DC-LAMP or sig-Nef-DC-LAMP encoding mRNA (GenBank AY936885, AY936884, AY936879, synthetic constructs derived from HIV-1 subtype B) [243]. Two weeks after the last vaccination, patients were submitted to ATI (Figure 1).

**FIGURE 1   DC-TRN study overview**

The leukapheresis procedure was scheduled 3–4 weeks before the administration of the first vaccine. Each vaccination cycle, indicated by a syringe cartoon, consisted of autologous mature dendritic cells electroporated either with Tat, Rev, or Nef mRNA. At week 14, all patients were submitted to analytical treatment interruption (ATI). Combined antiretroviral therapy (cART) was resumed when considered necessary. Immunological assays were performed at different time points during follow-up, as indicated below the timeline. Virus from plasma obtained before initial cART and at a number of visits post-ATI was sequenced for gag, pol, vif, vpr and vpu, tat, rev, and nef. Virus evolution at the gene level was studied for all sequences, whereas a selection of time points was used for analyses at the epitope level.

**Molecular HLA typing**

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) obtained at the eligibility-screening visit using the QiAamp DNA blood mini kit (Qiagen). PCR amplification and high resolution sequencing spanning exons 1 to 5 from human leukocyte antigen (HLA)-A and HLA-B antigens was performed using reagents from Applied Biosystems.

**HIV-1 RNA isolation, cDNA synthesis, PCR amplification, and sequencing**

HIV-1 genes were sequenced from plasma samples obtained before first cART initiation and during plasma viral load rebound following ATI. Viral RNA was isolated either using the extraction module of the ViroSeq genotyping system version 2.6 (Abbott) or manually using the Roche High Pure viral RNA isolation kit (Roche Applied Science, Almere). Viral RNA was reverse transcribed using the ViroSeq genotyping system or with AMV or Superscript III Reverse Transcriptase with outer primers for genomic regions of gag, vif to vpu, second exon of tat and rev, and nef. Two approaches with partially different primers for reverse transcription and amplification of the cDNA by (semi-)nested PCR were developed (Table S1). Purified bulk PCR products were sequenced directly with the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech) and the ABI Prism 3100 DNA analyzer or the 3130 XL genetic analyzer (Applied BioSystems), following the manufacturer’s instructions. All primers were obtained from Eurogentec. HIV-1 sequences from DC-TRN participants have been deposited in GenBank with accession codes KF301679-KF302031.

To test for correct patient-specific clustering, a maximum-likelihood phylogenetic tree was reconstructed from the concatenated nucleotide sequences of the open reading frames (ORFs) for all time points, using PhyML [289] implemented in Seaview version 4.2.6 [290] (Figure 2).

**Control sequences**

To compare viral sequence evolution in vaccinated and unvaccinated participants, a selection of the available HIV-1 sequences was made from the HIV Sequence Database of Los Alamos in September 2010 [31]. Subtype B sequences were retained when the following criteria were met: sequence spanning the entire ORF of vif, vpr, vpu, tat, rev, env or nef, availability of at least two time points with absolute or relative dating in the database or original publication; virus derived from plasma or PBMCs; and sample identifiable by a unique patient code. Sequences derived from the same date and patient were aligned using the alignment program MUSCLE (multiple sequence alignment by log-expectation) [291], subsequently a consensus sequence was made with Geneious Pro (v5.1) [292]. Sampling dates were expressed as the number of days since the first available sample. In case of unknown sampling day, we set the date to the 15th of the sampling month. Participants having at least two sequences collected at least 90
days after the reported seroconversion date were selected to represent the chronic phase of HIV infection.

**FIGURE 2**  Phylogenetic tree of the whole genome

Maximum-likelihood phylogenetic tree of gag, pol, vif, vpr, vpu, tat, rev, nef, and env sequences. The tree comprises nucleotide sequences as available from each time point for each study participant. Sequences are labeled with the participant ID-letter and the number of weeks after analytical treatment interruption (ATI); 000 represents the time point prior to combined antiretroviral therapy (pre-cART). The scale bar refers to the degree of sequence mismatch. Trees were constructed in Seaview version 4.2.6 [290]; 100 bootstrap replications, those larger than 40 are shown in gray. Parameter settings were as follows: Hasegawa, Kishino and Yano (HKY), transition/transversion ratio optimized, \( \gamma \) with four rate categories (optimized), and tree searching operator NNI (nearest neighbor interchange).

**Evolution at the whole gene level**

The potential effect of vaccination on viral evolution was explored using a fixed-effects extension of Bayesian hierarchical phylogenetic models (HPMs) [293, 294] implemented in BEAST (Bayesian Evolutionary Analysis Sampling Trees) [295]. By specifying hierarchical prior distributions, HPMs allow pooling information across different patients to improve estimate precision of within-host viral evolutionary parameters, in this case the HIV evolutionary rate, in individual patients. By incorporating fixed effects, differences in evolutionary rates can be assessed in different patient groups, and support for these differences can be formalized as Bayes factors [296].

Here, vaccination is the characteristic of interest that determines patient grouping and we tested whether evolutionary rate differences exist among these groups. Thus, keeping the terminology of [293], we set up the following fixed effects model for each gene:

\[
\log \theta_i = \beta_0 + \delta_i \beta_{\text{Vaccine}} \]

where \( \theta_i \) is the evolutionary rate in patient \( i \), \( \beta_0 \) is an unknown grand mean, and \( \delta_i \) is a binary indicator that allows for estimating the posterior probability for the inclusion of a vaccine effect and \( \text{Vaccine} \), defines the presence or absence of vaccination.

For each patient-specific viral population, we specify a Hasegawa, Kishino and Yano (HKY) model of substitution with discrete gamma rate variation among sites for all genes, and constant population size prior on the tree. Because of its correlation with tree height, and thus the rate of evolution [297], we also linked the constant population size parameter into an HPM. All Markov chain Monte Carlo analyses were run until convergence, as inspected with Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). If no proper mixing of the chain was observed within a reasonable amount of time, the mean of the initial hierarchical prior distribution on the evolutionary rate was informed by the independently estimated average rate for the other genes. To allow for reasonable variation, the variance of the hierarchical prior distribution was set to the largest observed range of values. These settings ensured proper mixing for all runs. For rev, viral evolution was calculated for exon 2 separately to increase the number of sequences from 93 for the complete gene to 186 (see Table S2 for the number of analyzed HIV-1 sequences).
HIV immunotherapy: virus sequence evolution

Difference between vaccine and autologous virus sequence
The sequence of the trial immunogens was designed based on consensus HIV-1 clade B and codon optimized to ensure high protein expression levels. Differences between vaccine and pre-cART autologous sequences were calculated and expressed as percentage of mutated amino acids.

Evolution at the epitope level
Sequences from three time points were analyzed for mutations in epitopes: before cART was initiated, denoted ‘pre-cART;’ when viral load first surpassed 10,000 copies/ml during ATI: ‘early-ATI;’ and at the last time point for which a sequence is available, at least 26 weeks after early ATI: ‘late-ATI.’ Sequencing errors (three or more X’s) were discarded and insertions and deletions were excluded from the analysis. In the context of HLA haplotypes, each position in the HIV-1 genome was defined as part of an epitope if it falls within an immunodominant epitope published by Streeck et al. [298] (see Table S5 listing selected epitopes). The HLA-binding affinities of Streek epitopes differing from the pre-cART sequence were predicted using the in silico prediction method NetMHCpan-2.4 [87]. If the difference in IC\textsubscript{50} values of pre-cART and Streek epitope exceeded 1000 nmol/l, the pre-cART sequence was not considered as an epitope. Additionally, the binding affinity of all nonamers derived from the autologous pre-cART virus sequences and from the DC-TRN vaccine were predicted using NetMHCpan-2.4 [87]. The nonamers among the top 1% with the highest binding affinity were selected as epitopes, and are further referred to as predicted epitopes. Epitopes with an X at anchor position 2 or 9 were discarded.

Association of immune responses and mutations
Study participants were classified as immune responder or nonresponder for pre and postvaccination time points, using both the dendritic cell-based ELISpot and CFSE (carboxyfluorescein succinimidyl ester) proliferation assays [288]. T-cell responses in the CFSE proliferation assay were considered positive when the number of SFU was more than 50 and more than two times SD of replicate negative controls. Proliferative T-cell responses in the CFSE proliferation assay were considered positive when the number of SFU was more than 50 and more than two times SD of replicate negative controls. Postvaccination responses were considered enhanced if the number of SFU was at least three times prevaccination (DC-ELISpot) or if the % of CFSE\textsuperscript{low} T cells was more than two times prevaccination (DC-ELISpot). For both assays, immune responses postvaccination were compared to prevaccination and patients were categorized as nonresponder postvaccination; responder pre and postvaccination; induced responder postvaccination; or enhanced responder postvaccination (Table S6).

Statistical analysis
Quantitative data for each patient were compared with the paired Wilcoxon signed-rank test for variables that do not follow a normal distribution. P values <0.05 were considered to be statistically significant.

RESULTS
HIV sequencing and phylogenetic reconstruction
The number of sequences obtained for viral evolutionary analyses per patient is shown in Table S2. We included a median of four sequences per patient per gene (range 1 – 10) with a median of the latest time point during ATI of 48 weeks (range 6 – 110). In four out of 17 patients, a pre-cART viral sequence for tat, rev, or nef was not available. Median plasma viral load and CD4\textsuperscript{+} T-cell count for the sampled time points and nadir CD4\textsuperscript{+} T cells pre-cART are reported in Table S3.

The phylogenetic tree reconstructed from the concatenated ORF sequences shows distinct clusters for each patient, except for patients B, E, and F (Figure 2). For patient B, the viral sequences pre-cART and at 6 and 20 weeks post-ATI cluster together, whereas those obtained at later time points diverge in two different clusters. This may have resulted from an HIV superinfection followed by recombination and, therefore, excluded patient B from further analyses. The taxa of patient E and F are interspersed most likely as a consequence of repeated HIV transmissions between these sexual partners. Sequences from these two patients were included in further analyses because the patients differ in their HLA haplotypes, which determine the repertoire of CD8\textsuperscript{+} T-cell epitopes.

Viral evolution at the gene level
Because the rate of evolution is not homogeneous across genes [299], vaccine-targeted and nontargeted genes cannot be compared directly. Therefore, a group of control sequences was selected from the HIV Sequence Database (Table S4). Using a Bayesian HPM approach incorporating fixed-effects (see methods), we tested whether there is any evidence for evolutionary rate differences between the vaccine-targeted sequences from DC-TRN vaccinated patients compared with sequences from the control group of chronically infected individuals. This analysis resulted in low Bayes factor, suggesting the absence of an immunotherapy effect on the viral evolutionary rate in all genes (Table 1). The same holds when the control group is extended with sequences from acutely infected patients. Although the Bayes factor for vpu is slightly greater than the frequently used cut-off value of 3 [296], an effect of immunotherapy on vpu is unlikely as vpu was not included in the vaccine. The clustering of patient E and F sequences appears to reflect multiple transmission events for this couple. Adaptation due to
the toggling between selective environments may increase substitution rates; however, their inclusion does not result in a noticeable effect on the evolutionary rate, thus adding weight to the robustness of our result. In summary, DC-TRN immunotherapy did not affect the rate of virus evolution as it was similar to vaccination-naive controls.

**TABLE 1** Effect of immunotherapy on viral evolution

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We report the 95% highest posterior density (HPD) interval for the conditional effect size (conditional on inclusion of the effect) on a log scale. In line with the absence of substantial Bayes factors, almost all intervals contain 0.

**FIGURE 3** Viral evolution in epitopes and nonepitopes

(A) Difference in percentage of mutated amino acids in epitope regions and nonepitope regions for vaccine proteins (Tat, Rev, and Nef) and nonvaccine proteins (Gag, Pol, Vif, Vpr, Vpu, and Env) when sequences obtained late after analytical treatment interruption (late-ATI) are compared to samples obtained prior to combined antiretroviral therapy (pre-cART). Because HIV-1 proteins differ in their capacity to mutate, all calculations of mutation percentages were done for each protein separately. Percentages more than 0 reflect more mutations in epitope than in nonepitope regions late-ATI, whereas percentages less than 0 reflect more mutations in nonepitope than in epitope regions late-ATI.
Viral evolution at the epitope level

To study evolutionary patterns at the epitope level, epitopes were selected for each patient based on their HLA-haplotype (Table S7) from the most extensive list of experimentally found immunodominant epitopes [298]. These epitopes are referred to as ‘Streeck epitopes’ (Table S5) and regions not containing epitopes are referred to as nonepitopes. Participants differ in the numbers of Streeck epitopes in vaccine proteins Tat, Rev, and Nef (median per patient at pre-cART is 8, range 3–11). Potential CTL epitopes were predicted by the peptide-HLA affinity prediction method NetMHC-pan [87] that was previously used to identify novel HIV-1 epitopes [300]. These epitopes are referred to as ‘predicted epitopes’ (Table S5).

Because of CTL pressure, one would expect that escape mutations occur more frequently in epitopes compared with nonepitopes. Consequently, the percentage of mutated amino acids in epitopes minus nonepitopes will be at least 0% postvaccination. However, the percentage of mutated positions at late-ATI in Streeck and predicted epitopes is not different from nonepitopes for both vaccine and nonvaccine proteins (Figure 3A). Because some mutations can occur soon or late after immunotherapy, we investigated the effect of sampling time. For epitope regions in vaccine proteins, the mutation rate was calculated both for early-ATI versus pre-cART and late-ATI versus pre-cART (Table S8) and shown in Figure 3B. At late-ATI, a nonsignificant higher percentage of amino acids in epitopes of vaccine proteins is mutated, reflecting ongoing viral evolution. Taken together, these results do not support increased selection pressure due to therapeutic immunization.

Immune responses related to epitope mutations

The variation within and outside epitopes was studied in relation to the immune responses measured with DC-ELISpot and CFSE proliferation assays as previously reported [288] (summarized in Table S6). Immune responses were induced or enhanced in eight of 15 patients tested for Tat, 10 of 15 for Rev, and eight of 15 for Nef in DC-ELISpot and in 11 of 16 for Tat, nine of 16 for Rev, and eight of 16 for Nef in CFSE proliferation.

Study participants were grouped based on their vaccine-specific immune responses pre and postvaccination. If immunotherapy did not affect responses, participants were subdivided into group 0 (nonresponders prevaccination) and 1 (responders prevaccination).
HIV immunotherapy: virus sequence evolution

We classified participants with induced responses in group 2 and with enhanced responses in group 3. The percentage of mutated positions at late-ATI in epitopes minus nonepitope regions of vaccine proteins is not increased for immune categories 2 and 3, which we consider as high responders compared with group 0 and 1 (Figure 4). No significant difference was found between the groups, tested by Wilcoxon signed-rank test, suggesting that there is no relation between immune response upon vaccination and virus evolution in epitopes. These results were independent of the immune assay used and the method of epitope identification.

DISCUSSION

The DC-TRN trial provided the opportunity to study a unique longitudinal dataset, comprising samples from up to 7 years before the administration of the immunotherapy, when cART was first initiated, and up to 2 years after vaccination. We performed bulk sequencing of the longitudinal dataset to monitor virus evolution at different levels. Our data show that the DC-TRN therapeutic vaccination did not significantly affect virus evolution either at the whole gene level, or in the genes targeted by immunotherapy or in the patients’ HLA-specific CD8+ T-cell epitopes within these genes.

The effect of immunotherapy on virus diversity may be variable, because of the complex interplay between vaccine, host, and HIV. The virus may counteract vaccine-induced and/or enhanced immune pressure by escape mutations. For the genes tat, rev, and nef included in the candidate vaccine, escape mutations have been described in natural infection [176, 299, 301, 302] and after vaccination [285, 303] or CTL transfer [304, 305]. Indeed, in one DC-TRN participant studied in detail, immune escape from Rev-specific immune pressure was observed in a newly defined CTL epitope [306]. The general picture, however, was that therapeutic vaccination did not result in positive selection increasing viral diversity, similar to what others reported [283, 287]. Although Li et al. [287] observed an increase in ambiguous bases resulting in increased epitope diversity after therapeutic vaccination, this was also found in placebo controls. Hoffmann et al. [286] found reduced nef sequence diversity in HIV in relation to strong Nef-specific CD4+ T-cell and CD8+ T-cell responses following MVA-nef immunotherapy. This reduction in sequence diversity was not observed in gag and vif sequences, from the same participants. The authors did not observe enhanced mutation rates in nef and hypothesized that a sieve effect on the present quasispecies eliminated viruses containing vaccine epitopes; this selection reduced virus divergence from prevaccination.

The major limitation of the datasets we used is the small number of sequences per patient per gene. This precludes the use of more flexible but also more parameter-rich coalescent models such as Skyride or Skyline [307, 308] and potentially biases the rate estimates toward lower values. However, both the vaccine and control group are parameterized with the same models; thus, any bias, if present, should be the same for both groups.

We investigated sequence variation using a list of immunodominant epitopes that has been described in acute and chronic infection [297]. As this list mainly comprises epitopes targeted in natural infection, it might not be representative of epitopes targeted by immunotherapy. The method of predicting epitopes is less restricted toward immunodominance in natural infection, but only about 15% of all epitopes presented after natural processing generates T-cell responses in vivo [309].

The median percentage of different amino acids in pre-cART compared to vaccine sequences was 19% for Tat, 12% for Rev, and 14% for Nef. This difference between vaccine and autologous virus sequences is considerable and could bias the read-out of the immune assays based on vaccine sequences. Detected immune responses may have had suboptimal efficacy against autologous viruses [233, 310] and may explain why HIV sequence variability did not differ between groups with or without vaccine-specific immune responses.

The absence of an effect of immunotherapy on virus evolution could be the result of the limited robustness of cellular immune responses after DC-TRN vaccination. Clinical parameters such as plasma viral load, kinetics of CD4+ T-cell counts, and time remaining off cART were not affected by vaccination, suggesting limited efficacy of the vaccine. However, immunogenic vaccines not associated with improved clinical outcome may also result in increased viral sequence divergence [287]. Immunogenicity of therapeutic vaccines is influenced by the impaired function and number of T cells in chronically HIV-infected patients [311]. However, DC-TRN vaccination did induce or enhance cellular immune responses in the majority of the individuals [288], although these may have limited in vivo efficacy. Furthermore, the occurrence and accumulation of CTL escape mutants depends on the balance between the advantage of escape from immune pressure and the disadvantage of loss of replication fitness. Fitness-balanced escape has mostly been studied for Gag. For Nef, no correlation between CTL frequencies and escape rates has been found [301]. Due to high fitness costs of escape mutations and due to an increase in the diversity of the T-cell response over time or after immunotherapy viral escape may be limited [128].
A diverse immune response can induce low-frequency escape mutations that go unnoticed by bulk sequencing [128]; therefore, we recommend the use of single-genome sequencing or deep sequencing for future vaccine trials. 

Despite the limited general effects of DC-TRN immunotherapy on sequence divergence and clinical parameters, the early expressed genes tat, rev, and nef deserve further testing as immunogens [312]. To improve their efficacy in immunotherapy, the immunogens should be better matched to autologous virus [233] or consist of highly conserved epitopes in HIV-1 [313] preferentially combined with novel vectors and vaccination strategies [233, 314]. For the evaluation of such immunotherapy approaches, sequence evolution studies will be of key interest to complement immune monitoring.

**ACKNOWLEDGEMENTS**

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**SUPPLEMENTARY DATA**

**TABLE S1 Primers for (semi)-nested PCR**

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**Table S2** Number of time points for which sequences are available

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Pre-cART defines whether or not the pre-cART sequence was present in the patient: y, yes, pre-cART sequence was present; n, no, pre-cART sequence was not present.

**Table S3** Time points, viral load and CD4+ T-cell count

| Time points used for evolution at the epitope level. Viral load and CD4+ T-cell count at the corresponding time points. Time points denoted as week number. CD4+, CD4+ T-cell count in cells/µl, pVL, plasma viral load in copies/ml, wk, week. | Page 104 - 105 >>
## HIV immunotherapy: virus sequence evolution

### Table: Virus Sequence Evolution

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### Table: Immune Response

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</tr>
<tr>
<td>N</td>
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<td>1.0×10^3</td>
<td></td>
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</tr>
<tr>
<td>O</td>
<td>5.3×10^2</td>
<td>1.0×10^3</td>
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</tr>
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<td>P</td>
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<td>1.0×10^4</td>
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<td>Q</td>
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<td>1.0×10^3</td>
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### Table: Immune Response (continued)

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<th>Patient</th>
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<th>early-ATI</th>
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<td>0</td>
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<tr>
<td>C</td>
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<td>49</td>
<td>7</td>
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<tr>
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<td>-570</td>
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<td>9</td>
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<tr>
<td>M</td>
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<td>6</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
<td>N</td>
<td>-478</td>
<td>4</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>Q</td>
<td>-204</td>
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<tr>
<td>Total</td>
<td>13</td>
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<td>9</td>
<td>10</td>
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TABLE S4  Control sequences from Los Alamos HIV sequence database

<table>
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<tr>
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<th>Sequences #</th>
<th>Patients &gt; 2 seq (%)</th>
<th>Patients #</th>
<th>Sequences #</th>
<th>Patients &gt; 2 seq (%)</th>
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</thead>
<tbody>
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<td>vpr</td>
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<td>43</td>
<td>54</td>
<td>10</td>
<td>23</td>
<td>30</td>
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<td>vpu</td>
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<td>40</td>
<td>50</td>
<td>9</td>
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<td>40</td>
<td>46</td>
<td>9</td>
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<td>rev</td>
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<td>40</td>
<td>46</td>
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<td>21</td>
<td>33</td>
</tr>
<tr>
<td>rev_exon2</td>
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<td>186</td>
<td>38</td>
<td>55</td>
<td>161</td>
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<td>env</td>
<td>55</td>
<td>174</td>
<td>38</td>
<td>51</td>
<td>148</td>
<td>33</td>
</tr>
<tr>
<td>env_gp41</td>
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<td>174</td>
<td>38</td>
<td>51</td>
<td>148</td>
<td>33</td>
</tr>
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<td>nef</td>
<td>26</td>
<td>93</td>
<td>65</td>
<td>21</td>
<td>64</td>
<td>48</td>
</tr>
</tbody>
</table>

TABLE S5  Streeck epitopes

Streeck epitopes and the corresponding sequences of the patient at the different time points; discarded Streeck epitopes; and the top 1% best predicted epitopes.

This table is provided online at http://links.lww.com/QAD/A394.

TABLE S6  Immune responses as previously reported [288]

<table>
<thead>
<tr>
<th>IMMUNE RESPONSE PREVAC VS POSTVAC</th>
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<tbody>
<tr>
<td>IN DC-ELISPOT</td>
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</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Response</th>
<th>Patient</th>
<th>Total # patients</th>
<th>Response</th>
<th>Patient</th>
<th>Total # patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>0</td>
<td>C, H, I, K, O</td>
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<td>0</td>
<td>C, I, K, N</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>A, P</td>
<td>2</td>
<td>1</td>
<td>O</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>B, F, G, J, L, N, Q</td>
<td>7</td>
<td>2</td>
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<td>3</td>
<td>M</td>
<td>1</td>
<td>3</td>
<td>A, H, J, L, P</td>
<td>5</td>
</tr>
<tr>
<td>Rev</td>
<td>0</td>
<td>I, K, Q, P</td>
<td>4</td>
<td>0</td>
<td>C, I, K, O</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>A</td>
<td>1</td>
<td>1</td>
<td>A, G, J</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>B, C, F, M, N, Q</td>
<td>6</td>
<td>2</td>
<td>B, E, F, M, N, P</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>G, H, J, L</td>
<td>4</td>
<td>3</td>
<td>H, L, Q</td>
<td>3</td>
</tr>
<tr>
<td>Nef</td>
<td>0</td>
<td>O</td>
<td>1</td>
<td>0</td>
<td>I, K, M, N</td>
<td>4</td>
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<tr>
<td></td>
<td>1</td>
<td>A, H, J, L, P, Q</td>
<td>6</td>
<td>1</td>
<td>A, J, L, O</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>B, I, K, M</td>
<td>4</td>
<td>2</td>
<td>C, E</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>C, F, G, N</td>
<td>4</td>
<td>3</td>
<td>B, F, G, H, P, Q</td>
<td>6</td>
</tr>
</tbody>
</table>

Assigned scores for vaccination response assayed by DC-based ELISPOT or DC-based CFSE proliferation of CD8+ T cells, with cut-off values for response as reported by [288]. Postvaccination versus prevaccination with score ‘0’ for N, non-responder; score ‘1’ for R, responder, and score ‘2’ for RE, responder, immune response enhanced after immunotherapy. Additional information on this table is provided online at http://links.lww.com/QAD/A395.
### TABLE 57  HLA-types of DC-TRN study subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA-A</th>
<th>HLA-A'</th>
<th>HLA-B</th>
<th>HLA-B'</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A*03:01</td>
<td>A*29:02</td>
<td>B*07:02</td>
<td>B*44:03</td>
</tr>
<tr>
<td>B</td>
<td>A*02:01</td>
<td>A*32:01</td>
<td>B*27:05</td>
<td>B*38:01</td>
</tr>
<tr>
<td>C</td>
<td>A*02:01</td>
<td>A*29:02</td>
<td>B*40:02</td>
<td>B*44:02</td>
</tr>
<tr>
<td>D</td>
<td>A*01:01</td>
<td>A*24:02</td>
<td>B*08:01</td>
<td>B*40:01</td>
</tr>
<tr>
<td>E</td>
<td>A*24:02</td>
<td>A*32:01</td>
<td>B*35:01</td>
<td>B*44:02</td>
</tr>
<tr>
<td>F</td>
<td>A*01:01</td>
<td>A*24:02</td>
<td>B*07:02</td>
<td>B*08:01</td>
</tr>
<tr>
<td>G</td>
<td>A*02:01</td>
<td>A*03:01</td>
<td>B*07:02</td>
<td>B*44:02</td>
</tr>
<tr>
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<td>A*11:01</td>
<td>B*11:02</td>
<td>B*57:01</td>
</tr>
<tr>
<td>I</td>
<td>A*03:01</td>
<td>A*29:02</td>
<td>B*40:01</td>
<td>B*57:01</td>
</tr>
<tr>
<td>J</td>
<td>A*03:01</td>
<td>A*03:01</td>
<td>B*07:02</td>
<td>B*07:02</td>
</tr>
<tr>
<td>K</td>
<td>A*01:01</td>
<td>A*32:01</td>
<td>B*14:01</td>
<td>B*37:01</td>
</tr>
<tr>
<td>L</td>
<td>A*02:01</td>
<td>A*24:02</td>
<td>B*14:01</td>
<td>B*52:01</td>
</tr>
<tr>
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<td>A*68:01</td>
<td>B*15:01</td>
<td>B*35:01</td>
</tr>
<tr>
<td>N</td>
<td>A*01:01</td>
<td>A*02:01</td>
<td>B*07:02</td>
<td>not determined</td>
</tr>
<tr>
<td>O</td>
<td>A*02:01</td>
<td>A*02:01</td>
<td>B*15:01</td>
<td>B*51:01</td>
</tr>
<tr>
<td>P</td>
<td>A*31:01</td>
<td>A*52:01</td>
<td>B*40:01</td>
<td>B*40:02</td>
</tr>
<tr>
<td>Q</td>
<td>A*03:01</td>
<td>A*29:01</td>
<td>B*35:01</td>
<td>B*51:01</td>
</tr>
</tbody>
</table>

### TABLE 58  Percentage of mutated amino acids

Mutations compared to the pre-cART sequence for Streeck epitopes and the top 1% best predicted epitopes. This table is provided online at [http://links.lww.com/QAD/A397](http://links.lww.com/QAD/A397).
Sequence evolution and escape from specific immune pressure of an

HIV-1 REV EPITOPE

with extensive sequence similarity to human nucleolar protein 6

Escape from a novel HIV Rev epitope

Sabine D. Allard*, Anna L. de Goede*, Brenda De Keersmaecker, Carlo Heirman, Patrick Lacor, Albert D.M.E. Osterhaus, Christian Demanet, Kris Thielemans, Rob A. Gruters# and Joeri L. Aerts#

* both authors contributed equally as first authors
# both authors contributed equally as senior authors
Chapter 4
Escape from a novel HIV Rev epitope

ABSTRACT

Antigen-specific immunity is crucially important for containing viral replication in human immunodeficiency virus (HIV)-1-infected hosts. Several epitopes have been predicted for the early expressed HIV-1 proteins Tat and Rev, but few have been studied in detail. We characterized the human leukocyte antigen (HLA)-B44-restricted Rev epitope EELLKTVRL (EL9) in an HIV-1-infected subject treated with antiretroviral therapy. Interestingly, a high sequence similarity was found between the EL9 epitope and the human nucleolar protein 6 (NOL6). However, this similarity does not seem to impede immunogenicity as CD8\(^+\) T cells, previously stimulated with EL9-pulsed dendritic cells, were able to specifically recognize the HIV-1 Rev epitope without cross-recognizing the human self-antigen NOL6. After the subject interrupted antiretroviral therapy and virus rebounded, mutations within the EL9 epitope were identified. Although the emerging mutations resulted in decreased or abolished T-cell recognition, they did not impair Rev protein function. Mutations leading to escape from T-cell recognition persisted for up to 124 weeks following treatment interruption. This study shows that the HLA-B44-restricted Rev CD8\(^+\) T-cell epitope EL9 is immunogenic notwithstanding its close resemblance to a human peptide. The epitope mutates as a consequence of dynamic interaction between T cells and HIV-1. Clinical status, CD4\(^+\) T-cell count and viral load remained stable despite escape from T-cell recognition.

INTRODUCTION

During the past 25 years, a multitude of data has been accumulated supporting the importance of cellular immunity in containing human immunodeficiency virus (HIV) replication in the infected host, recently reviewed by Goulder and Watkins and McMichael et al [61, 315]. However, T-cell responses are often insufficient to maintain durable control of HIV replication, and disease progression occurs in most patients. The effectiveness of the CD8\(^+\) cytotoxic T-lymphocyte (CTL) responses does not appear to depend solely on their magnitude. Rather the quality of the immune response, as determined by breadth (number of epitopes recognized) and functional attributes, seems to be decisive. Because epitope variability is high in HIV-1 infection, the induction of a broad repertoire of CTL responses, targeting multiple viral epitopes, could result in better suppression of HIV [316]. Indeed, analysis of CD8\(^+\) T-cell responses in untreated HIV-infected individuals showed a significant correlation between increasing breadth of Gag-specific responses and a decrease in plasma viremia [171]. Functional attributes affecting the quality of the immune response are polyfunctionality and cytotoxic potential. Polyfunctional CD8\(^+\) T-cell responses play a role in the control of acute HIV infection and protect from rapid disease progression [115, 137]. However, recent findings from HIV vaccine trials suggest that the HIV-specific cytotoxic potential of CD8\(^+\) T cells is a better correlate of HIV control [317]. It remains to be determined which functional attributes of CD8\(^+\) T cells correlate with control of HIV infection.

To boost and/or redirect immune responses against HIV by means of therapeutic vaccination, the choice of the antigen targeted by the vaccine is an important factor determining the success of controlling infection. Given the fast kinetics of HIV replication, early recognition of infected cells by CTL might increase the chance of target cell elimination before the release of progeny virus [19, 176, 177] and therefore influence CTL efficacy [171, 223]. The regulatory protein Rev, together with Tat and Nef, are expressed early in the lifecycle of HIV [178], making these antigens interesting candidates for HIV vaccine development.

Candidate vaccine antigens should ideally mount strong T-cell responses against virus-derived epitopes, while not giving rise to autoimmune responses because these would negatively affect safety. The immunogenicity of potential CTL epitopes depends on a number of factors [318, 319]. Efficient processing and major histocompatibility complex (MHC) binding of peptides derived from foreign antigens are the primary requirements for the initiation of a cellular immune response. Another important determinant for immunogenicity is the availability of CTL bearing a T-cell receptor (TCR) that recognizes the peptide-MHC complex with high affinity. This is, in a large part, dependent on selection mechanisms that take place in the thymus. By distinguishing self from non-self, the immune system walks a fine line to
preserve the integrity of the host. Deficits in this discrimination can result in susceptibility to infections on the one hand and over-reactivity to antigens, leading to immunopathology or autoimmunity, on the other hand [320]. Several mechanisms through which infections can initiate or maintain autoimmunity have been postulated in the literature. To date, many studies are available supporting the concept of molecular mimicry [321, 322]. In this model, it is proposed that sequence similarities between viral and human antigens can potentially lead to recognition of self-antigens, ultimately resulting in autoimmunity. Thus, responses to foreign antigens, bearing elements that are sufficiently similar in amino acid sequence or structure to self-antigen, could result in activation of either B-cells [323] or T cells [324, 325] that are cross-reactive with self-antigens. Rolland et al. [319] recently identified, by analyzing the extent of similarity between HIV-1 and the human proteome, 16 HIV-1 consensus B nonamers considered similar to human proteins.

We previously described the in vitro generation of HIV-specific T-cell responses following coculture with Tat-, Rev- and Nef-expressing dendritic cells (DCs) derived from blood monocytes of eight HIV-1 infected subjects [243]. In one of these subjects, we observed a strong Rev-specific CD8+ T-cell response. This Rev-specific response was further characterized and led to the identification of the human leukocyte antigen (HLA)-B44-restricted Rev epitope EELLKTVRL. This epitope was previously shown by Rolland et al. [319] to be highly similar to a host sequence present in the human nucleolar protein 6 (NOL6). In this article, we describe the effects of molecular mimicry between the Rev EELLKTVRL epitope and the human NOL6 peptide (EELLKEVRL) on T-cell activation, as well as the sequence evolution of the Rev EELLKTVRL epitope over time when the study subject interrupted antiretroviral therapy (ART).

**METHODS**

**Study subjects**

Approval for this study was obtained from the institutional review board of the Universitair Ziekenhuis Brussel, Brussels, Belgium, and informed consent was provided according to the Declaration of Helsinki. The study subject, diagnosed with HIV-1 subtype B infection in 2001, was recruited from the AIDS Unit of the Department of Internal Medicine. At the time of first blood sampling, the patient was on ART since 5 years, which was started shortly after seroconversion (time between last negative and first positive HIV serology: 40 weeks; time between diagnosis and initiation of ART: 8 weeks; time of infection: unknown). Pre-treatment viral load was >50,000 copies/ml and nadir CD4+ T-cell count was 410 cells/µl. The study subject was eligible to participate in a phase-I/IIa therapeutic vaccination trial [288]. In short, following four monthly vaccinations with autologous DCs electroporated with Tat-, Rev-, and Nef-encoding mRNA, ART was interrupted (analytical treatment interruption or ATI). This allowed us to collect and cryopreserve blood samples at different time points over a total period of 124 weeks post-treatment interruption, for which both the viral sequence and the T-cell responses could be analyzed.

**Molecular HLA typing**

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) by a routine procedure using the QiAamp DNA blood mini kit (Qiagen). High-resolution sequencing spanning exons 1 to 5 from HLA-A and -B antigens was performed. Both the polymerase chain reaction (PCR) amplification and sequencing reagents were purchased from Applied Biosystems.

**Plasmids**

The construction of the plasmids pGEM-sig-Tat-DC-LAMP and pGEM-sig-Rev-DC-LAMP and their in vitro transcription into capped mRNA was previously described [243]. The retroviral plasmids pMFG-sig-Tat-LAMP1-ires-tNGFR and pMFG-sig-Rev-LAMP1-ires-tNGFR and the plasmids pcDNA3.1(+)-Tat and -Rev were obtained using similar cloning strategies. The plasmids pcDNA-HLA-A1, -A3, -A29, -B44, and -Cw7 were kindly provided by Dr P. van der Bruggen (Ludwig Institute for Cancer Research). Expression plasmids for active Rev (Rev-BOA, wild-type [WT] epitope, GenBank AY936883), inactive transdominant Rev (Rev-BOT, GenBank AY936884), and the mutants E10D, E11D, and E11D,K14Q (Rev-BOA(E10D), Rev-BOA(E11D), and Rev-BOA(E11D,K14Q), respectively) were constructed by site-directed mutagenesis of pRev-BOA-eGFP [326] using the QuikChange multimutagenesis kit (Stratagene) and following the manufacturer’s protocol. Plasmids were checked for correct mutagenesis with primers S2-F and S3-R SDM, which are listed in Table 1.

**Cell lines**

The melanoma cell lines LB373-MEL (HLA-B*44:02), LB2518-MEL (HLA-B*44:03), and LB4-MEL (HLA-B*44:04) were kindly provided by Dr A. van Pel (Ludwig Institute for Cancer Research). HeLa cells containing a stably integrated Rev-deficient HIV-1 proviral genome (HLB) from Dr Barbara K. Felber and Dr George N. Pavlakis [327] were obtained through the NIH AIDS Research and Reference Reagent Program.

**Table 1** PCR and sequence primers

<table>
<thead>
<tr>
<th>PCR and sequence primers</th>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOL6, human nucleolar protein 6; PCR, polymerase chain reaction; SDM, site-directed mutagenesis.</td>
<td></td>
</tr>
<tr>
<td>§ GenBank accession number.</td>
<td></td>
</tr>
</tbody>
</table>

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### Synthetic peptides

A set of 27 overlapping 15-mer peptides spanning HIV-1 Consensus B (HXB2) Rev was obtained through the NIH AIDS Research and Reference Reagent Program. The Rev peptides EELLKTVRL, DELLKTVRL, EDLLKTVRL, and EDLLQTVRL were synthesized by the department of Immunohematology and Blood Transfusion of the Leiden University Medical Center. The peptide EELLKTVPL was synthesized by PepScan. The HLA-B*44:03-restricted MAGE-C2 peptide was kindly provided by Dr A. van Pel. All other synthetic peptides were purchased from Eurogentec.

### Retroviral transduction of EBV-transformed B-cells

EBV B-cells were transduced with retrovirus generated from vectors pMFG-sig-Tat-Lamp1-IRES-tNGFR and pMFG-sig-Rev-Lamp1-IRES-tNGFR following a previously described transduction procedure [328].

### Transfection of COS-7 cells

COS-7 cells (200,000 cells) were co-transfected using Lipofectamine™ 2000 (Invitrogen) with 300 μg of CDNA encoding the respective HLA molecule, β2-microglobulin (β2m) and Rev-BOT or Tat-BOM protein [243], according to the manufacturer’s instructions.

### Generation and electroporation of MDDC

The generation of monocyte-derived DCs (MDDC) from PBMCs and the mRNA electroporation of mature DCs was previously described [243].

### Generation of CD8+ T-cell clones

CD8+ T cells were enriched from PBMCs by magnetic sorting using CD8 MicroBeads (Miltenyi Biotec) and co-cultured with autologous DCs electroporated with sig-Rev-DCLAMP-encoding mRNA, as previously described [243]. On day 15 of bulk culture, CD8+ T cells secreting interferon-gamma (IFN-γ) upon re-stimulation with Rev-expressing DCs were enriched using the IFN-γ secretion assay (Miltenyi Biotec), according to the manufacturer’s instructions. These T cells were subsequently cloned by limiting dilution using irradiated allogeneic PBMC originating from three different HIV seronegative donors as feeder cells. On day 12 after cloning, wells were screened visually for clonal growth. Of the 60 wells where T-cell proliferation could be observed, a total of 13 exhibited Rev specificity in IFN-γ enzyme-linked immunosorbent assay (ELISA). Seven of these 13 T-cell clones (#8, 13, 15, 19, 44, 57, and 59) were successfully expanded.

### IFN-γ and TNF-α assays

Responder CD8+ T lymphocytes were seeded at 5 × 103 cells in 96-well round-bottom plates and co-cultured, in duplicate or triplicate, with 2 × 104 stimulator cells in a final volume of 200 μl lymphocyte medium supplemented with 25 IU/ml of interleukin (IL)-2 and incubated for 18 h at 37 °C. The IFN-γ or tumor necrosis factor-alpha (TNF-α) concentration in the supernatants was determined by ELISA, as described by the manufacturer (Endogen).

### Epitope recognition by standard 51Cr release assay

Peptides of interest were incubated in triplicate for 2 h at 0.1 nM with 5,000 51Cr-labeled target cells in a total volume of 50 μl IMDM without supplements. Subsequently, target cells were washed and co-cultured for 4 h with effector CD8+ T cells (ET ratio range: 2.5:1 – 25:1) before determination of 51Cr release by lysed cells. Labeling with 51Cr and the release assay were performed as described previously [243].
HIV-1 plasma RNA levels, HIV-1 RNA isolation, cDNA synthesis, PCR amplification, and sequencing

HIV-1 plasma RNA levels were determined using the Ultrasensitive Roche Amplicor HIV-1 RNA Monitor test (CAP/CA PHS; Roche Diagnostics), following manufacturer’s instructions. Viral RNA was isolated from plasma samples using the Roche High Pure viral RNA isolation kit (Roche Applied Science). RNA was transcribed into cDNA with 25 pmol of primer P1-R. After 2 min of denaturation at 80 °C, reverse transcriptase components were added and reverse transcription reaction was performed for 1 h at 50 °C. Rev cDNA was amplified using a nested PCR with outer P2-F plus P1-R and a nested primer set Pnest1-F plus Pnest2-R. PCR reactions were performed in a final volume of 50 µl containing cDNA or first PCR product and 12.5 pmol of each primer using the following amplification conditions: 7 min at 95 °C followed by 40 cycles of 40 s at 95 °C, 40 s at 50 °C, and 3 min at 72 °C, then 10 min at 72 °C. For the nested PCR, annealing temperature was 56 °C and elongation time 2 min. Molecular clones were obtained by cloning purified PCR products in the pCR4-Topo vector (Topo TA Cloning Kit; Invitrogen). Purified bulk and cloned PCR products were sequenced with Pnest2-R and S1-F using the Big Dye terminator sequencing kit version 3.0 and the 3130 XL genetic analyzer (Applied Biosystems), following the manufacturer’s instructions. PCR and sequence primers were obtained from Eurogentec and are listed in Table 1.

Sequencing of NOL6

Chromosomal DNA of the study subject was isolated from the non-plastic adherent fraction of PBMC using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The second and third exons, including the intermediate intron, of NOL6 (411 bp in total) were amplified by PCR with primers P3-F and P3-R (Eurogentec) (Table 1). Amplification conditions were as described for the HIV-1 amplification procedure, with an annealing temperature of 58 °C and elongation time of 40 s. Purified PCR product was sequenced with primer P3-F and P3-R using the HIV-1 sequencing procedure.

Sequence data analysis

Sequencing data were assembled by SeqMan (v 5.08, DNASTAR Inc.) with manual alignment of the sequences in the first exon of Rev using ClustalW, included in the software package BioEdit (v 7.0.9.0; Tom Hall Ibis Biosciences). Amino acid sequences were deduced from the nucleic acid sequences with the EditSeq program of DNASTAR.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Evolution of the EL9 sequence following ATIa</th>
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<tr>
<td><strong>WEEKS FOLLOWING ART INTERRUPTION</strong></td>
<td><strong>CD4+ T-CELL COUNT (CELLS/µL)</strong></td>
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<tr>
<td>-280</td>
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<td>131</td>
<td>591</td>
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<td>160</td>
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</table>

WT, wild-type viral sequence in the study subject before ART initiation.

a The emergence of mutations within the EL9 epitope is depicted in the amino acid alignment. Nucleotide substitutions resulting in amino acid mutations and sequences of clones with a frequency of >10% are shown. Therefore, percentages do not always add up to 100%. In all cases, viral RNA was isolated from plasma. ATI, analytical treatment interruption.

Rev complementation assay

HeLa cells containing a stably integrated Rev-deficient proviral genome (HLfB cells) [329, 330] were transfected with 1 µg of plasmid expressing Rev protein variants in frame with eGFP. Levels of HIV Gag p24 protein in culture supernatants were determined by ELISA (Aalto Bio Reagents). Intracellular expression of eGFP and HIV Gag proteins was assessed by flow
cytometry. Dead cells were excluded by staining with fixable LIVE/DEAD far red-fluorescent reactive dye (Invitrogen) prior to permeabilizing and fixing cells with BD Cytofix/Cytoperm kit (BD Biosciences). Cells were subsequently stained with a PE-labeled monoclonal antibody specific for Gag p55 (clone KC57, Beckman Coulter). Samples were acquired on a FACSCalibur and analyzed with CellQuest Pro software (both BD Biosciences). Both the p24 concentration of culture supernatant and the percentage of p55-expressing cells measured at 36 h post-transfection, were corrected for transfection efficiency (% eGFP-positive cells at 24 h post-transfection). The transactivation capacities of the Rev mutants were normalized to that of WT, active Rev (Rev-BOA, activity set at 100%).

RESULTS

The Rev EELLKTVRL antigenic peptide is HLA-B*44 restricted

In previous in vitro experiments, using CD8+ T cells originating from the study subject, we observed strong Rev-specific CTL responses [243]. To further characterize these CTL responses, we generated Rev-specific CTL clones by limiting dilution. Of the seven clones recovered, all showed high affinity for Rev. The location of the epitope(s) within the Rev protein was identified by stimulating CTL clone #59 with peptide-pulsed autologous EBV B-cells after which IFN-γ secretion was assayed. The peptides used were individual 15-mer pools of a set of 27 overlapping peptides covering Rev. The predominant Rev response was directed against peptide 3 (DEELLKTVRLIKFLY; Figure 1A). The remaining six CTL clones were stimulated likewise using peptides 2, 3, and 4 and all showed specificity for peptide 3 (data not shown).

The study subject was found to be HLA-A*03:01, -A*29:02, -B*07:02, -B*44:03, and -C*07 positive. To determine the HLA restriction element of peptide 3, COS-7 cells, HLA-B44 was identified as the restriction element (Figure 1B). The peptide-Rev. By analyzing the IFN-γ production of CTL clone #59 in response to these transfected COS-7 cells, HLA-B44 was identified as the restriction element (Figure 1B). The peptide-binding motif of the B44 supertype molecules contains an acidic residue at position 2 and a hydrophobic or aromatic residue at the carboxy terminus [331]. On the basis of this binding motif, four possible HLA-B44-restricted epitopes within peptide 3 were predicted nonamers DEELLKTVR and EELLKTVRL and decamers DEELLKTVRL and EELLKTVRLI. By measuring the cytolytic activity against these nona- and decamers, EELLKTVRL, denoted as EL9, was shown to be the shortest optimal peptide (Figure 1C). EL9 corresponds to position 10 – 18 of the Rev protein sequence according to HXB2 numbering.

To further dissect the subtype of the HLA-B44 restriction of EL9, both IFN-γ and TNF-α secretion by CTL clone #59 in response to EL9 peptide-loaded melanoma cell lines expressing HLA-B44 subtypes HLA-B*44:02, HLA-B*44:03, or HLA-B*44:04 were analyzed. Cytokine secretion was confined to the HLA-B*44:03 and, although to a much lower extent, the HLA-B*44:02 cell lines, as shown in Figure 1D. The presentation of the EL9 epitope by both HLA-B*44:02 and -B*44:03 molecules was confirmed by IFN-γ ELISA and 51Cr release assay using peptide-pulsed allogeneic EBV B-cells (Figure 1E and 1F).

Molecular mimicry between EL9 and NOL6 does not result in cross-reactivity of Rev-specific CTL

The sequence of the HIV-1 Rev epitope EL9 (EELLKTVRL) differs only by one amino acid from the peptide at position 89 – 97 of the human protein NOL6 (EELLKEVRL) [332]. The study subject’s genomic DNA was isolated, amplified, and sequenced at the NOL6 region with recovery of the WT EELLKEVRL sequence. The flanking regions of this peptide lacked single nucleotide polymorphisms and had no sequence similarity with the flanking regions of the EL9 epitope. CD8+ T cells, obtained 124 weeks following ATI, were co-cultured for 1 week with autologous DCs pulsed either with Rev EL9 peptide EELLKTVRL, with human NOL6 peptide EELLKEVRL, or with negative control peptide MAGE-C2. Bulk CD8+ T cells were then assayed for (cross)-recognition of these peptides. Following 1 week of co-culture with peptide-pulsed autologous DCs, no T cells specific for either NOL6 or MAGE-C2 were generated (Figure 2, data not shown for MAGE-C2 bulk stimulation).

EL9 sequence evolved following ART interruption

In the context of an immunotherapy trial, the study subject interrupted ART resulting in a rise in plasma viral load (pVL), which allowed us to determine plasma viral RNA sequences. Viral bulk and clonal sequences were obtained before ART initiation and at different time points following ART. Amino acid sequences were aligned in order to study sequence variability within the EL9 epitope over time (Table 2). Before ART initiation (i.e. 280 weeks prior to ATI), the EL9 sequence was found to be EELLKTVRL, further referred to as WT. The first mutations within the EL9 sequence evolved following ART interruption

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Figure 1
The Rev EELLKTVRL antigenic peptide is HLA-B44 restricted
(A) IFN-γ ELISA of CTL clone #59 against autologous EBV B-cells pulsed with the individual peptides of a set of 27 overlapping 15-mer peptides covering Rev. EBV B-cells transduced with Tat and Rev expressing retrovirus served as negative (neg) and positive (pos) control, respectively. Data represent the average of triplicate cultures. The ‘p numbers’ refer to the 27 different 15-mer peptides. (B) IFN-γ ELISA of CTL clone #59 against transfected COS-7 cells. Average values of duplicate cultures are displayed. Values for negative control Tat (ranging from 0.0 to 18.7 pg/ml) were subtracted. (C) Peptide-pulsed, 51Cr-labeled autologous DCs were co-cultured for 4 h in triplicate with Rev-specific CTL clone #57. Autologous EBV B-cells were used as positive control. Average values are displayed, values for negative control MAGE-C2 (ranging from 0.0 to 12.24%) were subtracted. DC, dendritic cell; CTL, cytotoxic T-lymphocyte; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha.

Figure 2
Molecular mimicry does not result in cross-reactivity of Rev-specific CTL
CD8+ T-cells obtained 124 weeks following ATI were co-cultured for 1 week with autologous DC pulsed with Rev EL9 peptide EELLKTVRL (circle symbols), human NOL6 peptide EELLKEVRL (triangle symbols), or irrelevant MAGE-C2 peptide SESIKKKVL (data not shown). Bulk T-cells were assayed in IFN-γ ELISA against autologous EBV B-cells pulsed with Rev EL9 peptide (magenta symbols), human NOL6 peptide (filled gray symbols), or MAGE-C2 peptide (open symbols). No cross-recognition of the human NOL6 peptide by the Rev EL9-specific CD8+ T-cells was observed. Data represent the average of duplicate cultures. CTL, cytotoxic T-lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha.
**Escape from a novel HIV Rev epitope**

**Chapter 4**

**E11D and E11D,K14Q mutations result in escape**

The E11D,K14Q mutant of EL9 remained present in the viral population for over 1 year, with its frequency slowly decreasing from 40% at week 72 to 20% at week 124. The E11D,K14Q mutation could reflect a viral escape of the EL9 epitope from immune pressure. To test this hypothesis, CD8+ T cells from three different time points (9 weeks before ATI and 60 and 110 weeks following ATI) were co-cultured with autologous DCs pulsed either with WT EL9 peptide or one of the mutants E10D, E11D, and E11D,K14Q. Bulk CD8+ T cells were then assayed in IFN-γ ELISA for (cross)-recognition of all of the four peptides (Figure 4 and data not shown). CD8+ T cells obtained prior to ATI, a time point at which none of the EL9 epitope variants were present, secreted IFN-γ in an antigen-specific manner upon co-culture with DCs expressing the WT peptide and, although to a lesser extent, the E10D mutant. Comparable data were obtained with CD8+ T cells sampled at 60 weeks (data not shown) and 110 weeks following ATI (Figure 4A and 4B). Following 1 week of co-culture with DCs pulsed with the E11D or E11D,K14Q mutant, no antigen-specific activation of CD8+ T cells could be induced, irrespective of the time point at which the T-cells were sampled (Figure 4C and 4D). CD8+ T cells obtained at 124 weeks post-ATI were tested in a similar assay for recognition of the L13I mutant. Compared with the WT EL9 peptide, recognition of the L13I mutant peptide was reduced. After bulk stimulation with WT EL9 peptide, L13I peptide concentration had to be increased 100× to obtain IFN-γ concentrations comparable with WT EL9 peptide (data not shown).

**EL9 mutants do not impair Rev protein function**

The impact of the EL9 mutants on Rev function was assessed by a Rev complementation assay, analyzing the capacity of the EL9 mutants to induce production of HIV Gag from a Rev-deficient provirus. Extracellular p24 protein (ELISA) and intracellular p55 protein (flow cytometry) were measured 36 h after transfection of HeLa cells (Figure 5). Following
transfection of plasmid expressing WT Rev protein (WT Rev-BOA), a concentration of 21 ng Gag p24/ml supernatant was detected and 7.5% of all living cells were positive for p55. The p24 concentration observed with the mutant Rev-BOAE10D) was slightly reduced compared with WT Rev-BOA, whereas for the mutants Rev-BOAE11D) and Rev-BOAE11D,K14Q) p24 concentrations were comparable with WT. The percentage of p55-positive cells did not differ between WT Rev and the Rev mutants, indicating that none of the three mutants was functionally impaired.

**FIGURE 5** Impact of EL9 mutants on Rev function

HeLa cells containing a stable integrated Rev-deficient provirus were transfected with Rev-GFP-expressing plasmids. Thirty-six hours post-transfection, Gag p24 protein secretion in the supernatant was detected by ELISA and intracellular Gag p55 protein was detected by flow cytometry. The transactivation capacities of the Rev protein mutants E10D, E11D, and E11D,K14Q were normalized to that of WT Rev-BOA (100%) and corrected for transfection efficiency by percentage of GFP-positive cells at 24 h post-transfection. The transfection efficiency, determined as the percentage of GFP-positive HeLa cells on flow cytometry at 24 h post-transfection, was comparable for the different conditions. For both assays, protein production was set at 100% for the positive control (WT Rev-BOA). As a negative control, transdominant inactive Rev-BOT was used. ELISA, enzyme-linked immunosorbent assay; WT, wild type.

**DISCUSSION**

In this article, we report the characterization of an HLA-B44-restricted Rev epitope, EELLKTVRL, denoted as EL9, which is highly similar to the peptide EELLKEVRL present within the human protein NOL6. The occurrence of sequence similarities between pathogen-derived self-peptides that results in cross-activation of autoreactive T- or B-cells by pathogen-derived peptides. This mechanism, known as molecular or host mimicry, was first put forward by Fujinami and Oldstone [321, 322]. Animal models in which molecular mimicry can trigger autoimmune disease are abundant [320]. Recently, data providing strong support for the role of molecular mimicry by antibody cross-reactivity in HIV-1-immunologic thrombocytopenia were published [333]. Although so far no direct evidence has been found for a link between HIV-1 T-cell epitope mimicry and autoimmunity, designing HIV vaccines including antigenic sequences with high similarities to the host proteome could result in cross-reactive responses with the risk of inducing autoimmunogenicity. Strong negative selection for T cells recognizing epitopes that are similar to self-antigens prevents autoimmunity. However, at the same time negative selection of autoreactive T cells could result in reduced immune recognition of regions of pathogen-derived proteins with high similarity with self-antigens, as was recently suggested for HIV infection by Rolland et al. [319] and Frankild et al. [318]. They show that HIV peptides frequently recognized by T cells have low similarity to the human host proteome. In addition, Rolland et al. show a trend toward negative correlation between the degree of similarity of HIV T-cell epitope sequences to the host proteome and their immunogenicity. The proposed mechanism for these findings is that CTL showing strong affinity for HIV epitopes with high similarity to human peptides have been tolerated via negative selection [318, 319, 334]. Although it has been advocated to remove host mimics from vaccine constructs to increase safety and efficacy, the impact of molecular mimicry on immune evasion, which plays a critical role in disease progression in HIV-1 infection, is unknown [319, 334]. In addition, the inverse relationship between similarity to the host proteome and frequency of immunologic recognition observed by Rolland et al. did not reach statistical significance. Moreover, for HIV-1, only 16 HIV-1 consensus B nonamers were found to have sequence similarity to human host proteins [319].

We report strong CTL recognition of a Rev-derived epitope that is highly similar to a human peptide. The T cells from despite its similarity to a self-antigen, can be explained by other interacting factors contributing to immunogenicity. Factors such as epitope entropy and representation of amino acids, not generally found at the C-terminus of CD8+ T-cell epitopes, might have more pronounced effects on immunogenicity than molecular mimicry [319]. Another possible explanation for the strong immune recognition of the EL9 epitope, despite its sequence similarity with NOL6, is the fact that its sequence is not completely identical to
the human NOL6 peptide. Rev EL9 bears a threonine (T) residue at position 6 of the epitope, whereas a glutamic acid (E) at this position, as seen in the human NOL6 peptide, has never been observed for HIV-1 subtype B Rev. In fact, residue 15 of the Rev protein is relatively well conserved with T, A, and I having been described [332]. Accumulation of random T to E mutations in EL9, resulting in 100% sequence similarity with the EELLKTVERL sequence within NOL6 and immune escape of the virus, is therefore not expected. Furthermore, the lack of sequence similarity between the flanking regions of the Rev EL9 and NOL6 peptide probably results in a different antigen processing and ensuing T-cell recognition [335]. Finally, the glutamic acid at position 6 in the EELLKTVRL sequence of NOL6 not only represents one amino acid mismatch compared with the EL9 epitope, it also results in a reduced affinity for HLA-B*44:02 and HLA-B*44:03. The latter is expected because the peptide-binding motif of HLA-B44 preferentially contains a G, T, I, or V at position 6 of the nonamer. The average relative binding of a nonamer, calculated by dividing the IC\textsubscript{50} (nM) of the positive control peptide by the IC\textsubscript{50} (nM) of the peptide tested, with a T at position 6 is 5.0 for HLA-B*44:02 and 4.0 for HLA-B*44:03, whereas for E it is reduced to 0.26 and 0.48, respectively [331]. It is unlikely that low expression levels of NOL6 protein would be responsible for not inducing tolerance as mRNA expression levels of NOL6 in the thymus are comparable with those observed in lymphoid cells [336].

Our results show that the EL9 Rev epitope is presented by HLA-B*44:02 and -B*44:03 molecules. The frequency of these HLA-B44 alleles varies depending on the population studied. In European Caucasians, the HLA-B*44:02 and -B*44:03 frequencies can be found in up to 20% and 26.5% of the population, respectively. Lower frequencies have been observed in sub-Saharan countries. Detailed frequencies can be found at www.allelefrequencies.net [337]. On the basis of these allele frequencies, it is likely that the EL9 epitope is recognized by a considerable fraction of the HIV-infected population in the Western world and to a lesser extent in sub-Saharan Africa. Epitope presentation by both HLA-B*44:02 and B*44:03 molecules is in accordance with previously published data showing that both HLA-B44 subtypes share a common peptide-binding motif. However, these HLA molecules can also present distinct antigens, owing to different conformations adopted by the same peptides in the two HLA molecules. Moreover, some peptides bind selectively to only one of the two molecules [338, 339].

We here show that autologous CD8\textsuperscript{+} T cells are activated to a higher extent when EL9 is presented by HLA-B*44:02 rather than by HLA-B*44:02. This can either be because of less efficient binding of EL9 to HLA-B*44:02 or to lower binding affinity of the TCR to heterologous peptide-presenting HLA molecules. To draw conclusions on the cross-reactivity of HLA-B*44:02 and HLA-B*44:03 for the EL9 epitope, CTL clones from different donors should be studied. Our results offer further insight into the interplay between viral evolution and cellular immune response against HIV. While on ART, the study subject entered a clinical trial in which four vaccinations of autologous DCs electroporated with mRNA encoding Tat, Rev (containing the EL9 epitope), and Nef were administered. Immunotherapy did not result in the control of viral replication. The dynamics of CD4\textsuperscript{+} T-cell count and pVL were evaluated in longitudinal samples until 124 weeks post-treatment interruption. Sequence evolution of Rev EL9 was studied and newly emerged epitope mutants were detected. To which extent the immunotherapy and re-initiation of antiretroviral treatment interruption have affected clinical and immunological parameters and viral sequences is unknown. The observed mutations in the EL9 epitope could theoretically be the result of the immunotherapy the patient was subjected to. Although this hypothesis seems unlikely because the mutant amino acids (aspartic acid at position 10 or 11 and glutamine at position 14) are present in HIV-1 subtype B isolates from Europe, with a prevalence of respectively 6%, 28%, and 4% [31], it is however likely that the emerging mutations result from a selective immune pressure on the virus. The Rev-specific CD8\textsuperscript{+} T cells from the study subject proliferated and secreted IFN-\gamma and TNF-\alpha upon antigen stimulation. Other factors contributing to the effectiveness of the immune response, such as CD2 and MIP-1\beta excretion, perforin expression, and maturation state, as previously described [137], were not assessed in our study. However, because the induced Rev-specific CTLs were cytotoxic, they can be characterized as functional T cells and can therefore exert a selective immune pressure on the virus [317]. The E10D mutation was first detected at 72 weeks post-ATI but disappeared 38 weeks later. Its recognition by CTL resulted in slightly reduced, but not abolished, IFN-\gamma production compared with WT EL9. In contrast, the E11D/K14Q mutation resulted in full escape from immune recognition. Up to 50 weeks after the appearance and persistence of the E11D/K14Q mutant, no CD8\textsuperscript{+} T cells with specificity for this mutant have been detected. The E11D mutation not linked to K14Q, also abrogated recognition by T cells. The mechanism of immune evasion by the prevailing E11D mutation has not been dissected in this study but is most likely because of a reduced binding of these peptides to the HLA-B*44:03 presenting molecule [340, 341]. The other escape mutations we observed do not include anchor residues and in our opinion most likely interfere with TCR interaction. The reduced binding of the E11D mutants to the HLA-B*44:03 molecule has been calculated using the epitope prediction program EpiToolKit [340] and a model described by Tenzer et al. The latter is based on in vitro experiments and combines predictions of proteasomal cleavage, transporter for antigen presentation (TAP) transport, and MHC class I binding. The glutamic acid at position 11 of the Rev protein (E11) is an anchoring residue of the HLA-B*44-restricted nonamer. Because the B44 supertype molecules in general and the HLA-B*44:03 subtype in particular prefer a glutamic acid at this position, a substitution of E11 will abrogate the anchoring of the epitope [342]. Because the E11D single mutation completely abrogated T-cell recognition, we hypothesized that
the E11D mutation could have a negative effect on viral replication capacity, compensated by the K14Q mutation [129, 343]. A wide range of mutations in rev, including mutations in EL9, can result in a reduced function of the Rev protein, but to date no mutants at amino acid position 11 or 14 have been studied [329]. The EL9 epitope is located in the helix-loop-helix region of the Rev protein [344]. Within this functional region, the amino acids L12, V16, and L18 have been identified as being important for the molecular interactions mediating the multimeric assembly of Rev after binding to the Rev-responsive element [344]. The mutations observed in the study subject do not affect the leucine motif and were therefore not expected to influence protein function. Indeed, we show that E10D, E11D, and E11D,K14Q mutations do not diminish Rev protein function, as assessed by the Rev complementation assay. From these results, we conclude that an effect of the observed mutants on Rev protein function is absent. Therefore, the simultaneous occurrence of the E11D mutation and the K14Q mutation cannot be explained by negative or compensatory effects on viral replication capacity. A possible linkage of E11D to K14Q is unlikely because it was not observed in a dataset of sequences of 68 HLA-typed HIV-1 subtype B-infected subjects (data from the Los Alamos sequence database [31]). Although five of these 68 subjects were HLA-B*44:03 positive, the frequency of E10D, E11D, or K14Q mutations in these subjects was the same as for the non-HLA-B*44:03 positive subjects.

It is well known that the dynamics of immune escape are complex. The possibility of viral evolution in epitope or epitope flanking regions is influenced by the functional constraint of the sequence comprising the epitope. If epitopes are located in conserved regions of the protein, escape from immune pressure by viral mutation might compromise replicative capacity [121, 129, 131, 345]. Mutants can appear not only shortly after the induction of a specific immune response, but also years later. It has been suggested that a slower appearance of escape mutants is associated with a larger fitness cost [131, 302, 346, 347]. An explanation for the relatively late emergence of mutations in EL9 is the additional functional constraints imposed by overlap of the open reading frame (ORF) of the first exon of rev with tat [348]. Rev consists of 116 amino acids encoded by two exons with ORFs fully overlapping Tat and/or Env, respectively. Mutations within Rev can therefore be accompanied by amino acid changes in Tat and/or Env, possibly resulting in deleterious effects on the virus [348, 349]. The mutation leading to the E10D substitution is synonymous in Tat, whereas the mutations resulting in the E11D and K14Q substitutions are non-synonymous in Tat: T58P and Q60P (HXB2 numbering). However, these two amino acid changes are located in a highly variable region of Tat and synthetic variants with the T58P and Q60P mutation had full transactivation activity [349]. Although the emerging EL9 mutants escape from immune recognition, the pVL values during ATI were comparable with the pVL values before initiation of ART, suggesting a limited effect on viral replication of the EL9-specific CD8+ T cells in the study subject. Nevertheless, the characterization of this epitope is of importance because it contributes to our understanding of immune recognition of the HIV proteome. In addition, identification of novel HIV-1 epitopes can lead to improvements of epitope prediction algorithms [240].

In conclusion, we characterized a HLA-B44-restricted CD8+ T-cell epitope in the early expressed HIV-1 Rev protein. Despite a high sequence similarity with the human nuclear protein NOL6, we detected no CTL cross-reactivity. Mutations in the EL9 epitope result in viral escape from CTL recognition and are largely tolerated with respect to viral replicative capacity.

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DC immunotherapy in HIV-1 infection induces

A MAJOR BLOOD TRANSCRIPTOME SHIFT

HIV immunotherapy: host transcriptome effects

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Anna L. de Goede, Arno C. Andeweg, Henk-Jan van den Ham, Maarten A. Bijl, Fatiha Zaaraoui-Boutahar, Wilfred F.J. van IJcken, Sofie Wilgenhof, Sabine D. Allard, Carel A. van Baalen, Brenda De Keersmaecker, Marchina E. van der Ende, Patrick Lacor, Joeri L. Aerts, Kris Thielemans, Rob A. Gruters and Albert D.M.E. Osterhaus

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PART II
STRATEGIES FOR
ANTIGEN DELIVERY
IN HIV IMMUNOTHERAPY

Chapters 6 and 7
Characterization of recombinant

INFLUENZA A VIRUS AS A VECTOR FOR HIV-1 P17Gag

Recombinant Influenza as a vector for HIV

Anna L. de Goede, Patrick H. Boers, Lennard J. Dekker, Albert D.M.E. Osterhaus, Rob A. Gruters and Guus F. Rimmelzwaan
ABSTRACT

We have generated a recombinant influenza A virus with the HIV-1 p17\textsuperscript{Gag} (rFlu-p17) gene inserted into the influenza virus neuraminidase (NA) gene. Expression of HIV-1 p17 protein was detected by conventional Western blot analysis and also by liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of rFlu-p17 infected cells. The latter method does not depend on protein-specific antibody preparations and proved to be a powerful tool to detect proteins of interest. Next, antigen presentation of p17 expressed after infection of antigen-presenting cells was determined. Cloned p17-specific CD8\textsuperscript{+} T cells were co-cultured with rFlu-p17 infected B-cells and produced IFN-\gamma upon stimulation. Furthermore, we showed that immunization with rFlu-p17 elicited a humoral immune response in mice. This study shows that replication-deficient rFlu-p17 is antigenic \textit{in vitro} and immunogenic \textit{in vivo} and warrants further development as a candidate vaccine vector.

INTRODUCTION

Human immunodeficiency virus (HIV) replication can be suppressed by highly active antiretroviral therapy (HAART). Despite the success of antiviral drugs in terms of reduction of mortality and morbidity associated with AIDS, HIV is still the leading viral cause of mortality worldwide. This is, amongst other reasons, caused by the limited access to HAART in resource poor countries. Vaccines against HIV would offer an alternative preventative or therapeutic strategy. An effective, prophylactic vaccine would protect against infection, prevent transmission and in those infected with HIV, it would slow down the rate of progression to AIDS. A therapeutic vaccine could control viral replication and minimize or eliminate the need of HAART exposure in infected individuals [217]. Despite substantial efforts, a vaccine for the prevention or therapy of AIDS will not become available shortly. Therefore, the development of new antiviral drugs and effective vaccines is urgently needed.

Various vaccine strategies have been considered for the development of an HIV vaccine. Especially vaccines that aim at the induction of both humoral and cellular virus-specific T-cell responses are considered important [217, 390]. Influenza viruses are attractive candidates as viral vaccine vectors since they elicit strong humoral and cell-mediated immune responses, can be manipulated by reverse-genetics techniques [391, 392] and can be administered needle-free at mucosal sites. For a wide range of infectious and non-infectious diseases, such as chlamydia, tuberculosis, malaria and cancer, recombinant influenza viruses have been tested as viral vaccine vectors [393-398]. Also, several research groups have tested recombinant influenza as a viral vector for various HIV-1 or SIV antigens in mouse models. It was shown that HIV-1 or SIV-specific antibodies and/or specific T lymphocytes were elicited after infection with recombinant influenza virus [399-406]. These results imply the value of recombinant influenza vector for HIV vaccination. In the present study we constructed a replication-deficient recombinant influenza vector for the antigen HIV-1 p17\textsuperscript{Gag} (rFlu-p17) and addressed the following questions: (1) are rFlu-p17-infected cells recognized by human p17-specific CD8\textsuperscript{+} T cells \textit{in vitro} and? (2) is rFlu-p17 immunogenic in mice? Additionally, we tested if expression of HIV-1 p17 from the recombinant vector could be detected by mass spectrometry as an alternative method for Western blotting.

The results of the present study show that mass spectrometry is an attractive method to detect viral proteins in infected cells. It enables the detection of proteins independent of the availability of antigen specific antibodies. Our study also demonstrates that the replication-deficient rFlu-p17 allows activation of human p17-specific CD8\textsuperscript{+} T cells \textit{in vitro} and induces antibody responses in mice.
METHODS

Plasmids and viruses
Recombinant influenza viruses were generated by reverse-genetics technology based on the gene segments of the strain A/PR/8/34, as described previously [392, 407]. A MluI endonuclease restriction site was introduced into the neuraminidase (NA) gene segment and the start codon of NA removed by site-directed mutagenesis (Figure 1). Most of the NA coding sequence was excised by digestion with MluI and SpeI and replaced by the coding sequences of either HIV-1 p17Gag (amino acid 1–132 of consensus Gag protein, GenBank accession number AAX39502.1) and HIV-1 Rev (GenBank accession number AAX39506.1) [326] or enhanced green fluorescent protein (eGFP) amplified from pEGFP-N1 (Becton and Dickinson), resulting in recombinant viruses designated rFlu-p17, rFlu-Rev and rFlu-GFP. HIV-1 genes were codon optimized synthetic genes (GeneArt). The NA segment was chosen for insertion of the transgene because it is a promising approach for the construction of recombinant influenza viruses [391, 399, 408]. Recombinant viruses were generated as described by Rimmelzwaan et al. and De Wit et al. [391, 392]. Of note, these viruses are replication deficient because they lack functional NA and therefore depend on an exogenous source of NA (0.3 mU/ml NA obtained from V. Cholera (Sigma–Aldrich)) for replication.

Demonstration of protein expression
Expression of p17 protein in MDCK cells infected with rFlu-p17 was tested by Western blot analysis. MDCK cells infected with rFlu-p17 or rFlu-GFP which was included as a negative control, and human 293T cells transfected with the NA-p17 gene segment were harvested in PBS containing protease inhibitor Complete Mini EDTA-free (Roche Applied Science). SDS-PAGE separated proteins were blotted onto Hybond-P (GE Healthcare Amersham) and membranes were incubated with α-p17 antiserum (AIDS Research and Reference Program, NIH) diluted 1:20,000 or an α-GFP monoclonal antibody (Chemicon, USA) diluted 1:1000 after blocking with PBS containing 0.05% Tween-20 and 5% nonfat dried milk. After washing, the membranes were incubated with horseradish peroxidase-conjugated swine α-rabbit (for polyclonal α-p17 serum) or rabbit α-mouse (for α-GFP) immunoglobulin antibody (Dako) diluted 1:200. Bound antibodies were detected using enhanced chemiluminescence (GE Healthcare Amersham) as recommended by the manufacturer.

In parallel, rFlu-P17 and rFlu-GFP infected MDCK cells were inactivated with Trizol (Invitrogen). To test the suitability of the LC-MS/MS method for the detection of arginin-rich proteins, 293T cells were transfected with a plasmid from which Rev is expressed, and inactivated with Trizol. Cells were precipitated by washing with cold acetone, treated with 0.1% RapiGest SF (Waters), sonified and incubated with DTT (Sigma–Aldrich) and iodo-acetamide (Sigma–Aldrich) at final concentrations of 5 and 3 mM respectively. Proteins were then digested with 1 µg trypsin (Promega) overnight at 37 °C. Samples were acidified with trifluoroacetic acid (0.5%) to degrade the RapiGest and supernatants were subsequently analyzed by nanoLC-LTQ-orbitrap (Finnigan LTQ-Orbitrap XL, Thermo Fisher Scientific) mass spectrometer. Separation was performed on a nanoscale liquid chromatography system (nanoLC Ultimate 3000, Dionex) with a 180 min gradient (5–50% acetonitrile/H2O, 0.1% formic acid). The injection volume was 5 µl of the tryptically digested protein pellet. Tandem mass spectra were extracted by the extract_msn tool of Xcalibur 2.0.7 (Thermo Fisher Scientific). All MS/MS samples were analyzed using Mascot (version 2.2; Matrix Science, UK). Mascot was set up to search the MSDB database (selected for Virus, 2006-08-03 version) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Oxidation of methionine was specified in Mascot as variable modifications and carboxymethylation of cysteine as a fixed modification. Peptide identifications that exceeded the specific database search engine thresholds (ion score greater than 25) were loaded into Scaffold (v 2.01.02; Proteome Software Inc.).

CD8+ T-cell antigenicity in vitro
Three different CD8+ T cell clones were used to test the antigenicity of rFlu-p17 in vitro: a Rev-specific clone directed against the HLA-B57 restricted Rev67-75 (SAEPVPLQL) epitope [99], a clone (G10) directed against the HLA-A2 restricted p1777–85 (SLYNTVATL) epitope [409] and a clone against the HLA-A*0201 restricted influenza matrix M158–66 (GILGFVFTL) epitope [410]. T-cell clones were stimulated with HLA-matched EBV-transformed B cells at an E:T ratio of 1:2. The B cells were infected with rFlu-p17 or rFlu-Rev at an MOI of 10 TCID50/cell or incubated

![Figure 1](image-url)
with 3 μM p17 peptide GSEEELRSLYNTAVL (ADP 703.8 Cambridge Research Chemicals) or 10 μM Rev peptide (Eurogentec). After co-culture for 6 h in the presence of GolgiStop (BD Biosciences), the T cells were incubated with monoclonal antibodies specific for CD3 (PerCP-labeled, BD Biosciences) and CD8 (FITC-labeled, DAKO), treated with Cytofix/Cytoperm and GolgiStop (BD Biosciences) and stained for intracellular IFN-γ (PE-labeled, Pharmingen). Subsequently the cells were analyzed by flow cytometry using a BD FACSCalibur flow cytometer. Data were analyzed with BD CellQuest Pro software (BD Biosciences).

### Immunogenicity in vivo

Pathogen-free C57BL/6 and BALB/c female mice, 6–8 weeks of age, were obtained from Charles River Laboratories. For all experiments, institutional care and use guidelines were followed. Mice were anesthetized with isoflurane and immunized either intranasally (i.n.) with 50 μl 1 × 10^6 TCID<sub>50</sub> or intraperitoneally (i.p.) with 800 μl 1 × 10^6 TCID<sub>50</sub> of rFlu-p17 or rFlu-Rev. Mice were given a booster immunization with the same dose and route of administration 4 weeks after the first infection, 8 days later mice were anesthetized and blood was drawn by orbita puncture.

For the detection of p17-specific serum antibodies an ELISA was used. EIA/RIA high binding plates (Costar) were coated with recombinant p17 protein (ARP 689, NIBSC), 200 ng/well, next, incubated with mice sera diluted in a range from 1:100 up to 1:110,000 and subsequently incubated with a HRP-labeled goat anti-mouse antibody (Dako). Plates were developed using tetramethylbenzidine substrate (Meddens), the colour reaction was stopped with 0.5N sulphuric acid and OD<sub>450</sub> values were read with the Infinite200 reader (TeCan). The mean OD<sub>450</sub> value of duplicate wells was calculated and the endpoint titer was expressed as the reciprocal of the highest serum dilution with an OD<sub>450</sub> value above an arbitrary cut-off value of 0.2.

For the detection of influenza virus specific serum antibodies a hemagglutination inhibition (HI) assay was performed using turkey erythrocytes and four hemaglutinating units of influenza virus A/PR/8/34, according to standard procedures [41][1]. The HI titer was expressed as reciprocal of the highest serum dilution which still completely inhibited the agglutination of the erythrocytes by influenza virus.

### Statistical analysis

GraphPad Prism 4 was used to prepare graphs and analyze data. A Mann–Whitney non-parametric test was used and differences were considered statistically significant for p < 0.05. For ELISA titers below 100, a value of 30 was used for the calculations.

### RESULTS AND DISCUSSION

In this study, we describe the construction and characterization of a recombinant influenza virus as a vector for HIV-1 p17 (rFlu-p17). We show that rFlu-p17-infected cells allow activation of human p17-specific CD8<sup>+</sup> T cells in vitro. In addition, upon vaccination of mice with rFlu-p17 virus, specific antibody responses were induced. Furthermore, we show that HIV-1 p17 protein expression from the recombinant influenza virus can be detected not only by conventional Western blotting but also by mass spectrometry.

P17 protein expression in cells infected with rFlu-p17 was demonstrated by the commonly used Western blotting method. As shown in Figure 2A, p17 protein was detected in cells transfected with a plasmid from which the NA gene is transcribed and in cells infected with rFlu-p17. After infection with rFlu-GFP, which was included as negative control, no p17 could be detected. In contrast, infection with rFlu-GFP resulted in the detection of GFP, which was not observed after infection with rFlu-p17. Although Western blot analysis of protein is a very useful method, it is quite laborious and dependent on the availability of antibody preparations specific for the protein of interest. As an alternative for Western blotting, we also used mass spectrometry for the identification of p17 in rFlu-p17 infected cells. The MS/MS analyses resulted in the identification of p17 with an amino acid sequence coverage of 45%, which is statistically significant (Figure 2B and 2C). LC-MS/MS analysis of 293T cells transfected with Rev expressing plasmid resulted in the identification of Rev with amino acid sequence coverage of 25% (data not shown). Tryptic digestion of the arginin-rich protein Rev theoretically results in five peptides with a mass suited for mass spectrometry, two of these peptides were detected by LC–MS/MS. From these experiments we conclude that detection of protein expression from a recombinant viral vector by mass spectrometry is feasible. Because specific antibodies are not required, this method is universal for the detection of proteins and therefore is an attractive alternative for the commonly used technique of Western blotting.

To test if infection with rFlu-p17 would drive endogenous antigen processing and MHC class I restricted presentation, the capacity of HLA-matched EBV-transformed B-cells infected with rFlu-p17 to activate p17-specific CD8<sup>+</sup> T cells was assessed by staining for intracellular IFN-γ production upon stimulation. As shown in Figure 3, the p17-specific CD8<sup>+</sup> T-cell clone responded to B-cells pulsed with the corresponding p17 peptide or infected with rFlu-p17. The Rev-specific CD8<sup>+</sup> T-cell clone, which was included as negative control, only responded to a Rev-derived peptide and not to rFlu-p17 infected antigen-presenting cells. As expected, the CD8<sup>+</sup> T-cell clone specific for the influenza matrix protein responded to rFlu-p17 infected cells and M1-derived peptides.
After two i.n. or i.p. immunizations with rFlu-p17, BALB/c and C57BL/6 mice developed p17-specific antibody responses, whereas immunization with rFlu-Rev did not, although both viruses induced influenza virus specific HI antibodies (Figure 4). The HI antibody response induced with rFlu-p17 was significantly lower than the response induced with rFlu-p17, suggesting that the insertion of the Rev gene had a modest negative effect (Figure 4A). We concluded that rFlu-p17 was immunogenic in mice.

Recombinant influenza viruses expressing SIV or HIV-1 genes have been described by others [399, 401-406, 412]. In most cases, the lentiviral nucleotide sequences were cloned into the HA gene of influenza viruses, although the NS1 [402] and NA gene [399, 401-406, 412] segments also have been used for insertion of foreign genes. Interruption of the NA gene caused by gene insertion results in a virus that is able to initiate infection, giving rise to expression of influenza and recombinant proteins but virus progeny cannot be released from the infected cell [413]. Furthermore, an eventual reassortment event with a wild type virus cannot result in viable, replication competent recombinant virus. Thus, in the case of rFlu-p17, p17 is expressed and processed not only by the MHC class II route of antigen processing but also by the class I pathway. The latter enables activation of CD8+ T cells, which was confirmed with the p17-specific CD8+ T-cell clone [390]. Immunization with the rFlu-p17 resulted in p17-specific antibody responses, confirming that these recombinant influenza viruses are immunogenic and may be used as viral vectors. Although not demonstrated, we anticipate that this vector also would induce p17-specific CD4+ and CD8+ T-cell responses.

In conclusion, recombinant influenza viruses are promising vector candidates that may be used for the induction of antibody and cell-mediated immune responses including CD8+ T-cell responses. More research on the induction of humoral and cellular immune responses is required to further develop recombinant influenza viruses as vaccine candidates. LC–MS/MS provides a universal method for the detection of specific proteins and offers several advantages compared to conventional Western blot analysis: the availability of protein-specific antibodies is not required and the method is less laborious. LC–MS/MS is a useful tool that may aid in the characterization of any recombinant viral vector, independent of the origin of the gene of interest that is expressed.

Detection of the HIV-1 p17Gag protein by Western Blot analysis and mass spectrometry (MS). (A) Lysates of 293T cells transfected with a plasmid from which HIV p17Gag is expressed (lane 1), rFlu-p17 infected MDCK cells (lanes 2 and 5) and rFlu-eGFP infected MDCK cells (lanes 3 and 4) were subjected by SDS-PAGE. Subsequently proteins were transferred to a Hybond-P PVDF membrane and probed with a rabbit polyclonal antibody specific for HIV p17Gag (lanes 1–3) or a mouse monoclonal antibody specific for GFP (lanes 4 and 5). M, lanes with protein standards; molecular masses (in kDa) are indicated by the numbers on the left. (B and C) MS analysis of trypsin cleaved proteins in rFlu-p17 infected MDCK cells identified p17Gag. Panel B shows the amino acid sequence of p17Gag. The bold and underlined depicted parts of the sequence have been identified by MS/MS resulting in a total sequence coverage of 45%. MS analysis of non-infected control cells did not result in the detection of p17Gag sequences (data not shown). Panel C shows an example of a selected MS/MS spectrum for one of the peptides that was identified, namely KAQQAAADTGNSSQYSQNY of p17Gag. For a selection of peaks, the exact m/z values are denoted in the spectrum.
Recombinant Influenza as a vector for HIV

**FIGURE 3**  Activation of p17-specific CD8⁺ T cells by rFlu-p17

Activation of p17-specific CD8⁺ T cells by rFlu-p17 infected HLA-matched EBV transformed B-cells. P17, Rev and influenza virus M1 protein-specific cloned CD8⁺ T cells were stained for intracellular IFN-γ after stimulation with B-cells infected with rFlu-p17 or pulsed with peptides as indicated. "*" indicates “not tested”. Error bars represent standard error of the mean of duplicate flow cytometry stainings and subsequent analysis.

**FIGURE 4**  rFlu-p17 induces p17-specific antibody responses in mice

*Influenza virus and HIV-1* p17-specific antibody responses after vaccination with rFlu-Rev and rFlu-p17 were detected by HI assay (A) and ELISA (B) respectively. The ELISA endpoint titers were defined as the highest plasma dilution resulting in an absorbance value at 450 nm higher than cut-off value of 0.2. BALB/c and C57BL/6 mice were immunized by the i.n. or i.p. route as indicated. Mice inoculated with PBS or rFlu-Rev were included as negative controls. p values > 0.05 were considered non significant, denoted by ns.
ACKNOWLEDGEMENTS

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Induction of humoral and cellular immune responses by

ANTIGEN-EXPRESSING IMMUNOSTIMULATORY LIPOSOMES

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Antigen-expressing liposomes

Maryam Amidi, Mary J.G. van Helden, Neda Rafiei Tabataei, Anna L. de Goede, Marijn Schouten, Volkert de Bot, Anastasia Lanzi, Rob A. Gruters, Guus F. Rimmelzwaan, Alice J.A.M. Sijts and Enrico Mastrobattista
Recently we have shown that liposomes can be used as artificial microbes for the production and delivery of DNA-encoded antigens. These so-called antigen-expressing immunostimulatory liposomes (AnExILs) were superior in inducing antigen-specific antibodies compared to conventional liposomal protein or DNA vaccines when tested in mice after i.m. immunization. In this study, we investigated the capacity of AnExILs to induce T-cell responses. By using a plasmid vector encoding a model antigen under control of both the prokaryotic T7 and the eukaryotic CMV promoter we hypothesized that antigen production could lead to CTL activation via two distinct routes:

1. production of antigens inside the AnExILs with subsequent cross-presentation after processing by APCs,
2. endogenous production of antigens after AnExIL-mediated transfection of the pDNA.

Although we were not able to demonstrate transfection-mediated expression of luc-NP in mice, i.m. injection of AnExILs producing luc-NP resulted in T-cell responses against the encoded NP epitope, as determined by tetramer staining. T-cell responses were comparable to the responses obtained after i.m. injection of naked pDNA. In order to find out whether CTL activation was caused by cross-presentation of the exogenous antigens produced inside AnExILs or by endogenous antigen production from transfection with the same pDNA source a second study was initiated in which the contribution of each of these effects could be separately determined. These results demonstrate that the observed T-cell responses were not exclusively caused by cross-presentation of the AnExIL-produced antigens alone, but were rather a combination of dose-dependent antigen cross-presentation and low levels of endogenous antigen production.

INTRODUCTION

Immunotherapy of cancer requires the conversion of weak tumor antigens into strong immunogens in order to overcome the often weakened or tolerized immune system of the cancer patients. Moreover, to prevent occurrence of tumor resistance to the immunotherapy, mixtures of antigens rather than single tumor associated antigens are preferred [414]. DNA vaccines have been extensively explored for cancer immunotherapy (reviewed by [415-417]). The popularity of DNA can be ascribed to its ease of manipulation, production, and formulation in addition to the possibility to incorporate multiple antigenic epitopes in one vector. Moreover, since DNA vaccination leads to antigen production within the cytosol, it often induces strong CTL responses needed for eradication of tumor cells [418]. However, in spite of high expectations based on their efficacy in preclinical models, immunogenicity of first generation DNA vaccines in clinical trials was shown to be poor, and despite recent improvements in delivery methods for DNA vaccines, no DNA vaccines have yet been licensed for human use [416].

Recently, we have developed an entirely new concept of vaccination that entails the production of protein antigens inside liposomes using cell-free protein synthesis systems. These so-called antigenexpressing immunostimulatory liposomes (AnExILs) combine the production and delivery of antigens in one system [419, 420]. It has two major advantages over conventional DNA vaccines. First, AnExILs do not only rely on the often inefficient transfection of antigen-encoding DNA into the vaccinee’s cells in order to be effective. In addition to expression of endogenous antigens in the vaccinee’s cells, the antigens will be exogenously produced inside the AnExILs prior to delivery. Second, since AnExILs make use of bacterial proteins (extracts or a mix of recombinant proteins) for cell-free protein synthesis, they have a strong adjuvant effect which is expected to strengthen the immune response against the produced antigens. Previous work indeed demonstrated that AnExILs, when compared to conventional DNA, protein or mixed DNA/protein liposomal vaccines, were superior in inducing antigen-specific antibody responses, which could be directly ascribed to the adjuvant-effect of AnExILs [420].

The objective of this study was to find out whether AnExILs are, besides generation of antibodies, also capable of inducing T-cell responses against the produced antigens, which is a prerequisite for further development of AnExILs as cancer vaccine. For this, a model CTL epitope was genetically fused to the C-terminus of reporter enzymes (luciferase or β-galactosidase) to assess antigen production, quality and degree of antibody production and epitope-specific T-cell responses. The results show (1) the capacity of AnExILs to induce NP-specific T-cell responses, (2) interesting clues to the mechanism of the T-cell induction with AnExILs.
Antigen-expressing liposomes

Methods

Materials

Egg-derived L-α-phosphatidylcholine (EPC), 1,2- distearoyl- sn-glycero- 3- phosphoethan- olamine- polyethylene glycol (PEG) 5000 (DSPE-PEG 5000) and 1,2- dioleoyl- sn- glycerol-3- (L-α-amino-L-carboxy- pentyl) iminodi- acetic acid succinyl (DOGS-NTA) were purchased from Avanti Polar Lipids, Inc. Luria Broth, 2-mercaptoethanol, adenosine-5'-triphosphate (ATP), phosphoenol-pyruvate (PEP), cytidine-5'-triphosphate (CTP), guanosine-5'-triphosphate (GTP), 3'-5'-cyclic adenosine monophosphate (cAMP), folinic acid, cholesterol (CHOL) and β-galactosidase enzyme (400 IU/mg) and each of the 20 encoded amino acids were purchased from Sigma -Aldrich. The fluorescein di-β-D-galactopyranoside (FDG) was supplied from Flucka. Rabbit polyclonal anti-β-galactosidase IgG and Cy-5 conjugated goat anti-rabbit immunoglobulin was from Abcam. Horseradish Peroxidase (HRP)-labeled goat anti-mouse total IgG and HRP-labeled rabbit anti-mouse IgG1 were purchased from Fermentas. Dithiothreitol (DTT), deMan-Rogosa-Sharpe (MRS) Broth and pyruvate kinase (PK) were from Flu ck. Rabbit polyclonal anti-β-galactosidase IgG and Cy-5 conjugated goat IgG anti-rabbit immunoglobulin was from Abcam. Horseradish Peroxidase (HRP)-labeled goat anti-mouse total IgG and HRP-labeled rabbit anti-mouse IgG1 were obtained from Invitrogen. HRP-labeled Rat monoclonal anti-mouse IgG2a was obtained from Abcam. PEG-8000 was from Promega. All other materials used were of analytical or pharmaceutical grade. 

Plasmids

Plasmid pVEX2.2EM-LacZ-NP encoding E. coli β-galactosidase fused to H3N2 influenza nucleoprotein epitope (NP) and under control of the prokaryotic T7 promoter, was used for in vitro production of β-galactosidase-NP, as a model antigen for vaccination studies. Plasmid pVAX-LacZ-NP, under control of eukaryotic CMV promoter was used in DNA vaccines for immunization studies. Furthermore, pDUAL GC-Luc-NP, encoding firefly luciferase fused to NP epitope, with both prokaryotic T7 and eukaryotic CMV promoters, which allows in vitro and in vivo expression of Luc-NP protein using single plasmid DNA, was used for in vivo antigen expression and immunization against influenza NP epitope.

Preparation of cell-free protein synthesis system and AnExILs

The E. coli Rosetta-gami™ strain, which contains pRARE encoding rare tRNA codons and is devoid of endogenous β-galactosidase enzyme (Novagen), was used to make S30 bacterial extract as described previously [419]. A coupled in vitro transcription/ translation reaction mixture (further referred to as IVTT mix), consisted of 30% (v/v) S30 extract, 175 µg/ml tRNA, 250 µg/ml creatine kinase, 5.8 mM magnesium acetate, 260U T7 polymerase, and 50% (v/v) low-molecular-weight mix (LM mix) containing: 110 mM HEPES, 3.4 mM DTT, 2.4 mM ATP, 1.6 mM CTP, 1.6 mM GTP, 1.6 mM UTP, 0.8 M creatine phosphate (CP), 0.65 mM cAMP, 0.05 mM folinic acid, 0.21 M potassium acetate, 27 mM ammonium acetate, 2 mM each of the 20 amino acids, and 8% (v/v) PEG-8000, was used for protein synthesis.

Neutral PEG-liposomes consisting of EPC, CHOL and DSPE-PEG 5000 with a molar ratio of EPC:CHOL:PEG 5000 = 1.60.9:0.025 were prepared as described previously [419]. For preparation of AnExIL formulations with antigen expressed inside/outside and inside liposomes (further referred to as AnExIL and AnExIL-In), 100 µl of IVTT mixture and pDNA, was used to rehydrate a batch of 6 µM of PEG-lipid cakes in order to form liposomes encapsulating IVTT mix and pDNA. The liposomes were incubated on ice for 10 min to complete the rehydration process. For AnExIL-In protein expression outside liposomes was inactivated by adding RNase with a final concentration of 10 µg/ml, to the liposomal suspension. Both AnExIL and AnExIL-In were incubated at 30 °C for 3 h to allow protein synthesis to complete.

Mice for immunization and in vivo imaging of antigen expression

Female C57BL/6 (B6) mice, 6-8 weeks old (Charles River, the Netherlands), were housed in groups of 5 and maintained in the animal facility of Utrecht University with a 12 h day and night schedule, while food and water were ad libitum. The experiments were approved by the Ethical Committee for Animal Experimentation of Utrecht University.

Immunization with AnExIL-pDUAL vaccine and imaging of in vivo antigen expression

AnExIL-pDUAL vaccine containing pDUAL-Luc-NP, which can be expressed both in bacterial cell-free expression system and in vivo in mammalian cells, was prepared as described earlier in the section of preparation of AnExILs. Briefly, 100 µl of IVTT mix with either 50 µg (100 nM) or 100 µg (200 nM) of pDUAL-LUC-NP was used to rehydrate a batch of freeze-dried liposomes and incubated for 3 h at 30 °C to produce Luc-NP antigen in vitro. For imaging of in vivo antigen expression and immunization, mice (5 per group) were vaccinated i.m. twice with three-week intervals. The animals received 100 µl of different formulations (Table 1), divided over two injections of 50 µl, which were injected in each of the hind leg muscles. Seven days after the last immunization, mice were sacrificed, spleens were dissected and single cell suspensions of splenocytes were analyzed for cellular immune responses against NP epitope.

For imaging of in vivo antigen expression in vaccinated mice, at the days 0, 1 and 4 post immunization, mice were anesthetized with isoflurane (Abbott Laboratories) and injected intraperitoneally with 100 µl solution of the substrate luciferin (25 mg/ml, Promega) supplemented with 1.25 mM ATP and 10 min later the luminescence produced by active luciferase was acquired during 5 min with a photon imager (Biospace Lab). The signal from the areas of interest (ROIs) was analyzed using the Biospace Lab M² vision software.
TABLE 1  First immunization study design

<table>
<thead>
<tr>
<th>Description</th>
<th>Amount (μl)</th>
<th>DNA dose (μg)</th>
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<tr>
<td>AnExIL–pDUAL vaccines (n = 5, 2 immunizations i.m.)</td>
<td></td>
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</tr>
<tr>
<td>AnExIL–Luc-NP (pDUAL–Luc-NP)</td>
<td>(2 × 50)</td>
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<tr>
<td>AnExIL–Luc-NP (pDUAL–Luc-NP)</td>
<td>(2 × 50)</td>
<td>(100)</td>
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<tr>
<td>Control naked DNA (pDUAL-Luc-NP)</td>
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<td>(100)</td>
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TABLE 2  Second immunization study design

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<tr>
<th>Description</th>
<th>Amount (μl)</th>
<th>DNA dose (μg)</th>
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<tbody>
<tr>
<td>AnExIL–DNA mixed vaccines (n = 6, 2 immunizations i.m.)</td>
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<tr>
<td>AnExIL–Lipo (pVEX-LacZ-NP) + (pVAX-LacZ-NP)</td>
<td>DNA vaccine + protein</td>
<td>(50 + 50)</td>
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<tr>
<td>AnExIL–In–Lipo (pVEX-LacZ-NP) + (pVAX-LacZ-NP)</td>
<td>DNA vaccine + protein</td>
<td>(50 + 50)</td>
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<tr>
<td>Control vaccines (n = 6, 2 immunizations i.m.)</td>
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<tr>
<td>Liposomal DNA (pVAX–LacZ-NP)</td>
<td>Only DNA vaccine</td>
<td>(50)</td>
</tr>
<tr>
<td>AnExILs (pVEX-LacZ-NP)</td>
<td>No DNA vaccine, only protein</td>
<td>(50)</td>
</tr>
<tr>
<td>Liposomal DNA + electroporation (positive control) (pVAX-LacZ-NP)</td>
<td>Only DNA vaccine</td>
<td>(50)</td>
</tr>
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</table>

b pVAX-Lac-Z-NP is a codon-optimized eukaryotic plasmid for expression of Lac-Z-NP in mammalian cells in vivo.
c AnExILs with only prokaryotic vector, pIVEX-Lac-Z-NP.

Immunization with AnExIL–DNA mixed vaccines

To be able to determine the effect of endogenous antigen production and exogenously delivered protein antigen on CTL induction, we have prepared AnExIL–DNA mixed vaccines. In this immunization study, β-galactosidase-NP protein was used as a model antigen. AnExILs were loaded with pIVEX-LacZ-NP, which produces β-galactosidase-NP in AnExILs under the control of a T7 promoter (further referred to as AnExILs). Liposomes were loaded with pVAX-LacZ-NP (further referred to as Lipo–DNA), which only produces μ-galactosidase-NP antigen after successful transfection in mice. To prepare AnExIL–DNA mixed vaccines, 50 μl of AnExIL containing 3 μg (20 nM) of pIVEX-LacZ-NP was mixed with 50 μl of liposomes containing 50 μg (100 nM) of pVAX-LacZ-NP and used for vaccination. By mixing these two formulations potential synergistic effects of these two separate antigen processing routes can be investigated.

In the first AnExIL–DNA mixed vaccine, the in vitro production of β-galactosidase-NP antigen was performed inside and outside of liposomal compartments. This formulation is further referred to as AnExIL–Lipo. In the second AnExIL–DNA mixed vaccine (further referred to as AnExIL–In–Lipo), the antigen was only produced inside liposomes and the outside production was inhibited by adding RNase to outside of the liposomes. The amount of produced antigen of AnExIL–In–Lipo was 10% of that of AnExIL–Lipo. Mice (6 per group) were intramuscularly (i.m.) immunized with AnExIL–DNA mixed vaccines and controls (Table 2) twice with three-week intervals. The animals received 50 or 100 μl of different formulations (Table 2), divided over two injections of 50 μl, which were injected in each of the hind leg muscles. For the positive control group DNA immunization was directly followed by electroporation of the injected area (100 V, ten pulses of 50 ms with 1 s interval) using tweezer electrodes attached to an Electro Square Porator device (ECM830, BTX). Before each immunization, blood samples were taken and individual serum samples were separated from blood cells and coagulated proteins by centrifugation for 5 min at 3000 × g and 4 °C, and stored at −20 °C for further analysis of antibody response against β-galactosidase. Seven days after the last immunization, mice were sacrificed, spleens were dissected and single cell suspensions of spleens were analyzed for cellular immune responses against NP epitope.

NP-specific CD8+ T-cell response

Single-cell splenocyte suspensions were prepared from spleens using 100 μm cell strainers (BD Biosciences) (from six animals of each group). Red blood cells were removed using red blood cell lysis buffer (Roche). The cells were washed with 2% FCS in PBS and stained for flow cytometry with Live/Dead Aqua cell stain (Invitrogen), PerCP labeled anti-CD3, APC-labeled anti-CD4, FITC-labeled anti-CD8 antibody (BD Biosciences) and PE-labeled H-2D* tetramer with the NP epitope (Sanquin Research) in the first immunization study with AnExIL-pDUAL. 
vaccines and in follow-up immunization with AnExIL–DNA mixed vaccines, cells were stained with APC-labeled anti-CD8 antibody (BD Biosciences) and PE-labeled H-2D^b pentamer with the NP epitope (ProImmune). After staining, in the first experiment, cells were analyzed using a FACS Canto-II, with FACS Diva software. Live, CD3^+CD8^+ cells were selected, background signals were subtracted and the percentage of live, CD3^+CD8^+PE^+ cells was reported. In the second experiment, cells were analyzed with FACS Calibur with a high throughput sampler in combination with Platemailer and Cellquest Pro software (BD Pharmingen). The percentage of CD8^+PE^+ cells in live, CD3^+ population was calculated. Comparison between mice of different groups with NP-specific positive T cells was made by a one-way ANOVA test with a Bonferroni post-test and p-values of <0.05 were considered significant.

β-Galactosidase-specific antibody responses

β-Galactosidase-specific antibody responses were determined by using an enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates (high binding capacity; Greiner) were coated overnight at ambient temperature with commercially available β-galactosidase (100 ng in 100 μl/well) in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6). After coating, plates were washed and blocked by incubation with 2.5% (w/v) milk powder in coating buffer (200 μl/well) for 1 h at 37 °C. Subsequently, the plates were washed with PBS containing 0.05% Tween, pH 7.4 (PBS/Tween). Appropriate dilutions of sera of each individual mouse were applied to the plates, serially diluted two-fold in PBS/Tween and incubated for 2 h at 37 °C. Plates were then washed and incubated either with horseradish peroxidase (HRP)-conjugated goat antibody against mouse IgG or HRP-conjugated rabbit antibody against mouse IgG1 or HRP-conjugated rat antibody against mouse IgG2a (all diluted 1:5000 in PBS/Tween, 100 μl/well) for 1 h at 37 °C. Thereafter, the plates were washed twice with PBS/Tween and once with PBS. Specific antibodies were detected by adding 100 μl of 3,3',5,5'-tetra methyl benzidine (TMB, 0.1 mg/ml) in 10 mM sodium acetate pH 5.5 buffer also containing 0.06% (v/v) hydrogen peroxide to each well. After 10 minutes, the reaction was stopped by adding 50 μl 2 M H₂SO₄ to each well. Total IgG, IgG1 and IgG2a antibody titers are expressed as the reciprocal sample dilution corresponding with an A₄₅₀ of 0.2 above the background (420). Comparison between mice of different groups with positive titers was made by a one-way ANOVA test with a Bonferroni post-test and p-values of <0.05 were considered significant.

RESULTS

Cell-free protein synthesis system and AnExILs

In order to determine the capacity of AnExILs to induce epitope-specific T-cell responses in mice, the NP ASNENMDAM epitope was chosen as a model epitope since it is known to induce good T-cell responses in mice with an H2-D^b haplotype [421, 422]. The S30 extract used for cell-free protein synthesis was prepared from the E. coli strain BL21 Rosetta-gami™, which is devoid of endogenous β-galactosidase. This was important to avoid interference of endogenous β-galactosidase in the experiments. According to literature, for intramuscular immunization a minimum dose of 100 μg DNA is needed to achieve detectable in vivo antigen expression and NP-specific T-cell response [423]. In our first in vivo experiment, we used two doses of DNA, 50 and 100 μg in AnExIL-pDUAL vaccines. The 50 μg DNA dose was chosen because the level of in vitro antigen production in AnExILs with 50 μg DNA was higher than that with 100 μg DNA. Moreover we investigated the impact of the amount of the exogenously and endogenously produced antigen on induction immune responses.

Imaging of in vivo antigen expression and induction of NP-specific T-cell response by AnExIL-Luc-NP vaccines

The potential of AnExILs containing pDUAL-Luc-NP plasmid DNA (AnExIL-Luc-NP) to induce NP-specific T-cell responses after primeboost i.m. immunization in mice was examined. Moreover, a relation between the in vivo antigen expression and the degree of the NP-specific T-cell response was studied for AnExIL-Luc-NP as well as for naked pDUAL-Luc-NP vaccine (Table 1). For this, bioluminescence imaging of mice was used to monitor the conversion of i.p. administered luciferin in time by Luc-NP protein produced either inside the AnExILs or via transfection of the pDNA after i.m. injection of the AnExIL-Luc-NP or pDUAL-Luc-NP formulations. At day 0 of the in vivo imaging of Luc-NP expression, a strong luminescent signal of luciferase was seen immediately after i.m. injection of freshly prepared AnExIL-Luc-NP vaccines with two doses of pDNA (50 and 100 μg), which revealed the presence of in vitro produced Luc-NP antigen (Figure 1).

The luminescent signal and therefore the level of Luc-NP expression of AnExIL-pDUAL vaccine with 50 μg of pDNA was higher than that with 100 μg DNA. The naked DNA vaccine (100 μg of pDUAL-Luc-NP) did not show any luminescent signal at day 0 since no endogenous antigen was produced right after injection of plasmid DNA. The luminescent signal was further monitored at day 1 and day 4 post immunization for in vivo Luc-NP expression (i.e. transfection-mediated endogenous antigen production). None of the two groups immunized with AnExIL-Luc-NP vaccines showed any detectable in vivo antigen expression on days 1 and 4, however, 2/5 mice that had received naked DNA vaccine showed a moderate signal at day 4 (Figure 1).
In vivo imaging of exogenous and endogenous antigen (Luc-NP) production in mice (n = 5) after intramuscular immunization with AnExIL-Luc-NP (50 μg), AnExIL-Luc-NP (100 μg) and pDUAL-Luc-NP (control) vaccine two times. At day 6 post-second immunization, spleens were dissected and splenocytes were collected. Each dot represents an individual mouse, and the average is depicted as a line. One-way ANOVA test followed by Bonferroni’s multiple comparison test was used to compare AnExILs with control after boost immunization.

* p<0.05 and ** p<0.001 were considered as significant and highly significant.

To evaluate the potency of AnExIL-Luc-NP vaccines to induce cellular immune responses, we measured the percentages of NP-specific CD8+ T cells in splenocytes of the mice in this in vivo antigen expression study. As shown in Figure 2, mice vaccinated with either AnExIL-Luc-NP vaccine showed NP-specific T-cell responses whereas mice that received 100 μg of naked DNA vaccine showed a weak T-cell response, which was significantly lower than that of AnExIL-Luc-NP at 50 μg DNA dose (p <0.008) (Figure 2).

The AnExIL-Luc-NP vaccine, containing 50 μg DNA, induced higher numbers of NP-specific CD8+ T cells than the vaccine containing 100 μg of DNA in immunized mice. This correlates with the level of antigen expression by these two AnExIL-Luc-NP formulations as measured with bioluminescence imaging (Figure 1). Empty AnExILs and control pDUAL vector showed no detectable NP-specific T-cell responses (data not shown). Taken together, we can conclude that AnExIL-Luc-NP vaccine (50 μg) was more effective than the AnExIL-Luc-NP (100 μg) and conventional DNA vaccines. The extent of T-cell responses induced by AnExIL-Luc-NP vaccines relates to the amount of protein antigen (Figure 1 and 2).
Induction of NP-specific T-cell immunity by AnExIL–DNA mixed vaccines

Immunization with AnExIL-Luc-NP as described above can lead to T-cell activation via (1) production of antigens in the AnExILs and subsequent cross-presentation after processing by APCs, (2) endogenous production of antigens after AnExIL-mediated transfection of the pDUAL-Luc-NP plasmid entrapped in AnExILs, or (3) a combination of these two routes.

To investigate which of these mechanisms is dictating the induction of T-cell responses and to further explore the role of DNA and protein antigen in CTL responses, we have set up an experiment with AnExIL–DNA mixed, which allowed us to discriminate between these different mechanisms. Quantification of T-cell responses shows that among all groups, only the mice vaccinated with a mixture of AnExIL-pIVEX-LacZ-NP and Lipo-pVAXLacZ-NP (further referred to as AnExIL–Lipo) and Lipo-pVAX-LacZ-NP vaccine in combination with electroporation (further referred to as a positive control) developed NP-specific T-cell responses that were significantly higher than those induced by the control formulations (p <0.001). The relative proportions of NP-specific T cells in the positive control group were higher than those in the AnExIL–Lipo's group (p<0.05) (Figure 3). Mice immunized with AnExIL-In mixed with Lipo-pVAX-LacZ-NP vaccine (further referred to as AnExIL-In–Lipo) showed slightly higher NP-specific T-cell responses than other control groups. Neither AnExIL nor Lipo–DNA could induce any NP-specific T-cell response in immunized mice (Figure 3). Moreover, following immunization with an AnExIL control producing irrelevant antigen (AnExIL-EGFP), no NP-specific T-cell response was observed (data not shown). These results strongly suggest that endogenous production and processing of antigen from pDNA templates is needed for induction of detectable CTL responses, but in case of poor transfection this can be compensated by co-delivering the protein antigen. Strikingly, the degree of T-cell activation is then related to the amount of exogenous protein antigen co-delivered with the AnExILs. Thus, co-delivery of DNA vaccine and high amount of exogenous protein antigen in AnExIL–Lipo vaccine showed an enhanced effect for induction of NP-specific T-cell responses.

Systemic antibody responses after i.m. immunization in mice

To investigate the potency of AnExIL–DNA mixed vaccines to induce antigen-specific antibodies, we compared systemic humoral responses of mice immunized i.m. with AnExIL, AnExIL–Lipo, AnExIL-In–Lipo and controls. After two i.m. immunizations, Lipo–DNA (Lipo-pVAX-LacZ-NP) was poorly immunogenic and induced no serum IgG response, which is related to the poor transfection capability of neutral liposomes as opposed to cationic liposomes (Figure 4). In contrast to Lipo–DNA vaccine, all other formulations induced antibody responses, among which those induced by AnExIL and AnExIL–Lipo were significantly higher than those induced by the others (Figure 4) (p <0.001). There were no significant differences between the total-IgG (T-IgG) antibody titers of positive control and AnExIL-In–Lipo vaccine. Importantly, AnExIL

and AnExIL–Lipo showed similar IgG antibody levels, which indicate that the addition of Lipo–DNA did not affect antibody titers (Figure 4). Mostly, in order to induce prolonged and strong immune responses (memory response), there is a need for booster vaccinations. After booster immunization all groups of mice showed significantly higher IgG titers than those after prime immunization (Figure 4). Taken together, the results point to a strong immunostimulating effect of AnExIL formulations on induction of humoral immune responses, which greatly relies on the quantity of administered protein antigen rather than DNA.

We further looked at the quality of the systemic antibody response, IgG1 and IgG2a subclasses of β-galactosidase-specific antibodies (Figure 4B and 4C). After i.m. immunizations with Lipo–DNA vaccine, there were no detectable levels of IgG1 or IgG2a titers in the sera of the vaccinated mice. AnExIL and AnExIL–Lipo vaccines induced substantially higher IgG1 titers than the positive control (electroporated Lipo-pVAX-LacZ-NP) and AnExIL-In–Lipo vaccine (p <0.001) (Figure 4B). Importantly, mice received the AnExIL, AnExIL–Lipo and positive control

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vaccines showed elevated levels of IgG2a antibodies, indicative of T-helper 1 immunity, while IgG2a antibodies were present but reached substantially lower levels in mice vaccinated with AnExIL-In–Lipo (p < .001) (Figure 4C). In contrast to the IgG1 immune response, IgG2a antibody levels, induced by the AnExIL and AnExIL–Lipo vaccines and positive control, were comparable (Figure 4C).

**DISCUSSION**

The objectives of this study were

1. to determine the capacity of AnExILs to induce specific T-cell responses after immunization in mice,

2. to determine which antigen source of the AnExIL formulation (pDNA or protein antigen produced within the AnExILs) was most important for inducing these CTL responses.

Two separate experiments were performed. In the first experiment, CTL responses were measured after prime-boost immunization with AnExIL-Luc-NP, which contained a single plasmid DNA capable of producing antigens in vitro and, after transfection, in vivo. In the second study, mice were immunized separately with AnExILs (producing exogenous antigen), liposomal DNA (endogenous antigen), and a mixture of AnExILs and liposomal DNA to further study the impact of endogenous antigen and cross presentation of an exogenous antigen on CTL and humoral immune response. The results presented here demonstrate that in both cases the combined AnExILs and DNA vaccine resulted in substantial T-cell responses.

In the first in vivo study, we investigated the level of in vivo antigen expression of AnExIL formulation and its relation to induction of a cell-mediated immunity (Figure 1 and 2), which is a prerequisite for an effective immunotherapy against cancer or persistent viral infections. In order to be able to monitor in situ antigen production and study T-cell responses, we used AnExIL formulations loaded with an antigen-encoding pDNA under control of a dual (both prokaryotic and eukaryotic) promoter, which enables exogenous antigen production within the AnExIL formulations as well as endogenous antigen production after transfection in mammalian cells. This also allowed us to prepare AnExIL vaccine, as a single formulation without adding liposomal DNA vaccine. Luciferase-NP was chosen as a model antigen, which gave us the possibility to monitor in vivo antigen expression in time by checking luminescence signal from expressed luciferase (Luc-NP) and subsequently to look at NP-specific T-cell responses in vaccinated mice. The results show that mice received AnExIL-Luc-NP vaccines (with both 50 and 100 μg of pDNA) did not show any detectable Luc-NP expression up to 5 days post immunizations but could elicit NP-specific CD8$^+$ T cells (Figure 1 and 2). In contrary, 2 out of 5 mice immunized with naked DNA showed weak in vivo Luc-NP expression but no NP-specific CD8$^+$ T cells (Figure 1 and 2). These results indicate that AnExIL-Luc-NP vaccines with no detectable endogenous antigen expression showed much higher immunostimulatory effects than the naked DNA vaccine thus there was probably not a direct relation between the level of endogenous antigen expression and induction of T-cell response. This observation can also be found in other studies where the level of endogenous antigen expression did not necessarily correspond to the strength of T-cell response and other factors such as adjuvanticity, route and method of immunization were crucially important for

**FIGURE 4** Serum anti-β-galactosidase antigen-specific antibodies

Serum anti-β-galactosidase antigen-specific total serum IgG (A), IgG1 (B) and IgG2a (C) responses in mice immunized intramuscularly with AnExIL-Lipo, AnExIL-In-Lipo, AnExIL, Lipo-DNA and Lipo-DNA + electroporation two times. Sera were collected 21 days after each immunization. Antibody titers are expressed as mean of n=6 and the bars represent the 95% confidence intervals. One-way ANOVA test followed by Bonferroni’s multiple comparison test was used to compare titers of different groups after boost immunization. * p < 0.05 and ** p < 0.001 were considered as significant and highly significant.
transfect cells with pDNA to produce sufficient endogenous antigen and thereby to staining (Figure 3). We used relatively strong electroporation, as a tool, to efficiently induce of an antigen specific T-cell response [423, 424]. To examine whether T-cell responses could be further improved by increasing the dose of DNA, AnExIL-Luc-NP vaccine with a 100 μg pDUAL-Luc-NP was used for immunization in mice. Surprisingly, increasing the dose of DNA from 50 to 100 μg within the AnExIL formulations resulted in lower exogenous antigen expression and relative numbers of antigen-specific T-cell responses in immunized mice. Importantly, these results show that

1. DNA vaccine alone is not sufficient to induce detectable T-cell response in mice; and
2. in AnExILs, where DNA vaccine is combined with exogenous protein antigen, the level of antigen-specific T-cell response is dependent on the amount of exogenously produced antigen co-delivered.

Therefore the potency of AnExIL vaccines to induce T-cell response, relies on both DNA and protein antigen dose. The lower exogenous antigen expression within the AnExIL vaccine using a high dose of DNA can be explained by oversaturation of the coupled transcription and translation reaction with DNA template potentially leading to truncated transcripts and concomitant protein antigens. Therefore, because of lower production of full-length Luc-NP antigen in AnExILs with 100 μg DNA, the NP-specific T-cell response was substantially lower (Figure 1 and 2).

To further get insight into the mechanism of induction of immune responses by AnExIL formulations, in particular cell-mediated immunity, we looked at the impact of endogenously and exogenously produced antigen as well as each component of the AnExIL–DNA mixed vaccine on induction of cell-mediated and humoral responses. For this purpose, groups of mice were vaccinated with AnExILs containing only prokaryotic plasmid DNA mixed together with liposomal DNA vaccine prior to immunization. As controls, mice received either only AnExILs, liposomal DNA vaccine or liposomal DNA vaccine, by electroporation. In this way we could determine whether the immune response is mainly provoked by in vivo transfection of pDNA and/or cross-presentation of the exogenous antigen. The results of T-cell response show that as expected, our positive control (electroporated DNA vaccine) could elicit high level of NP-specific CD8+ T cells, measured by pentamer staining (Figure 3). We used relatively strong electroporation, as a tool, to efficiently transfect cells with pDNA to produce sufficient endogenous antigen and thereby to obtain a high detectable CTL response. Besides the positive control, AnExIL–Lipo was the only group that showed significantly higher levels of NP-specific CD8+ T cells than the other groups (Figure 3) and AnExIL, with no DNA vaccine but similar amounts of protein antigen as AnExIL–Lipo could not generate any NP-specific CD8+ T cells in mice (Figure 3).

These results demonstrate that

1. neither exogenous protein antigen nor DNA vaccine alone is sufficient for inducing T-cell responses;
2. the synergetic effect of combined DNA and protein antigen in adjuvanted AnExIL formulation on generation of robust T-cell responses.

After evaluation of cell-mediated immune responses, the impact of each component/factor of AnExIL–DNA mixed vaccines on induction of humoral response was also studied. In this respect, AnExIL and AnExIL–Lipo, with the same amounts of protein antigen (β-galactosidase-NP) showed comparable, strong humoral immune responses against β-galactosidase after i.m. immunization in mice (Figure 4). This indicates that mainly the amounts of protein antigen determine the strength of antibody responses and that the DNA vaccine did not play any role in the induction of humoral immunity. Normally, to induce a robust immune response, a DNA vaccine needs adjuvants and/or strong in vivo DNA transfection [425-429]. Non-adjuvanted liposomal DNA failed to induce any humoral responses in mice whereas mice vaccinated with electroporated pDNA most probably had enough endogenous antigen production to induce reasonable antibody responses (Figure 4). To get insight into the quality of humoral responses by AnExIL– DNA mixed vaccines, we looked at the antibody subtype profile induced by AnExIL formulations and controls. In most infectious diseases, neutralizing antibodies are mainly responsible for bacterial or viral shedding through attaching to viral or bacterial antigens [430-435]. In mice, both IgG1 and IgG2a/b immunoglobulins are contributing to virus neutralization, where IgG2a is mainly involved in cell-mediated immunity and is more effective than IgG1 in protection against intracellular bacterial infections and viral infections, by preventing pathogen replication [436, 437]. For an effective humoral immunity against pathogens, the absolute concentration of IgG and thereby, induction of a combined IgG2a/IgG1 (T-helper1/T-helper2) response is important [436, 438]. In this study, i.m. immunizations with positive control vaccine resulted in a strong biased Th1 immune response, however, AnExIL and AnExIL–Lipo, regardless of adding DNA vaccine, could markedly enhance both Th1 and Th2 responses (Figure 4B and 4C), which may be beneficial for protection against viral or intracellular bacterial infections. These results clearly show the quality of the immune response to β-galactosidase is substantially affected by the characteristic of vaccine formulations and the immunostimulatory effects of AnExILs over non-adjuvanted protein-based or DNA-based vaccines, with limitation in induction of both arms of T-helper response [426, 428, 439, 440].

There have been several reports on combined protein and DNA immunizations, which resulted in enhanced immune responses as compared to immunizations with protein or DNA vaccines alone [439, 441-444]. However, the authors mainly looked at induction
of neutralizing antibodies and mostly the DNA vaccines used in these studies, encoded
different antigens as the protein vaccines and sometimes the route of administration and
immunization regimen for DNA and protein vaccine were different [439, 442-444]. In this
study, it is demonstrated that the AnExILs containing eukaryotic pDNA, pre-expressed
protein antigen and bacterial extract as an adjuvant was highly effective in stimulating
humoral and cell-mediated responses and no separate preparation/injection of protein and
DNA vaccines were needed. AnExILs are a versatile system and combine antigen-production,
DNA vaccine and adjuvanticity in one system and can circumvent current problems of low
immunogenicity with DNA vaccines, which mainly rely on the poor transfection of DNA.
There are only few studies that show the impact of simultaneous injection of a DNA vaccine
and its cognate protein antigen on induction of cell-mediated immunity, which mainly
focused on generation of regulatory T cells [439, 445, 446].

The specificity of AnExILs, as a tailor-made vaccine, is only defined by its genetic input. The ease
of preparation of AnExILs and instant production of protein antigen, simply by adding DNA
template to IVTT mix and liposomes, makes AnExILs an interesting candidate for therapeutic
vaccines against severe viral infections or cancer, in which the antigens are optimized for
each individual. Moreover, such a versatile vaccine platform has the flexibility of a DNA
vaccine in terms of encoding multiple antigens and instant production of the antigens but
avoids poor immunogenicity of naked DNA vaccines. This is very important when there is an
urgent need for a potent therapeutic vaccine in case of cancer or any other life-threatening
infectious diseases, where multi component vaccines are highly desired. The flexibility of
AnExILs may allow us to efficiently express pathogen-derived membrane proteins, which
are extensively used for vaccination. Recent studies show efficient folding and incorporation
of various membrane proteins into liposomes after cell-free production [447-452]. We only
showed production of different individual proteins in AnExIL, nevertheless, multiple proteins
and/or immunostimulatory molecules such as cytokines/chemokines can be co-expressed
in AnExIL, where a specific type of immunity or more robust immune response is required.

In conclusion, we have shown the potential of AnExIL in the formulation of an all-in-one
vaccine for the induction of both T-cell and humoral immune responses. This warrants further
investigation in the applicability of AnExILs for the development of vaccines to prevent or
treat infectious diseases and cancer.
SUMMARIZING DISCUSSION
Although the number of new HIV-1 infections has started to decline, HIV-1 infection remains the leading cause of morbidity and mortality worldwide [58]. Infection with HIV is characterized by a complex interplay between the virus and the immune system of the host. During the acute phase of a primary infection the virus is partially controlled by a robust host immune response which is not sufficient to eradicate the virus and viral reservoirs are established early after infection. Thereafter, the immune system weakens in the chronic phase, eventually leading to AIDS. Antiretroviral drugs can decrease viral replication and prevent progression to AIDS but are not sufficient to eradicate the virus or to induce a strong immune response to the virus. These drugs must be continued lifelong resulting in high cost, adherence problems, the risk of side effects and the risk of emergence of resistance. Because host cellular immunity plays a central role in containing HIV replication, therapeutic immunization may be a valuable method to boost or re-direct HIV-specific immune responses in HIV-infected patients. The induction and expansion of potent virus-specific cellular immune responses upon acute infection can constrain HIV replication in chronic HIV infection. A safe and efficacious therapeutic vaccine for immunotherapy will offer a valuable alternative to antiretroviral drugs. The research described in this thesis studied the effects of HIV immunotherapy on the host immunity and on evolution of HIV.

DC-TRN IMMUNOTHERAPY TRIAL

To evaluate the effect of dendritic cell (DC)-based therapeutic vaccination in HIV infection, we conducted a phase I/IIa DC-based immunotherapy trial in HIV-1 infected patients stable on combined antiretroviral therapy (cART). In this non-randomized, non-blinded trial, 17 cART-treated HIV-infected adults with plasma viral loads below the detection limit were administered four vaccinations of a DC-based vaccine. The vaccine consisted of autologous monocyte derived mature DCs electroporated with mRNA encoding Tat, Rev, or Nef (DC-TRN). The vaccines were administered with four-weekly intervals and two weeks after the fourth vaccination, cART was interrupted (analytical treatment interruption, ATI). During the follow-up phase of 96 weeks, clinical and laboratory parameters were closely monitored and cART was resumed based on current guidelines for antiretroviral treatment of adults.

DCs FOR IMMUNOTHERAPY

DCs have ideal characteristics for therapeutic vaccination. They have the capacity to directly activate CD8+ T cells as well as to induce CD4+ T cell help to CD8+ T cells [200]. Moreover, DCs can induce naïve and enhance pre-existing T-cell responses. We obtained an immunogenic DC-based product for vaccination through ex vivo generation of monocyte derived DCs that were subsequently loaded with HIV antigens by mRNA electroporation. The properties of the DC-based therapeutic vaccine were improved by adding a DC-LAMP HLA class II targeting sequence to the mRNA for optimized presentation to CD4+ and CD8+ T cells [209]. We used a pro-inflammatory cytokine cocktail consisting of GM-CSF, IL-4, IL-6, IL-1β, TNF-α and PGE2 to induce maturation of these DCs [306]. This cytokine cocktail has the disadvantage that cytokines required for the induction of Th1 responses are produced in relatively low amounts during a short period of time, resulting in the skewing of the induced T-cell response toward Tregs [453]. In order to overcome the negative effects associated with DC maturation with a cytokine cocktail, a novel approach has been developed whereby mRNAs encoding activation signals are co-electroporated with mRNAs encoding antigens. This approach enhances the maturation status and at the same time facilitates the procedure for preparation of a DC-based vaccine [212]. Implementation of methods to optimize the stimulatory capacity of DCs is anticipated to improve the clinical outcome of DC-based vaccines. In a novel approach direct targeting of DCs in vivo by intranodal administration of mRNA encoding HIV antigens and co-stimulatory signals may have important advantages. This concept will be explored in a follow-up clinical trial from the DCTRN team in collaboration with international partners (www.ihivarna.org).
**IMMUNE MONITORING**

To date, it remains unclear which immunological responses should be induced by vaccination in order to protect from infection or disease progression [317] and how these parameters could best be measured. To date, in HIV vaccine research the question is not only ‘what to measure’ but also: ‘how to measure’. Measurements of vaccine-induced T-cell responses can be directly obtained from cells in the blood or mucosa. Cellular immune responses against HIV can be measured by a wide variety of techniques, each technique having its own unique properties. T-cell immune assays can be subdivided into several classes which are:

1. detection of cell frequency;
2. epitope mapping;
3. cell phenotyping;
4. detection of effector function such as cytokine secretion, cytolytic activity, proliferation in response to antigen, virus inhibition; and
5. systems biology including transcriptomics and proteomics.

**DC-TRN immune responses**

We evaluated HIV-specific cellular immune responses at varying time points in 16 out of 17 patients using 3 different immune assays: a peptide-based IFN-γ ELISpot assay, a DC-based IFN-γ ELISpot assay and a CFSE proliferation assay. In all but one patient an induction and/or enhancement of the vaccine-specific T-cell response was observed. The majority of the responses were directed against more than one viral antigen. In some patients, a response to non-vaccine proteins HIV-Gag and/or influenza Flu-NP was observed, possibly due to a general immune activation induced by the DC-TRN vaccine or an undefined bystander effect. In a regression analysis, the responses to vaccine antigens were significantly increased compared to those against non-vaccine antigens. Our results demonstrate that DC-TRN immunotherapy elicits HIV-specific cellular immune responses.

**ELISpot assay**

The ELISpot assay is widely used in the context of clinical vaccine trials to measure cytokines secreted by CD4+ and/or CD8+ T cells. Many laboratories have ample experience with the ELISpot assay as it has a number of advantages: it is a rapid and simple quantitative assay that can easily be performed in a high-throughput manner and can be validated by standardized stimuli and controls [454]. However, the ELISpot assay also has a number of drawbacks. It is not easy to distinguish true positive from negative responses. Experienced users and statistical methods are needed to define criteria for positivity [455]. In addition, when using mock stimuli to define negative responses, we may overlook the non-specific responses as we have shown for Gag and Influenza-NP antigens not included in the DC-TRN vaccine (Chapter 2). Several studies have indicated that the breadth or magnitude of HIV-specific CD8+ T cells secreting IFN-γ as detected by ELISpot with massive antigenic stimulation do not correlate with viral control and do not reflect in vivo T-cell efficacy [277, 456, 457]. In addition to secretion of IFN-γ, other cytokines such as TNF-α and IL-2 can be detected by ELISpot or intracellular cytokine staining (ICS) and flow cytometry as markers of CD8+ T-cell effector function. The antigenic stimulus for ELISpot or ICS can be provided by cells pulsed with DNA, mRNA, peptides, but also viral or cell lysates. We demonstrated that a higher sensitivity in IFN-γ is obtained with DCs pulsed with mRNA (Chapter 2). Autologous sequences for read-out will further improve the sensitivity and prevent the detection of responses that may be irrelevant in vivo [189, 310].

**Phenotypic and functional assays**

Polyfunctional CD8+ T cells capable of a multitude of functions, including cytokysis and the production of several cytokines and chemokines may have a better functional quality for control of viral replication than monofunctional CD8+ T cells [137]. They are defined by secretion of chemokines such as MIP-1β (CCL4) and the expression of cell surface markers CD107a and CD154 [137]. Additional phenotypical analysis of antigen-specific CD8+ T cells can include activation markers as HLA-DR and CD38 [113], exhaustion markers including PD-1, LAG-3, TIM-3, CTLA-4, CD160 [458] and markers of effector memory and central memory phenotype (CCR7, CD28 [172, 179, 459]). Detailed information about T-cell phenotype may provide additional information on the functional state of vaccine-induced T cells. The cytotoxic potential of CD8+ T cells can be assessed by a variety of functional assays including the 51Cr-release and fluorescent antigen-transfected target cell cytotoxic T lymphocyte (FATT-CTL) lysis assay [326, 460]. Viral inhibition by CD8+ T cells possibly provides the most direct measure of antiviral function in vitro and may reflect CTL immunity in vivo more closely than other phenotypical and functional assays [461-463]. Additionally, antigen-specific T cells can be quantified and isolated for epitope mapping and further analysis by HLA class I or II tetramer staining [464]. In addition to these well-established assays, transcriptional profiling has been added to the repertoire of monitoring assays [464], providing the advantage of giving a helicopter view on the breadth of antigen-specific T-cell immunity. Measuring changes in activity of the host genes in response to vaccine stimuli will provide insights into gene signatures that could predict adaptive immune responses to vaccines [356, 385].

So far, vaccine-elicited immune responses did not correlate with clinical effects suggesting that what we measure does not reflect the clinically relevant aspects of vaccine responses. We therefore propose to stop wasting precious clinical samples on the IFN-γ and IL-2 ELISpot assay for which ample evidence has shown that it does not predict vaccine efficacy. We suggest instead a multi-faceted immune monitoring approach relying on more than one
single functional output and that includes at least a viral inhibition assay and systems biology assays, i.e. transcriptomics and/or proteomics to increase in the identification of T-cell immune responses correlated with vaccine efficacy.

**DC-TRN VIRUS EVOLUTION AND REV EL9 EPITOPE**

Throughout HIV infection, the virus is subjected to selective pressures, including host immune mediated responses. HIV can escape from immune pressure by genetic mutations that can occur within epitope regions targeted by host CTL or in epitope flanking regions. In general, these mutations abolish the binding of the epitope to MHC molecules. Often, escape mutations can result in decreased viral replicative fitness and compensatory mutations accompanying escape mutations can restore viral fitness. Escape mutations in Tat, Rev and Nef have been described during natural infection [176, 299, 301, 302] as well as after vaccination [285, 303] or CTL transfer [304, 305]. In *Chapter 3* we tested whether vaccine-induced HIV-specific T-cell responses affect HIV evolution within the host. We analyzed virus evolution at the whole gene level as well as in single CTL epitopes. We did not find evidence for increased evolution of HIV after DC-TRN vaccination in immunogens compared to non-vaccination proteins (other than Tat, Rev of Nef) nor compared to vaccination naive HIV-infected control patients. With a multitude of anti-HIV CTL responses present, the advantage of escaping a single CTL response is small because there are still several other T-cell responses targeting the virus [465-467]. Our findings are in line with recent literature reporting that therapeutic vaccination, which induces T-cell responses, does not necessarily result in mutational pressure thereby increasing viral diversity [283, 287].

For the total DC-TRN study population, HIV-specific T-cell responses were detected by ELISpot and CFSE proliferation assays with either peptide pools or whole antigen mRNA and therefore did not dissect these responses into single peptides. For one patient included in the trial, we defined a new CTL epitope (*Chapter 4*). This Rev EL9 epitope (EELLKTVRL) was shown to be presented by both *HLA-B*44:02 and *B*44:03 molecules and had a high sequence similarity to the human NOL6 protein [319]. Sequence similarity between pathogen-derived and self-peptides poses the risk of cross-reactive responses possibly resulting in autoimmunity [318, 321]. We did not find evidence that such molecular mimicry resulted in a breakdown of discrimination between self and non-self antigens in this particular patient since the Rev EL9-specific T cell clone from the study patient did not cross-recognize NOL6 in vitro. It was recently suggested that negative selection of auto-reactive T cells recognizing HIV-derived epitopes not only prevents autoimmunity but could also result in immune evasion by HIV [318, 319]. In contrast, we showed that escape from immune pressure does occur, whereas in vitro protein function was not impaired. The escape from a single CTL targeting the EL9 epitope had no detectable effect on the plasma viral load (pVL). Possibilly there is substantial immune pressure by responses targeting other regions of the virus [465] or the in vitro test for protein function has limited predictive value for in vivo replicative capacity of the mutated EL9. CTL recognition of the EL9 epitope results in escape mutation with a limited effect on viral load.

**DC-TRN TRANSCRIPTOMICS**

Monitoring a limited number of immune parameters may be insufficient to predict vaccine efficacy. More accurate assays have been developed to characterize HIV-specific polyfunctional CD8+ T cells (simultaneous production of IL-2, IFN-γ, TNF-α, CD107a and MIP-1β) [137] or a combination of markers for memory phenotype, cytokine production, cytotoxicity, transcriptional control, activation status, exhaustion, tissue trafficking, and regulatory function, as well as for CTL cytototoxicity and viral infection. An integration of these immunological and antiviral assessments will contribute to the identification of correlates of vaccine efficacy [223, 317]. To this end, gene expression profiling has been added to the arsenal of evaluating vaccine responses as it has been shown to be instrumental in capturing immune response events and quality of vaccine-induced B- and T-cell responses [356, 363]. It puts fewer constraints on measurement of vaccine responses and can lead to unexpected insights. Microarrays can be used to measure changes in the activity of large gene sets before and after vaccination.

*Chapter 5* reports on the effects of DC-TRN vaccination on the gene expression profile of the peripheral blood compartment. Given the deteriorating effects of chronic HIV infection on the immune system that cannot be fully reconstituted by cART [111, 372, 468], the baseline transcriptome of HIV-1 infected patients on cART differed from uninfected controls. The transcriptome shift induced by DC-TRN vaccination in HIV-infected patients was not observed after vaccination of HIV seronegative melanoma patients with a DC-based vaccine. At baseline, melanoma and cART-treated HIV-infected patients have a different immune status [378] probably contributing to the distinct transcriptome observed. Studies to determine whether the DC-TRN induced transcriptome shift in HIV-infected patients is truly HIV-specific or specific for vaccination in the setting of a chronic infection, are warranted. We conclude that the effects of DC-based immunotherapy on the transcriptome are comparable to those of a replicating virus since the DC-TRN vaccination induced shift is not further affected by analytical treatment interruption (ATI) and subsequent active viral replication. Furthermore, the transcriptome after DC-vaccination is highly enriched for genes that are also induced by vaccination of healthy adults with a live attenuated influenza virus vaccine is enriched.
for T-cell- and monocyte-specific genes [356], which is replicating and confers protection. Upregulation of a number of activation marker genes and genes associated with T-cell exhaustion [78, 110, 374], may be indicative of disease progression and/or immune activation and exhaustion after DCiTRN vaccination. Elucidation of the mechanisms of vaccine-induced general immune activation and exhaustion will provide tools for the development of immunotherapy strategies that either prevent or counteract these unwanted effects. Moreover, extensive monitoring with applicable assays including blood transcriptome profiling of adverse immune activation effects in immunotherapy trials is warranted.

**Efficacy of DC-based Immunotherapy**

DCiTRN vaccination was safe and well-tolerated (Chapter 2). Safety of HIV immunotherapy is not to be taken for granted as was recently shown in a first-in-human trial that tested the ex vivo administration of Opal-HIV-Gag(c) peptide vaccine and was terminated due to a serious adverse event [469]. The potential therapeutic efficacy of the vaccine was assessed by comparing CD4+ T-cell counts, plasma viral load after ATI and the proportion of patients remaining off cART in our study population with historical controls (Chapter 2). Ninety-six weeks after ATI, 6 of 17 (35%) patients remained off antiretroviral therapy. No significant therapeutic efficacy of the vaccine was observed, although results from re-calculation with pre-cART pVL values that became recently available, showed a decrease of 0.5 log10 in viral load after ATI. The virological response to a therapeutic vaccine against HIV can only be assessed when the trial design includes a treatment interruption phase after vaccination. If control of viral replication, albeit partially, is demonstrated after ATI, a proof-of-concept of antiviral efficacy of the vaccine is provided that justifies investigation of these immunotherapeutic strategies against HIV [151].

The numerous prototype HIV-1 prophylactic and therapeutic vaccine candidates that have been evaluated in human clinical trials to date cover a wide range of approaches to induce antibody and/or T-cell responses in terms of vector systems (viral, non-viral), adjuvant formulations, antigens, and administration regimens. To date, results from over 80 therapeutic vaccination trials have been reported for multiple vaccine strategies including inactivated whole virus, peptides, naked DNA, viral vectors and dendritic cells. Most trials failed to demonstrate improvement of CD4+ T-cell count, control of HIV RNA or delay of clinical progression. In a number of trials, modest effects on clinical parameters or vaccine elicited HIV-specific immune responses were detected and these are summarized in Table 1. The most commonly used endpoints for therapeutic vaccine efficacy studies are the viral load as measured by levels of HIV RNA in plasma, CD4+ T-cell counts, and the time off cART after ATI. Assays for immune responses applied in the therapeutic vaccine trials with positive clinical effects, comprise lymphocyte proliferation assays on PBMC or subsets thereof, IFN-y and IL-2 ELISPOT or ICS, and assays for (neutralizing) antibodies (Table 1). All studies reported vaccine-elicited immune responses that were detected with one or more assays. In roughly half of the studies, some responses correlated with the efficacy parameter(s) but clearly not all. Thus, no consistent picture of an immune correlate of protection emerges from these trials.

**General Immune Activation and Exhaustion**

The effects of vaccine-induced HIV-specific T cell immunity could be counteracted by a generalized systemic immune activation that is associated with CD4+ T-cell dysfunction and loss and may induce increased cellular susceptibility to HIV infection [107, 492]. If immune activation persists, non-AIDS linked morbidities resulting from inflammation can occur and the immune system may deteriorate as characterized by exhaustion of T cells and a global decline of immune resources [80, 108, 109]. Indications of DCiTRN induced general immune activation were found in the analysis of T-cell immune responses because T-cell responses to non-vaccine antigens Gag- and/or Influenza-NP were increased compared to prevaccination (Chapter 2). In the transcriptomics study (Chapter 5) a number of activation
TABLE 1  Therapeutic vaccination studies with (modest) positive clinical effects

<table>
<thead>
<tr>
<th>VACCINE</th>
<th>IMMUNOGEN</th>
<th>EFFICACY PARAMETER</th>
<th>ASAY USED</th>
<th>ASAY POSITIVE</th>
<th>CORRELATE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaxSyn; rec. prot</td>
<td>Env</td>
<td>CD4*</td>
<td>CD4* LPR, IFN-γ IL-2</td>
<td>LPR, IFN-γ IL-2</td>
<td>nr</td>
<td>Valentine [473]</td>
</tr>
<tr>
<td>Vac-01; VaxSyn</td>
<td>Env</td>
<td>CD4*</td>
<td>LPR</td>
<td>LPR vs CD4*</td>
<td>Leanderson [474]</td>
<td></td>
</tr>
<tr>
<td>Vac-04; VaxSyn</td>
<td>Env</td>
<td>CD4*</td>
<td>LPR</td>
<td>LPR vs CD4*</td>
<td>Sandstrom [475]</td>
<td></td>
</tr>
<tr>
<td>Vac-04</td>
<td>Env</td>
<td>CD4* survival</td>
<td>IFN-γ</td>
<td>IFN-γ</td>
<td>nr</td>
<td>Bostrom [476]</td>
</tr>
<tr>
<td>Vac-05</td>
<td>Env</td>
<td>CD4*</td>
<td>LPR, Ab (ELISA), DTH</td>
<td>LPR</td>
<td>nr</td>
<td>Haydeman [477]</td>
</tr>
<tr>
<td>Remune inact. HIV</td>
<td>Gag/Pol/Vpr/gp41</td>
<td>CD4* pVL</td>
<td>Ab (WB)</td>
<td>Ab</td>
<td>nr</td>
<td>Churdboon-chart [478]</td>
</tr>
<tr>
<td>Remune</td>
<td>Gag/Pol/ Vpr/gp41</td>
<td>pVL ATI</td>
<td>LPR, DTH</td>
<td>LPR, DTH</td>
<td>nr</td>
<td>Fernandez-Cruz [479]</td>
</tr>
<tr>
<td>ALVAC-HIV canarypox</td>
<td>Env/Gag+CTL-epi Nef/Pol</td>
<td>ATI</td>
<td>LPR, CD8* IFN-γ</td>
<td>LPR vs ATI</td>
<td>tubiana [266]</td>
<td></td>
</tr>
<tr>
<td>ALVAC-HIV + lipo GT</td>
<td>Env/Gag + CTL-epi Nef/Pol</td>
<td>CD4* pVL (trend) ATI</td>
<td>CD4* LPR, CD8* IFN-γ, LPR vs CD4* and AT1</td>
<td>Levy [481, 482]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALVAC-HIV + Remune</td>
<td>Env/Gag + CTL-epi Nef/Pol, Gag/Pol/ Vpr/gp41</td>
<td>ATI</td>
<td>LPR, IFN-γ IL-2, LPR, IFN-γ IL-2</td>
<td>IFN-γ IL-2 vs pVL</td>
<td>Angel [264]</td>
<td></td>
</tr>
<tr>
<td>ACTG 5197; Ad5</td>
<td>Gag</td>
<td>pVL</td>
<td>LPR, IFN-γ (ICS)</td>
<td>LPR, IFN-γ</td>
<td>CD4* IFN-γ vs pVL</td>
<td>Schooley [483]</td>
</tr>
<tr>
<td>VacCiter; MVA</td>
<td>Nef</td>
<td>CD4* pVL</td>
<td>IFN-γ</td>
<td>IFN-γ</td>
<td>IFN-γ vs pVL</td>
<td>Harper [285]</td>
</tr>
<tr>
<td>Vac-4x peptide</td>
<td>p24Gag</td>
<td>pVL AT1</td>
<td>LPR, IFN-γ, IL-4 (ICS), IL-2, TNFα, MIP-1β, CD107a, PD-1, IL-10 (FACS), DTH</td>
<td>CD4* + CD8* + pVL, IFN-γ, IL-10, CD107a vs CD4*</td>
<td>Yran [484, 485], Lind [486]</td>
<td></td>
</tr>
</tbody>
</table>

* if no cell type is specified for the lymphoproliferation (LPR) assay, PBMC were used; if no technique is stated for the detection of cytokines IFN-γ, IL-2 or TNF-α, ELISpot was used. Ab, antibody; AT1, analytical treatment interruption; CD, dendritic cell; DTH, delayed-type hypersensitivity; FACS, fluorescence activated cell sorting; GzB, granzyme-B; ICS, intracellular cytokine staining; inact, inactivated; LPR, lymphoproliferation; nAb, neutralizing antibody; nr, not reported; pVL, plasma viral load; rec, recombinant; WB, western blotting.
marker genes and genes associated with T-cell exhaustion [78, 110, 374] were upregulated. Our findings stress the importance of the monitoring of deleterious nonspecific immune activation in therapeutic vaccination studies. They also underscore the need to rethink the use of adjuvants and co-stimulatory molecules to increase immunogenicity of (therapeutic) vaccines because they could cause general immune activation at the same time. The latter poses the infected individual at risk for disease worsening due to the increased availability of virus target cells.

The limited clinical efficacy of DC-immunotherapy that was observed, despite the fact that potentially favorable T cell responses were induced, could be the result of viral escape from vaccine-induced T-cell responses. However, we found no indication of increased viral evolution at the whole gene or epitope level (Chapter 3). This limited efficacy of DC-based immunotherapy may also result from functional defects of CD4+ and CD8+ T cells during chronic HIV infection. Vaccine-boosted HIV-specific T cells may lack functional quality for viral control [137]. It has been shown that they may lack the capacity to survive, could be restricted in their antiviral and cytotoxic capacities, have a high expression of co-inhibitory molecules, have a reduced avidity to HIV antigens, inefficiently traffic to the lymph nodes or could be suppressed by the presence of Tregs [137, 491]. Some of these defects of vaccine-induced T cells may be corrected or alleviated by the expression of co-stimulatory molecules that enhance DC-induced CD8+ T-cell responses [212] and is worth further exploration. In the DCTRN immunotherapy trial, the use of a consensus HIV subtype B sequence was preferred to autologous virus sequences but may have hampered success through the inclusion of epitopes from which the virus system had already escaped. Peptides and mRNA sequences for read-out were also based on consensus B sequence and could have detected ineffective T cells targeting epitopes not present in the autologous virus [202]. The diversity of the DCTRN vaccine and the autologous virus of the included patients before initial start of cART was 19% for Tat, 12% for Rev, and 14% for Nef (median percentage of different amino acids per protein, deliberately introduced mutations excluded). This difference between vaccine and autologous virus sequences is considerable and could bias the read-out of the immune assays based on vaccine sequences.

VACCINE AND STUDY DESIGN

Although virtually all HIV proteins have been regarded as immunogens, the decision about which antigen(s) to include in a T-cell HIV vaccine requires careful consideration of criteria such as kinetics of protein expression, immunodominance, sequence conservation and number of possible epitopes increasing the breadth of the induced response. In the DCTRN trial, the early expressed HIV-I proteins Tat, Rev and Nef were used as vaccine antigens. We deliberately introduced mutations in the consensus subtype B sequences of Tat, Rev and Nef of the DCTRN vaccine in order to abrogate some of the protein functions that could be deleterious to the host. Mutant Tat lacks the potential to activate the LTR, to translocate to the nucleus and to be taken up by other cells [493-495]. Mutant Rev retains normal RNA but cannot multimerize thereby inhibiting wild-type Rev function [496]. Mutant Nef has its myristoylation signal deleted and leucine zipper mutated and therefore it lacks the potential to downregulate CD4 and HLA class I molecules on the cell surface to decrease the presentation of peptides to T cells.

Potential immunogens

The immunogens Tat, Rev, and Nef were chosen based on encouraging preclinical studies, showing that CTL recognition of the early expressed antigens increased the chance of target cell elimination before progeny virus was released [19, 241]. Alternatively, since Gag protein is introduced into the cytoplasm during viral entry, its epitopes may be cross-presented earlier than Tat, Rev and Nef that require protein synthesis in the infected host cell. This may be invalid for all cell types since it was not observed in vitro experiments with a Gag epitope translocated into the early protein Nef [497]. Gag has additional advantages as an immunogen including high expression levels, relatively good conservation across HIV strains and the presence of dominant immune responses targeting many different epitopes in chronic infection [276, 498]. Genes encoding Env and Pol proteins have been widely used as vaccine antigens in humans and macaques [173, 179] and were selected for their ability to induce a broad immune response targeting multiple epitopes. A rational choice of antigen(s) is hindered by the complex interactions of the aforementioned factors (kinetics of protein expression, immunodominance, sequence conservation and number of epitopes present) and their relative contributions to immunogenicity. To date, the discussion on which factors are most relevant is ongoing.

Sequence diversity

The sequence diversity of HIV constitutes a major hurdle for vaccine development. Several approaches to deal with HIV sequence diversity have been proposed. For example, consensus sequences could be used to reduce genetic diversity between vaccine and circulating virus. Consensus sequences are constructed on the basis of an evolutionary model and are most central and most similar to currently circulating strains. Secondly, conserved regions from the viral genome can be chosen instead of variable regions in order to decrease sequence diversity within the antigen in circulating viruses. A disadvantage of conserved regions is that they have been evolutionary selected, may be poorly immunogenic and have to compete for recognition with rapidly changing immunodominant determinants [499]. Thirdly, it has
been proposed that the sequence from a virus with a record of viability should be used [500]. Multivalent strategies that use combinations of sequences, i.e. consensus and viable isolates or proteins derived from different strains, give a better coverage of autologous viral quasispecies [500]. Artificial, computed sequences provide an alternative way to increase sequence similarity between vaccine and circulating virus sequence [500]. For therapeutic vaccines, the issue of sequence diversity is somewhat less critical because it can be overcome by use of the autologous virus sequence. However, CTL escape mutations can rapidly predominate the viral quasispecies and epitope sequences inducing important early responses may go undetected when using autologous sequences based on isolated from later time points [122, 130, 298, 301]. Moreover, in the setting of the clinical trial it was thought cumbersome and expensive to produce patient-specific batches of high-quality clinical grade mRNA with autologous virus sequence.

**VECTOR STRATEGIES**

Both the choice of the immunogen and its route of delivery are critically important for the success of vaccination. Optimization of vectors may contribute to HIV vaccine design. Features of vectors that are beneficial for vaccine efficacy include the duration of antigen exposure, the engagement of adaptive immunity, the ability to target dividing and non-dividing cells and (for prophylactic vaccines) to target mucosal tissue [501]. In addition, optimized vectors induce greater breadth and/or magnitude of T-cell responses and when viral vectors are used, viral encoded proteins may manipulate CD8+ T-cell priming and redirect T-cell responses [175]. Recombinant viral vectors have been applied rather extensively in therapeutic vaccination trials against HIV. In most cases poxviruses (e.g. canary pox, fowl pox and MVA) and occasionally adenoviruses were used. Recombinant, non-integrating, influenza viruses present a promising antigen delivery system because they have features that are of benefit in the field of vaccination. Firstly, they elicit both humoral and cellular immune responses and induce CTLs by direct infection of immature DCs and monocytes facilitating both local and systemic antigen presentation [502, 503]. Secondly, influenza can be easily manipulated by a reverse genetics strategy. With the reverse genetics technique, it is feasible to adjust the NA and HA influenza segments to create different strains and subtypes to overcome previous immune responses directed to the vector [402]. Lastly, the potential routes of administration provide an additional advantage, especially in the setting of HIV: influenza vectors can be administered needle-free at mucosal sites. Antigens of interest can be fused and systemic antigen presentation [500] and its potential to elicit T-cell and antibody responses. Replication-competent vectors are anticipated to have major assets because they enhance immunity at the mucosal barrier, induce broader and/or stronger T-cell responses, or they elicit protective antibodies [505]. The introduction of gene(s) encoding immunological adjuvants such as IL-2 is anticipated to improve immunogenicity of recombinant influenza viral vectors [504]. More research is required to further develop recombinant influenza viruses as HIV vaccine candidates.

In addition to viral vectors, non-viral vectors have been developed for antigen delivery in vaccination strategies. Non-viral vectors have several advantages such as safety, almost unlimited transgene size and the possibility of repeated administration without vector-directed immune responses [506]. Liposome-based non-viral vaccine vectors improve DNA stability and delivery [507]. AnExILs combine antigen-production, DNA vaccine and adjuvanticity in one system [419, 420]. The substantial T-cell and antibody responses induced by AnExILs, as demonstrated in Chapter 7, indicate that the non-viral vector system of antigen-expression immunostimulatory liposomes is a promising vaccine candidate for T-cell targeting vaccines. AnExIL induced CTL responses resulted from a synergistic effect of combined DNA and protein antigen in the AnExIL formulation. Furthermore, the ease of preparation of AnExILs and the possibility to mix off-the-shelf liposomes and transcription/translation mix with a DNA template creates a versatile vaccine platform. The obtained results in Chapter 7 warrant further investigation in the applicability of AnExILs as a vector system for therapeutic vaccines against HIV. Taken together, gene delivery for vaccination is an emerging area and our work will have value for the development of both viral and non-viral vectors for HIV therapeutic vaccines.

**HIV IMMUNOTHERAPY: FUTURE PERSPECTIVES**

In the absence of a prophylactic HIV vaccine, providing sterilizing immunity as the ultimate goal, a therapeutic vaccine would be of great value. Therapeutic vaccination that provides a containment of HIV replication and prevention of disease progression or complete elimination will reduce the need for lifelong antiretroviral therapy and secondary transmission [151]. Providing a functional cure by immunotherapy may be closer at hand than a preventive vaccine and there is a scientifically sound rationale for further studies on HIV therapeutic vaccines. The main barriers to successful vaccine development are the sequence diversity...
of HIV, the lack of knowledge on natural immune responses, the lack of a validated animal model, the differences in functional immunity between animals used for efficacy studies and humans, the lack of correlates of protection or control, and the viral reservoir formed in latently infected cells [508].

Clues for HIV immunotherapy approaches come from recent studies in nonhuman primate models. A new generation of anti-HIV monoclonal antibodies possessing extraordinary potency and breadth of neutralizing activity were shown to suppress viremia in simian human immunodeficiency virus (SHIV) infected macaques and destroy virus-producing cells through antigen-dependent cellular cytotoxicity or complement cell lysis [509, 510]. It has been hypothesized that breakdown of B-cell tolerance may be important, if not required, for the generation of broadly neutralizing antibodies in HIV-infected persons. If such HIV-neutralizing antibodies that are at the same time autoreactive are administered or elicited by vaccination, there is a potential risk of inducing clinical autoimmunity [511] although broad and neutralizing antibodies are not necessarily autoreactive [512]. Another recent breakthrough obtained in the nonhuman primate model comes from the use of a persistent and replicating CMV-based vector expressing SIV antigens. Prophylactic vaccination with this vector elicited and maintained effector memory CD8+ T cells which resulted in durable control of SIV infection in 50% of the challenged macaques. Long-term, immune-mediated clearance from latent reservoirs was observed [459] and therefore this approach may be applicable to immunotherapy. Additional studies are needed on the contribution of effector memory T cells to HIV immunotherapy strategies for a functional cure in humans. Also, the use of replicating, persistent viral vectors for continuous antigen expression should be applicable to immunotherapy. Additional studies are needed on the contribution of effector memory T cells to HIV immunotherapy strategies for a functional cure in humans.

Immune activation, caused by chronic HIV infection significantly contributes to its pathogenesis. However, vaccination itself possibly also triggers a state of general immune activation at sites of HIV infection. If so, vaccination could not only lead to protection but also to adverse immunopathology shifting the balance from protection or viral control to infection or increased replication and inflammation. Indications of such an adverse effect come from the combined analysis of three trials using recombinant adenoviral vectors of 7 different serotypes, showing that pre-existing antibodies to the vector were not intrinsically associated with increased risk of HIV acquisition (STEP; VAX003 and VAX004) [513, 514]. Ideally, a vaccine induces optimal specific immune responses with minimal general immune activation. A major step that should be taken in HIV vaccine research field is to establish which vaccine characteristics correlate with the induction of general immune activation and exhaustion and how this potential side effect can be overcome or prevented. We propose intensive monitoring of HIV vaccine trials for systemic immune activation and exhaustion.

Development of an HIV vaccine is challenged by the difficulties in monitoring and evaluating effects of vaccination on the immune system. While lacking mechanistic correlates of immune control or protection, we lack immunological methods to demonstrate an efficacious immune control in vivo. In the past decades it has become clear that the classical, well-established and most commonly used immune assays have serious shortcomings in predicting immune control [314]. We suggest the use of a comprehensive and integrated immune monitoring strategy for vaccine evaluation, that relies on more than one single functional output and includes at least a viral inhibition assay and systems biology assays, i.e. genomics and/or proteomics.

Multiple other steps are to be taken in therapeutic vaccine research field to optimize current vaccine strategies and to develop new ones. For T-cell responses, we have to dissect the required strength, breadth and duration of the response. Studies should address the balance between CD4+ and CD8+ T cells and their polyfunctionality, avidity, affinity, and kinetics. Knowledge on the required location and regulation of the vaccine-induced immune response is necessary. The establishment of a reservoir of latently infected cells forms a challenge for therapeutic vaccines and studies to determine the effect of anti-latency strategies, possibly combined with immunotherapy, are warranted.

The DCTRN therapeutic vaccine was shown to be safe, to be T-cell immunogenic and to induce a transcriptome shift. The results from the comprehensive monitoring of the DCTRN trial with a wide variety of assays, including IFN-γ ELISPOT, a lymphocyte proliferation assay, virus sequencing and genomics analysis, provided valuable insights into immune monitoring strategies and potential adverse effects of immune activation. For the development of an effective and safe therapeutic vaccine for HIV, a better understanding of the mechanism of protection, control of virus replication and of general immune activation and exhaustion is required.

CONCLUSIONS

The research described in this thesis has provided novel insights into HIV-1 immunotherapy and its effects on both host and virus. DC-based immunotherapy against HIV-1 was evaluated in a clinical trial in which 17 HIV-1 infected patients were vaccinated with autologous DCs electroporated with mRNA of the early expressed HIV proteins Tat, Rev and Nef (DCTRN) and subsequently interrupted antiretroviral treatment. We have demonstrated that DCTRN is safe and induces in most patients CD4+ and CD8+ T-cell responses specific for the vaccine immunogens when assayed with well-established methods (Chapter 2). We showed that DC-
TRN therapeutic vaccination did not have a detectable impact on the virus sequence evolution in whole genes and CD8+ T-cell epitopes (Chapter 3). When we extended our analysis to a novel single CD8+ T-cell epitope that we identified in Rev, we found evidence for mutations in the virus allowing it to escape from host immunity (Chapter 4). DC/TRN vaccination had a profound effect on the gene expression profile of the peripheral blood compartment (Chapter 5). This transcriptome shift was sustained upon treatment interruption, was specific for DC-based immunotherapy in chronic HIV-1 infection, and was highly enriched for genes that are also induced by vaccination of healthy adults with a live attenuated influenza virus vaccine. The latter indicates that induced immune responses were indeed T-cell skewed and resembled the gene signature of an effective, replicating virus vaccine. Although the DC/TRN vaccine was shown to be T-cell immunogenic and to influence the transcriptome of PBMC, it did not result in overall clinical efficacy, when compared to a historical control group, or resulted in altered virus evolution. The effects of vaccine-induced HIV-specific T cell immunity could be counteracted by a generalized immune activation and exhaustion, for which we found indications in the vaccine-induced transcriptome. Further development of therapeutic vaccines should avoid generalized immune activation and T-cell exhaustion that are considered to limit vaccine efficacy and safety.

Optimization of immunogen delivery is important for the success of vaccination. We have evaluated the immunogenicity of a replication-deficient recombinant influenza viral vector and an antigen-expressing immune-stimulatory liposomal non-viral vector. Such vectors might overcome feasibility issues related to the manufacturing of autologous DCs for DC-based immunotherapy. Both vectors were demonstrated to induce antibody formation and activated cytotoxic T cells (Chapter 6 and 7). The data warrant additional studies on the immunogenic potential of these vector systems for therapeutic vaccination and it will be necessary to use immune assays that measure clinically relevant aspects of immunity including potential deleterious general immune activation and exhaustion.

Our research has contributed to a better understanding of the immunogenic potential of viral vector systems and to the impact of a DC-based therapeutic vaccine on host immunity and virus evolution by a thorough analysis using both well-established assays and a systems biology approach. Additional studies on vector optimization, correlates of immune protection and immune monitoring will aid in the development of therapeutic successful vaccines for HIV.


Lauring AS and Andino R. Quasispecies theory


Donaghy H, Gazzard B, Gotch F and Patterson S. Dysfunction and infection of freshly isolated


211Yang OO. Will we be able to ‘spot’ an effective HIV-1 vaccine? Trends Immunol 2003; 24: 67-72.

212Appay V. 25 years of HIV research! ... and what we don’t know, what we should know. Nat Rev Immunol 2009; 9: S169-77.


References


282. Moodie Z, Price L, Janetzki S and Britten CM. Response determination criteria for ELISPOT.
328. NCBI.
332. BiogPS.


425. Ho GV, Stedronsky Y, Hall CB, et al. Risk factors for...


469. Sandstrom E and Wahren B. Therapeutic


NEDERLANDSE SAMENVATTING
Het humaan immunodeficiëntie virus type 1 (HIV-1) is het virus dat AIDS veroorzaakt. HIV infecteert witte bloedcellen, met name CD4-positieve T-lymfocyten (CD4 T-cellen). CD4 T-cellen zijn helpercellen van het immuunsysteem en spelen een cruciale rol in de afweer tegen allerlei infecties en tumoren. Wanneer het virus een gastheercel geïnfecteerd heeft, vermenigvuldigt het zich daarin waardoor de cel vernietigd wordt. Doordat de HIV-geïnfecteerde CD4 T-cellen vernietigd worden, zal een belangrijk onderdeel van het immuunsysteem langzaam verdwijnen. Bij een onbehandelde HIV-infectie leidt dit in gemiddeld 10 jaar tot de dood als gevolg van AIDS. Sinds de eerste beschrijving van patiënten met AIDS in 1981, zijn ruim 35 miljoen mensen overleden aan de gevolgen van een HIV-infectie. Wereldwijd zijn op dit moment meer dan 35 miljoen mensen met HIV geïnfecteerd. Van deze HIV geïnfecteerden leeft 70% in Afrika ten zuiden van de Sahara en is 9% jonger dan 18 jaar. Volgens een schatting van de WHO hebben in 2012 ongeveer 2,3 miljoen nieuwe infecties met HIV plaatsgevonden en zijn 1,6 miljoen mensen overleden als gevolg van een HIV-infectie.


In het onderzoek dat is beschreven in dit proefschrift hebben wij de effecten van immuuntherapie in HIV-1-geïnfecteerde personen bestudeerd. Wij hebben onderzocht of HIV-immuuntherapie leidt tot het opwekken van T-cellen gericht op HIV. Daarnaast hebben we getest of HIV-immuuntherapie de activiteit van de genen van de gastheer beïnvloedt. Ook hebben wij het effect van immuuntherapie op het aanwezige virus in kaart gebracht. Naast het onderzoek naar de effecten van immuuntherapie in de mens werd een tweetal dragersystemen voor HIV-immuuntherapie ontwikkeld en getest op hun eigenschappen om immuunresponsen op te wekken.
Hoofdstuk 2
Het onderzoek naar de effecten van immuuntherapie is uitgevoerd binnen een klinische studie waarbij gebruik gemaakt werd van dendritische cellen (DCs). DCs vormen een onderdeel van het immuunsysteem en zijn ‘antigeen presenterende cellen’ die ziekteverwekkers zoals virussen en bacteriën (pathogenen) die het lichaam binnendringen, kunnen opsporen. DCs raken geactiveerd wanneer ze een pathogeen tegenkomen en zullen zich daarna naar de lymfeknopen begeven en stukjes van pathogeen-eiwitten (peptiden) presenteren aan T-cellen. Op deze manier wordt onder normale omstandigheden een adequate immuunrespons opgewekt tegen het pathogeen. Het doel ons toegepaste vaccin maakt gebruik van de goede antigeen presenterende eigenschappen van DCs om hiermee enkele HIV-eiwitten aan te bieden aan het immuunsysteem. Met de DC-vaccinatie werd beoogd om HIV-specifieke immuunresponsen op te wekken of aanwezige responsen te versterken. Wij hebben de eiwitten Tat, Rev en Nef (TRN) geselecteerd voor het vaccin DC-TRN omdat deze eiwitten in een vroeg stadium van de replicatiecyclus van HIV geproduceerd worden. Het is bekend dat het opwekken en versterken van immuunresponsen tegen deze drie eiwitten een belangrijke rol kan spelen bij het inperken van de vermeerdering van HIV.

In de klinische DC-TRN studie werden HIV-1-geïnfecteerde personen gevaccineerd met als doel om T-cellen te stimuleren. Deze cytotoxische T-cellen zouden de HIV-1-geïnfecteerde cellen kunnen uitschakelen en het virus dusdanig onder controle houden dat het veilig is om de anti-HIV medicatie te stoppen. vanuit lichaamseigen bloedcellen van HIV-geïnfecteerde deelnemers werden in het laboratorium DCs gekweekt die werden bewerkt zodat ze bepaalde HIV-eiwitten gingen maken. Deze bewerkte cellen werden als DC-TRN therapeutisch vaccin teruggegeven aan dezelfde persoon en na vier van zulke vaccinaties staakten de proefpersonen hun anti-HIV medicatie. Wij konden niet aantonen dat de vaccinatie tot een herstel van de gezonde T-cell-populatie leidde en de vaccinatie zorgde voor een duidelijke verschuiving van het genexpressieprofiel. De DC-TRN immuuntherapie zoals toegepast bij 17 HIV-geïnfecteerde personen bleek veilig en zonder noemenswaardige bijwerkingen. We vonden na vaccinatie zowel een inductie als kwalitatieve verbetering van T-cel responsen gericht tegen de HIV-specifieke delen uit het vaccin: Tat, Rev en Nef. De klinische studie omvatte geen placebo-controle groep en er was niet opgezet om statistisch significante verschillen in klinische responsen te meten. Om een indruk te krijgen van de effectiviteit van de DC-TRN immuuntherapie vergeleken wij daarom de proefpersonen met een historische controle groep van ongevaccineerde HIV-geïnfecteerde personen die hun medicatie hadden staakten en daarna de vaccinatie kregen tegen huidkanker, had de vaccinatie echter geen effect gesleept op activiteit van genen in witte bloedcellen. Chronische HIV-infectie is daarmee bepalend voor het effect van DC-vaccinatie op de geneesexpresse. Het geneesexpresseprofiel van DC-TRN vaccinatie is vergelijkbaar met dat van een levend verzwakt influenza virus vaccin. Wij hebben bij vergeleken studies bij echter geen effect gevonden op activiteit van genen in witte bloedcellen. Chronische HIV-infectie is daarmee bepalend voor het effect van DC-vaccinatie op de geneesexpresse. Het geneesexpresseprofiel van DC-TRN vaccinatie is vergelijkbaar met dat van een levend verzwakt influenza virus vaccin. Wij hebben bij vergelijken gevonden dat DC-TRN vaccinatie op de geneesexpresse lijken op die van een replicerend virus. Wij vonden ook een effect op genen betrokken bij algemene immuunactivatie, die niet specifiek is voor HIV infectie en op genen niet langer opgeruimd en het virus is door zijn mutaties de gastheer dan te slim af. In Hoofdstuk 3 hebben wij onderzocht of het virus is opgeleverd met DC-TRN vaccinatie. We hebben de patiënt-specifieke HIV-pezetenen vergeleken voor en na de vaccinatie. Het blijkt dat in de peptiden die dankzij het vaccin beter herkend worden door de T-cellen, net zoveel veranderingen optreden als in de peptiden waarop het vaccin geen invloed had. Ook wordt de evolutie van het totale erfelijke materiaal van het virus niet beïnvloed door de vaccinatie. Het virus ontsnapt dus niet aan de immuunrespons die met de vaccinatie is opgewekt. Dit kan betekenen dat de immuunrespons niet zo sterk is dat er virussen met mutaties kunnen ontstaan. Het is minder waarschijnlijk dat de immuunrespons gericht is tegen region’s in het virus waar het moeilijk kan muteren of dat onderdelen van de respons tegen zoveel verschillende gebieden gericht zijn dat een enkele mutatie geen voordeel biedt.


Hoofdstuk 5 beschrijft het onderzoek naar de effecten van DC-TRN immuuntherapie op de activiteit van genen in witte bloedcellen van de gastheer. Genexpressioprofilen laten zien welke genen actief zijn in de immuuncellen in bloed en wij hebben onderzocht wat het effect van DC-TRN vaccinatie is op de genexpressie. De chronisch HIV geïnfecteerde patiënten hadden voor vaccinatie al een andere gen-activiteit dan gezonde vrijwilligers en de vaccinatie zorgde voor een duidelijke verschuiving van het genexpressioprofiel. Gedurende de periode dat de anti-HIV medicatie gestaakt werd en het virus actief ging vermeerderen, was dit effect vrijwel ongewijzigd. In patiënten die niet geïnfecteerd waren met HIV en die DC immuuntherapie kregen tegen huidkanker, had de vaccinatie echter geen effect op activiteit van genen in witte bloedcellen. Chronische HIV-infectie is daarmee bepalend voor het effect van DC-vaccinatie op de genexpressie. Het genexpressioprofiel van DC-TRN vaccinatie is vergelijkbaar met dat van een levend verzwakt influenza virus vaccin. Wij hebben bij vergelijken gevonden dat DC-TRN vaccinatie op de geneesexpresse lijken op die van een replicerend virus. Wij vonden ook een effect op genen betrokken bij algemene immuunactivatie, die niet specifiek is voor HIV infectie en op genen
die betrokken zijn bij uitputting van het immuunsysteem. Dit is een mogelijk nadelig effect van immuuntherapie bij HIV-geïnfecteerde patiënten en behoeft verder onderzoek. De analyse van genexpressie heeft daarmee inzichten verschaft die complementair zijn aan die van klassieke meetmethodes. Het toepassen van genomics in het kader van immuun monitoring in vaccinatie studies heeft daarom meerwaarde.

Celtherapie met DCs heeft een aantal nadelen, waaronder de bewerkelijkheid van het kweken en manipuleren van de cellen die lichaamseigen moeten zijn en de toediening via injecties. Hoofdstuk 6 en 7 beschrijven het onderzoek naar alternatieven voor DCs als dragermateriaal van HIV-1 antigenen. Omdat virussen van nature gericht zijn op het overdragen van genetisch materiaal naar (menselijke) cellen, kunnen ze na aanpassing goed gebruikt worden als drager (vector) van vaccin-antigenen. Bij de aanpassing van een virus voor gebruik als vector moet er met name op gelet worden dat het virus niet meer ziekteverwekkend is voor de mens. Het influenza virus is een aantrekkelijke kandidaat als virale vector want het is makkelijk te manipuleren, kan sterke immuunresponsen opwekken en is via inhalatie toe te dienen. De constructie en karakterisatie van recombinant influenza als vector voor stukjes van HIV-1 is beschreven in Hoofdstuk 6. De virale vector is uit veiligheidsoverwegingen verzwakt en kan zich niet meer vermenigvuldigen. Met deze vector konden in vitro in het laboratorium T-cellen worden geactiveerd die ofwel de stukjes HIV-1 in de vector herkennen ofwel stukjes van het influenza virus zelf. In muizen, gevaccineerd met de vector, bleek het mogelijk om antistoffen tegen HIV-1 en influenza virus op te wekken. Een tweede manier om antigenen aan te bieden aan het immuunsysteem is door ze in te sluiten in kleine vetbolletjes (liposomen). Voordelen hiervan ten opzichte van virale vectoren zijn de grotere draagcapaciteit, de mogelijkheid om grote hoeveelheden op voorraad te produceren en de afwezigheid van een (verzwakte) pathogene vector waaraan veiligheidsbezwaren kunnen klemmen. In het kader van dit proefschrift werd het liposomale AnExIL dragersysteem getest (Hoofdstuk 7). Doordat het antigen in AnExILs zowel in de vorm van DNA als in de vorm van eiwit geproduceerd in het liposoom wordt aangeboden, werden antigen-specifieke antistoffen en T-cel responsen opgewekt.

Het onderzoek beschreven in dit proefschrift heeft geleid tot een beter inzicht in de veiligheid van DC immuuntherapie in HIV-1-geïnfecteerde mensen en de effecten van deze immuuntherapie op het immuunsysteem en ook op het virus. De resultaten laten zien dat DC-TRN therapeutische vaccinatie een virus-specifieke immuunrespons opwekt en de expressie van genen in de gastheer sterk verandert. Daarnaast is informatie verkregen op het gebied van vaccin-dragersystemen voor het opwekken van humorale en cellulaire immuniteit. De verkregen kennis is van belang bij het verder ontwikkelen van een vaccin tegen HIV-1.
ABOUT THE AUTHOR

11.1 Curriculum Vitae
11.2 PhD Portfolio
11.3 List of Publications
About the author

11.1 CURRICULUM VITAE

Anna de Goede was born on the 5th of January 1981 in Nijmegen. She graduated high school with the distinction cum laude at the Stedelijk Gymnasium Nijmegen in 1999 and started the study Pharmacy at the University of Groningen in the same year. After her master internship in the field of gene therapy at the University of Kuopio, Finland, where she studied the transcriptional regulation of the human VEGF-D gene, she obtained her Master’s degree with the distinction cum laude in June 2004. She received the “most promising pharmacy student” award of the Royal Dutch Pharmacist Association and passed the examination in Pharmacy in January 2006. For one year Anna worked as a pharmacist at the Royal Dutch Pharmacist Association on pharmacogenetics and drug interactions projects.

In January 2007 she started working at the Erasmus Medical Center, Rotterdam where she combined training at the department of Hospital Pharmacy to become a hospital pharmacist with a PhD research project at the department of Viroscience. The PhD research project was supervised by prof. dr. Ab Osterhaus and dr. Rob Gruters and focused on immunotherapy strategies for HIV and the effects on both the virus and its host and has resulted in the present thesis. During the hospital pharmacy training she was supervised by prof. dr. Arnold Vulto and specialized in biotechnology and advanced therapy medicinal products. She received her degree as a hospital pharmacist in February 2014.

As of March 2014 Anna holds a position as a hospital pharmacist at the department of Clinical Pharmacy at the Radboud UMC in Nijmegen with a focus on advanced therapy medicinal products.


11.2 PhD PORTFOLIO

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In-depth courses

2010
- Workshop Photoshop, postgraduate school, Molecular Medicine, Erasmus MC, Rotterdam

2009
- International bioinformatics workshop on virus evolution and molecular epidemiology, Katholieke Universiteit Leuven, Belgium and postgraduate school Molecular Medicine, Erasmus MC, Rotterdam.
- Intensive course scientific writing in English for publication, Erasmus MC, Rotterdam

2008
- Course on Immunology, Leiden Institute for Immunology (LUMC), Leiden
- Course in Virology, postgraduate school Molecular Medicine, Erasmus MC, Rotterdam
- Course on Laboratory Animal Science, Erasmus MC, Rotterdam

2007
- Course on Molecular diagnostics, postgraduate school Molecular Medicine, Erasmus MC, Rotterdam
- Molecular Immunology course, postgraduate school Molecular Medicine, Erasmus MC, Rotterdam
- Next generation sequencing, postgraduate school Molecular Medicine, Erasmus MC, Rotterdam

2007-2010
- Internal and external presentations at the department of Viroscience, Erasmus MC, Rotterdam (twice a week)

Presentations

2010
- "Dendritic-cell-based immunotherapy for HIV is safe, immunogenic and alters blood transcriptome profile" at the AIDS Vaccine Conference, Atlanta, United States (poster)
- "Dendritic cell based immunotherapy for HIV: results of a phase II clinical trial" at the Dutch Annual Virology Symposium, Amsterdam (oral)
- "Dendritic cell based immunotherapy for HIV: results of a phase II clinical trial" at the Molecular Medicine day, Rotterdam (oral)

2009
- "HIV sequence evolution after dendritic cell-based immune therapy" at the 3rd Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam (poster)
- "HIV-1 sequence evolution after dendritic cell-based immune therapy in a phase I/II clinical trial" at the European Congress of Immunology, Berlin, Germany (poster)
- "Evaluation of recombinant influenza virus as a vaccine vector for the induction of immunity to HIV-1 p17" at the Molecular Medicine day, Rotterdam (poster)
- "HIV-1 sequence evolution after dendritic cell-based immune therapy" Annual Dutch Hospital Pharmacist Days, Nunspeet (poster)

2008
- "Impact of immune therapy on HIV genotypes" at the Molecular Medicine day, Rotterdam (poster)

Attended conferences and symposia

2010
- AIDS Vaccine, Atlanta, United States
- Dutch Annual Virology Symposium, Amsterdam
- 4th Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam, the Netherlands
- Molecular Medicine day, Rotterdam

2009
- European Congress of Immunology, Berlin, Germany
- Dutch Annual Virology Symposium, Amsterdam
- 3rd Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam, the Netherlands
- Molecular Medicine day, Rotterdam
- Virology meeting, Dakar, Senegal

2008
- 2nd Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam, the Netherlands
- Molecular Medicine day, Rotterdam

2007
- 1st Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam, the Netherlands
- Molecular Medicine day, Rotterdam
- Go out of your lab days, postgraduate school Molecular Medicine, Heemstede

• "Blood transcriptome analysis in dendritic cell-based HIV-1 immune therapy" at the 4th Netherlands Conference on HIV pathogenesis, prevention and treatment, Amsterdam (poster)
• PhD day, Erasmus MC, Rotterdam
• Virology meeting, Greece

Award

2010
• Travel grant for AIDS Vaccine Conference, by Erasmus Trustfonds

Supervision and teaching

2012
• Lecture on Therapeutic Drug Monitoring of Antivirals, Wintercourse Master Infection and Immunity, Erasmus MC, Rotterdam

• Lecture plus exam on gene therapy and pharmaceutical aspects of it for master students medicine, Erasmus MC, Rotterdam

2010
• Co-supervision of bachelor students medicine in writing a practical review: Why do HIV-1 vaccines not work? Erasmus Journal of Medicine 2010, 1:34-37

11.3 LIST OF PUBLICATIONS

Published
• HIV-1 evolution in patients undergoing immunotherapy with Tat, Rev, and Nef expressing dendritic cells followed by treatment interruption

• Induction of humoral and cellular immune responses by antigen-expressing immunostimulatory liposomes

• Sequence evolution and escape from specific immune pressure of an HIV-1 Rev epitope with extensive sequence similarity to human nucleolar protein 6

• A Phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption

• Characterization of recombinant influenza A virus as a vector for HIV-1 p17Gag

Submitted for publication
• DC-based immunotherapy in HIV-1 infection induces a major blood transcriptome shift

Not related to this thesis, peer-reviewed
• Insufficient serum caspofungin levels in a paediatric patient on ECMO
About the author

- Development and validation of a paediatric oral formulation of clonidine hydrochloride

- Stability of sildenafil (Revatio*) dilutions in dextrose 5%

- Pharmacogenetics: from bench to bedside

- Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterialization and sprouting angiogenesis

Non peer-reviewed professional literature

- Emerging mupirocin resistance in staphylococci

- Recombinant influenzavirus als vector voor hiv-antigenen
  De Goede AL, Boers PHM, Dekker LJM, Vulto AG, Osterhaus ADME, Rimmelzwaan GF, Gruters RA.

- Occupational risk of anticancer monoclonal antibodies

- Advanced therapy medicinal products – entering the hospital pharmacy arena

- Dendritic cell based immunotherapy for HIV is safe and immunogenic: results of a phase I/II clinical trial

* both authors contributed equally
DANKWOORD
En dan: het dankwoord!
Ruim zeven jaar geleden startte ik bij het Erasmus MC met de niet-alledaagse combinatie van promoveren binnen de afdeling Virologie én de opleiding tot ziekenhuisapotheker. Ik was de uitdaging aangegaan en voelde opwinding, spanning en onzekerheid. De weg naar dit proefschrift bleek een (z)ware beproeving. De hulp, ondersteuning en inzet van heel veel mensen waren onmisbaar de afgelopen jaren. Dankzij jullie is het proefschrift af, en daar ben ik maar wat blij mee. Bedankt allemaal!

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Rob, in het bijzonder wil ik jou bedanken voor je jarenlange, zeg maar gerust eindeloze, inzet voor het HIV-onderzoek en onze pogingen om werkelijk alle aspecten van de DCTRN immuuntherapie trial onder de loep te nemen. Ik heb veel van je mogen leren, waaronder het structureren van data en hypotheses tot een duidelijk verhaal. Dank voor je optimisme en je kritische blik op mijn ‘schrijfwerk’, maar ook voor het delen van je passie voor uitstekend eten en drinken.

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Anna