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“Wrapped Up” Vaccines in the Context of HIV-1 Immunotherapy

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1. Introduction

The Human Immunodeficiency Virus-1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), was described for the first time in 1983 [1, 2]. In the meantime, various classes of anti-retroviral drugs have been developed and combination therapy has improved the quality of life for millions of people affected. At the end of 2010 more than 34 million people were living with HIV infection worldwide [3]. Despite the increased access to antiretroviral therapy, an extensive treatment gap persists between the low-/middle-income countries and well-developed ones. This resulted in 1.8 million HIV related deaths and 2.6 million newly infected persons in 2009 [3]. Even for those who have access to treatment, there is no cure, as current therapy regimens cannot eradicate the virus. Therefore, the control and ultimate eradication of this pathogen remains one of the most important challenges in today's biomedical research.

HIV belongs to the family of *Retroviridae* and the genus *Lentivirus* (*lenti*, Latin for “slow”), which is characterized by a long incubation period causing long-lasting illnesses [4]. An HIV particle has a spherical shape and a size of about 100 nm. It consists of an outer coat, called the viral envelope, and an inner capsid enclosing two copies of positive single stranded ribonucleic acid (RNA). The RNA genome is 9.5 kB large and is composed of nine genes encoding structural (Gag, Pol and Env), regulatory (Tat and Rev) and accessory (Nef, Vif, Vpr and Vpu) proteins [5]. HIV-1 mainly infects CD4⁺ cells such as CD4⁺ T cells, macrophages and dendritic cells (DCs). Infection is initiated by the binding of the viral envelope glycoprotein 120 (gp120) to the CD4 receptor of the host cell, resulting in a conformational change that allows gp120 to interact with one of the co-receptors, CCR5 or CXCR4.

Binding of gp120 to the co-receptor induces further conformational changes that lead to the exposure of the fusion domain on glycoprotein 41 (gp41). Fusion of this domain with the

lipid cell membrane allows entry of the viral core into the host cell cytoplasm. This is followed by reverse transcription of the single-stranded RNA into double-stranded DNA, which becomes integrated into the host genome [6]. After DNA integration, HIV remains present as a latent DNA provirus which becomes active upon cell activation [7]. Transcription of the viral proteins eventually leads to the formation of mature and infectious virions [8].

HIV-1 can be transmitted “horizontally” through hetero- or homosexual contact or blood-blood contact (e.g. blood transfusion or intravenous drug use) as well as “vertically” from mother-to-child [9]. The transmission of HIV strongly depends on the concentration of virus in the body fluids (genital secretions, plasma or breast milk), viral “fitness” properties and the host susceptibility at both the immunological and the cellular level [10].

Clinically, an HIV-1 infection course can be divided in three stages: the primary or acute infection phase, the chronic phase (first asymptomatic and later non-AIDS defining symptoms) and the terminal AIDS defining illness. The first days after infection, the virus spreads from the portal of entry via regional lymph nodes throughout the body. It readily infects CD4⁺ T cells, producing new virions, which results in a high plasma viral load (VL). The virus finds its way to all lymphoid organs, but with a particularly massive viral production by the gastro-intestinal associated lymphoid tissue (GALT) is often observed. Remarkably, only a proportion of the newly infected patients experiences a clinical “acute phase syndrome”, characterized by mononucleosis- or flu-like symptoms, including fever, fatigue, sore throat, skin rash, enlarged lymph nodes, diarrhea, nausea and general malaise. In the first three to six weeks a rapid decline of CD4⁺ T cells is observed in the peripheral blood and even more pronounced in the GALT, which nonetheless remains an important HIV reservoir [11]. The appearance of HIV-1 specific cellular immune responses and the subsequent production of HIV-1 specific antibodies results in a sharp drop of viral load reaching a steady state viraemia, called the viral setpoint (usually within six months after infection). A dynamic equilibrium is then established between viral replication (fitness) and viral suppression by the immune system. Nevertheless, because of the limited regeneration capacity of the immune system (including thymic atrophy in adults) the number of CD4⁺ T cells will continue to gradually decrease during the chronic phase. This stage can last up to ten years and is characterized by lack of clinical symptoms of illness or relatively mild symptoms that often do not raise suspicion of HIV infection. Eventually the immune system becomes exhausted due to chronic immune activation and T-cell depletion as a result of direct cytopathic effects of infected cells, but even more by induction of apoptosis of uninfected bystander cell (CD4⁺ and CD8⁺ T cells) and degeneration of lymphoid organs. Opportunistic diseases, including serious infections or malignant tumors that are no longer controlled due to a loss of immune surveillance are the cause of AIDS-related deaths [10, 12, 13].

The majority (> 90 %) of infected individuals progresses to AIDS within about ten years after primary infection (normal progressors). Some individuals (around 5 %) remain asymptomatic for more than ten years with stable numbers of CD4⁺ T cells and low to intermediate viral loads (long term survivors and long term non progressors) [14]. Less than

1 % of infected individuals have viral loads below 50 copies per ml for at least 1-2 years while untreated (elite controllers or HIV controllers) [15]. Some individuals remain uninfected, despite being highly exposed to HIV-1 (exposed seronegatives) [16].

The first effective antiretroviral drugs (all nucleoside analogues) became available at the end of the nineties. They were used in single and later in dual combinations, but could suppress the viral load (VL) only temporarily. This was due to the appearance of drug resistance [17]. Triple drug combinations called “highly active antiretroviral therapy” (HAART), are able to suppress VL in a more sustained way and hence can prevent the emergence of drug resistance. For a number of years viral suppression was only possible at the cost of a high medication burden and many side effects. During the last decade, however, HAART has become less complicated and better tolerated, which has converted HIV-1 infection into a chronic but treatable disease [18].

It should be kept in mind, however, that HAART is not a treatment devoid of shortcomings. Firstly, a life-long commitment to the therapy remains mandatory to keep the virus under control and delay disease progression. Secondly, the treatment may cause toxic drug-related adverse effects such as cardiovascular complications [19], renal and hepatic diseases [20], lipodystrophy and diabetes mellitus, collectively called “metabolic syndrome” [21]. Thirdly, even though HAART restores the number of circulating CD4⁺ T cells to near normal levels, responses against HIV itself remain deficient [11, 22]. Finally, this costly treatment is not available for all infected persons, especially not in low- and middle-income countries in Africa, Asia and Latin America, where the numbers of patients are the highest and still increasing [3].

Therefore, there is a clear need for cheaper and more widely available therapies that can suppress and/or eliminate the viral reservoir even if the treatment is stopped or interrupted. Improving HIV-specific cell-mediated immunity by therapeutic vaccination is a generally accepted approach to tackle the problem.

During the last decade immunotherapeutic vaccination strategies have been sought after to boost the immune system in order to control virus replication and to eliminate infected cells. These vaccines are largely based on ex vivo loading of dendritic cells with antigens and immune-stimulating molecules. This personalized process is time-consuming, labor intensive, and requires strict quality control. It should be stressed that high costs of the procedure together with the need to use sophisticated equipment, restrains its application in less developed countries.

Recently, particulate antigen vehicles have been introduced in the field of vaccine design with the purpose to improve antigen delivery and to induce antigen specific immune responses. A variety of nano- and micro-carriers has been developed to deliver protein, peptides and/or nucleic acids to cells of the immune system. The intracellular fate of these particles depends on their physicochemical characteristics such as size, stability and charge. These in turn determine the efficiency with which the specific cargo is delivered to antigen-presenting cells (APC) and the extent of antigen-specific immune responses induced.

2. HIV and the immune system

2.1. Immune activation

Chronic HIV-related immune activation is characterized by the inappropriate production of pro-inflammatory cytokines and overexpression of cellular activation and exhaustion markers. Most of these inflammatory responses induced by HIV are not directed toward HIV. They rather enhance susceptibility of target cells to HIV infection and enhance virus replication in already infected cells, which accelerates disease progression. This chronic, non-specific T cell activation leads to T cell exhaustion and apoptosis of CD4⁺ and CD8⁺ T cells [23]. Increased expressions of HLA-DR and CD38 molecules on CD8⁺ T cells correlate with a higher level of immune activation and constitute markers for bad prognosis, which are partly independent from actual CD4 T count and VL [24].

It remains unclear whether there is a single key mechanism behind this HIV-associated immune activation. A so-called “leaky gut syndrome” hypothesis proposes that massive loss of CD4⁺ T cells in the GALT may affect the protective barrier of the intestinal mucosa, allowing bacterial toxins such as lipopolysaccharide (LPS) to enter the bloodstream [25]. This “microbial translocation” could in consequence induce a pathological over-activation of both the innate and adaptive immune system. Another hypothesis puts more emphasis on intrinsic regulation of type I interferon (IFN) [26]. It has been shown indeed that patterns of type I IFNs produced by plasmacytoid DCs (pDCs) are different in non-pathogenic SIV infections of natural hosts (like sooty mangabeys, African green monkeys and mandrills) and pathogenic SIV infections of rhesus macaques. High and robust type I IFN responses are observed in natural hosts during acute infection. Expression of type I IFNs is, however, down-regulated during the chronic infection phase. By contrast, the type I IFNs are persistently produced in SIV infected rhesus macaques [27]. Sooty mangabeys, the natural hosts of SIV, show no immune activation and rarely progress to AIDS, despite high levels of virus replication and severe CD4⁺ T cell depletion in the GALT. In contrast, rhesus macaques, infected with the same or closely related SIV, progress to AIDS [28].

Another enigma remains the role of CD25 and forkhead box (FOX) P3 expressing regulatory CD4⁺ T (Treg) cells. On the one hand, they may suppress chronic immune activation. On the other hand, they could undermine the effective T cell responses [29]. It has been shown that the number of Treg cells increases in the GALT, but not in the peripheral blood, during HIV infection in untreated individuals [30]. Whether this accumulation of Treg cells delays disease progression by inhibition of immune activation or increases the susceptibility of the gastrointestinal tract to opportunistic infections remains a matter of debate [29].

2.2. HIV-specific humoral immune response

The humoral immune response is mediated by antibody producing B cells (**figure 1**). In general, by preventing infections of the host cells, virus-specific antibodies play an important role in the control of many viral infections [31]. This arm of the adaptive immune system is activated after uptake of viral proteins by antigen presenting cells (DCs,

macrophages and B cells) that digest the proteins into small peptides and present them on MHC II molecules to CD4⁺ T helper (Th) cells. Specifically activated Th2 cells that produce B cell stimulating cytokines (including IL-4, IL-5, IL-6, IL-10, TGF-β) will activate naive B cells. The latter are recognized by specific epitopes or intact virus through their surface IgM and promote B cell differentiation into plasma cells producing large amounts of IgG, IgA, IgE antibodies and memory B cells. During HIV-1 infection antibodies against gp120, gp41, the nucleocapsid (p24) and the matrix (p17) arise few weeks to several months after infection. This process is commonly referred to as seroconversion.

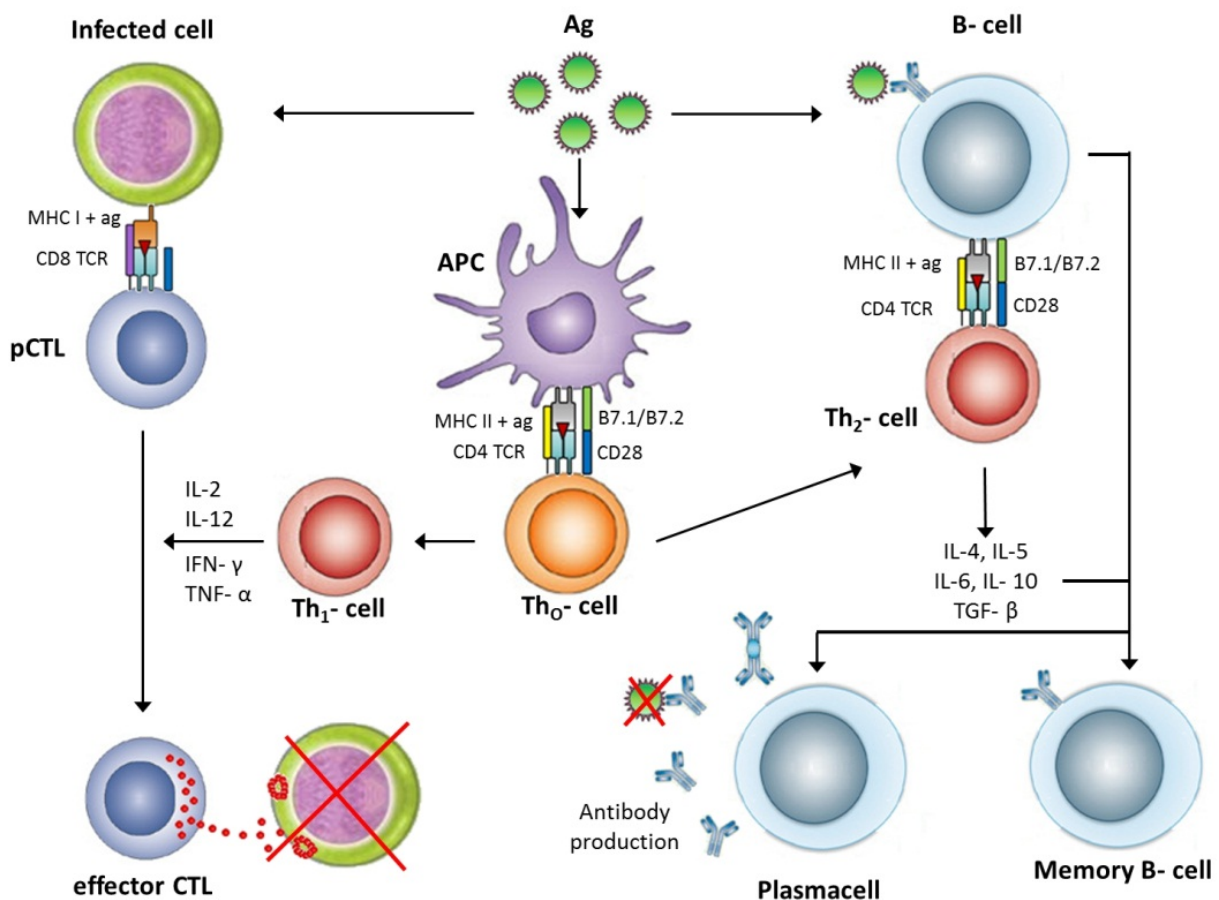


Figure 1. Overview of the adaptive immune responses after virus recognition by antigen presenting cells. Virus antigens are presented by dendritic cells and B cells to T cells. Infected cells present peptides together with MHC I molecules on the plasma membrane. The peptide-MHC I complex is recognized by precursor cytotoxic CD8⁺ T lymphocytes (CTLs). Th₁ cells, induced by antigen presenting cells, produce IL-2, IFN-γ, and TNF-α. This results in activation and differentiation of the precursor CTLs into memory or effector CTLs. Effector CTLs can directly kill infected cells by the production of perforines and granzymes. Activated Th₂ cells, also induced by antigen presenting cells, produce B cell stimulating cytokines (including IL-4, IL-5, IL-6, IL-10, TGF-β) that activate naive B cells. This facilitates/induces B cell differentiation into memory B cells and plasma cells that produce large amounts of IgG, IgA, IgE antibodies that prevent further virus infection. Ab: antibody, Ag: antigen, APC: Antigen Presenting Cell, DC: Dendritic Cell, IL: Interleukin, TCR: T Cell Receptor, Th: CD4⁺ T helper cell.

The virus neutralization is characterized by the interaction of specific antibodies with the viral envelope spikes. This interferes with virus attachment or viral entry in target cells and results in the inhibition of infection. Only a minority of anti-HIV Env antibodies, at any time, exerts immune pressure by autologous neutralization. However, the virus easily mutates and readily escapes from these potentially protective immune responses [32]. During the chronic course of infection only 20% of the infected individuals will generate broadly neutralizing antibodies (bNAbs) having the ability to neutralize heterologous viruses [33]. In addition to classical neutralization, antibodies can attach to HIV infected cells and kill them via antibody dependent cellular cytotoxicity (ADCC) mediated through their Fc moiety and natural killers cells (NK) [34, 35].

2.3. HIV-specific cellular immune response

The cellular immune response is the other arm of the adaptive immune system (**figure 1**) and it is crucial to combat viral infections. CD8⁺ cytotoxic T lymphocytes (CTLs), which eliminate infected cells, play a key role in this process. The initial step involves processing of intracellular antigens by the proteasome. The resulting peptides are then presented together with MHC I molecules on the membrane of infected somatic cells. The peptide-MHC I complex is recognized by precursor cytotoxic CD8⁺ T lymphocytes (CTLs). Also in that case a CD4⁺ T cell help, induced by antigen presenting cells, is crucial. In this case so-called Th1 cells, producing IL-2, IFN- γ , and TNF- α , activate and differentiate the CTLs into memory or effector CTLs. Effector CTLs can directly kill infected cells by the production of perforines and granzymes (**figure 2**) [36]. Alternatively, CTLs can induce apoptosis of the infected cells after interaction of Fas ligand on CTLs with Fas receptor on infected T cells [37]. CD8⁺ T cells also display a non-cytotoxic antiviral activity involving several cytokines, chemokines and a yet unidentified soluble CD8⁺ cell antiviral factor (CAF) [38].

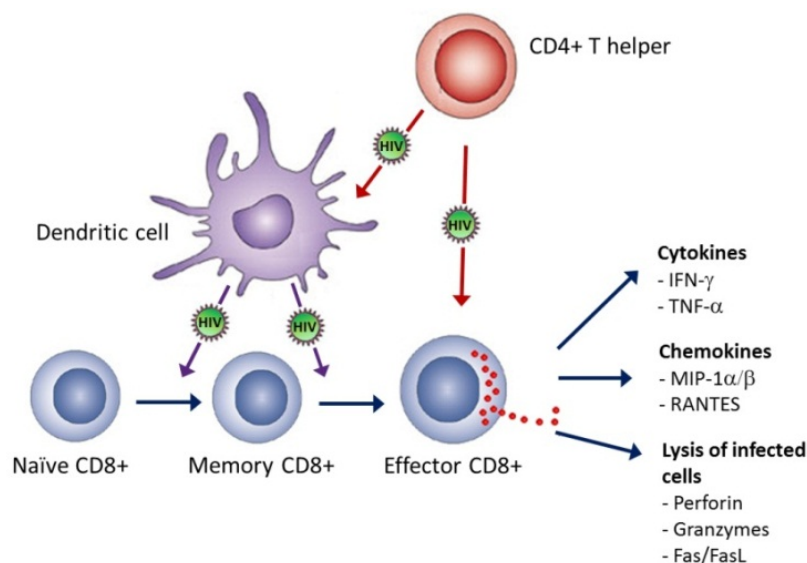


Figure 2. HIV-induced T cell responses. HIV specific CD8⁺ effector cells produce chemokines and cytokines in order to eliminate infected cells. CD4⁺ T helper cells help to stimulate both dendritic cells and CD8⁺ T-cells to maintain a CD8⁺ T-cell memory response. HIV interferes with this supportive function of CD4⁺ T-cells.

The first T cell responses during HIV infection arise when the viraemia peak is approached and reach maximum 1-2 weeks later. In non-controllers, the virus evades the CD8⁺ mediated T cell response by introducing mutations in CTL epitopes [39], by Nef-mediated down-regulation of MHC I and by influencing cytokine production and T-cell signaling [40]. Since an optimal CD8⁺ T cell response, similar to the B cell response, depends on help of CD4⁺ T lymphocytes, the deterioration of CD8 mediated viral control is also related to the weakening of CD4⁺ T cell function [41, 42].

There are many indications that HIV-specific CD8⁺ T cell responses are responsible for at least partial VL control. In the macaque model, depletion of CD8⁺ T cells during SIV-infection resulted in an increased viral load [43, 44]. In HIV-infected human subjects, who initially control the virus, escape mutations in specific CD8⁺ T cell epitopes were responsible for the loss of control and increase in VL [45, 46].

3. Correlates of protection

To date no definite biological markers have been unambiguously shown to correlate with patients' ability to control HIV infection by suppressing virus production and eliminating infected cells. Defining these factors would be crucial for the development of preventive and therapeutic vaccination strategies. For that reason, research groups focusing on preventive vaccines carefully study individuals that can avoid infection (exposed seronegatives) or partly control the virus load without the need of HAART.

3.1. Natural resistance and genetic factors of the host

Individuals that have the homozygote deletion $\Delta 32$ in co-receptor CCR5 are largely resistant to HIV-1 infection [47]. It has been recently reported that an HIV patient, who received CCR5 $\Delta 32/\Delta 32$ stem cells for transplantation, remained without viral rebound for several years [48, 49].

Certain intracellular molecules, expressed by the host, can at least partly protect against cellular infection or virus release. The most important factors identified so far are APOBEC3G, APOBEC3F, TRIM5 α and tetherin. APOBEC3G is a cytosine deaminase that incorporates adenosine instead of guanosine during synthesis of the viral DNA, which results in defective proviral DNA [50]. In addition APOBEC3G promotes natural killer cell-mediated lysis [51]. Individuals expressing large amounts of APOBEC3G have lower viral loads during acute infection phase [52]. TRIM5a binds to the viral capsid, blocking replication early in the viral life cycle [53]. Tetherin interferes with the virion release by attaching the mature virions to each other and to the host membrane [54].

Certain polymorphisms in the human leukocyte antigen (HLA type) and T or NK cell receptor can affect the cellular HIV-specific immune responses. It has been shown that human leukocyte antigens B*27, B*57 and B*58 are associated with better control of HIV-1 and slower disease progression [55-57]. Interestingly HLA B*57 also plays a role in the innate protective immune responses, acting as a natural ligand for inhibitory killer

immunoglobulin-like receptors (KIRs). KIR3DL1 and KIR3DS1 are also associated with delay in disease progression [58, 59].

3.2. Suggested immune correlates

High and broadly neutralizing antibody titers at the port of the virus entry are likely essential to prevent (new) infections of host cells. Indeed high levels of HIV-neutralizing IgA were detected at mucosal surfaces of some exposed seronegative individuals [60-62]. Moreover, in several models of transmission, passive immunization with neutralizing monoclonal antibodies could protect macaques from infection. In contrast, once infection has been established, neutralizing antibodies seem to be unable to control the virus spread [63]. In order to eliminate infected cells, strong CD8⁺ T cell responses seem to be of importance. As already discussed, most infected individuals show strong CD8⁺ T cell responses in reaction to the first viraemia peak, resulting in a decline of the viral load in early infection. Unfortunately, in most cases (except for elite controllers) these responses are not able to maintain full control, mainly due to iterative immune escape [39] and chronic immune activation [23], ultimately resulting in T cell exhaustion [64]. In contrast, HIV-specific CD8⁺ T cells preserve their function and new effective CD8⁺ T cell responses can arise against viral escape variants in elite controllers [55]. Additionally, a strong avidity of the T cell receptor for the epitope-MHC-I-complex has been shown to promote polyfunctional CD8⁺ T cells [65] and to initiate more rapid lysis of the target cell [66, 67]. Furthermore, the presence of polyfunctional CD8⁺ T cells, that have the capacity to exert different effector functions by producing IFN- γ , TNF- α , IL-2, MIP-1 β , perforines and/or granzymes and to proliferate upon antigen stimulation, has been associated with the "controller" status [68, 69]. Another important observation came from the study of Geldmacher and colleagues who reported that responses directed against Gag epitopes are dominant and potentially protective in long term non progressors and elite controllers [70]. One of the reasons for this observation could be escape mutations, in particular HLA-restricted epitopes of Gag, that come at a cost of great loss in viral fitness [71-73].

As already explained, maturation and differentiation of CD8⁺ T cells into functional memory and effector subsets are also dependent on functional CD4⁺ T helper cells. The remaining CD4⁺ T cells, after massive depletion during acute infection, need to be polyfunctional by producing at least both IFN- γ and IL-2 in order to proliferate upon antigen stimulation [41] and provide help to CTL. This Th1 function is impaired in HIV non-controllers [74].

Unfortunately, none of these factors can truly predict protection against HIV infection [75]. Therefore, at present it seems wise to conclude that all potential correlates (**table 1**) should be taken into account while designing HIV therapies. This includes the preservation of functional Th1 HIV-specific CD4⁺ T cells and the availability of central memory and memory effector HIV-specific CD8 T cells, with strong avidity for particular difficult-to-mutate epitopes. In addition also a broad functional activity, including production of several effector cytokines and lytic factors are important to result in high and broad HIV-suppressive immune responses [75].

Level of protection	Suggested correlates of protection
Viral factors	Deletion in Nef [76]
Host genetic factors	CCR5 D32/D32 [48, 49]
Host restriction factors	High levels of antiviral factor APOBEC3G [51, 52] High production of TRIM5 α [53] Up-regulation of tetherin [54] HLA types B*27, B*57, B*58 [55, 56] KIR3DL1, KIR3DS1 [58, 59]
Humoral immunity	Neutralizing Abs: IgA antibodies at the mucosal surfaces [60-63]
Cellular immunity	Polyfunctional T cells [66, 69] Proliferative CD4 ⁺ and CD8 ⁺ T cells [68, 70] Avidity of HIV specific T cell responses [65, 66, 74]

Table 1. Suggested correlates of protection.

4. Vaccination strategies against HIV-1

Many infection-related hurdles complicate the development of an HIV vaccine. These include the high genetic variability, the potential of cell-to-cell transmission and other evasion strategies such as down-regulating MHC I in infected cells and latency of the virus [77]. In addition, correlates conferring protection against HIV remain to be established. A number of potential markers have been suggested to prevent or control HIV infection. These comprise: production of high titers of neutralizing antibodies with broad specificities, concomitant HIV-specific activation of CD4⁺ and CD8⁺ T cells, polyfunctional T cell responses (production of several immune mediators by the same T cell) and induction of long- term memory cells [78].

4.1. Prophylactic vaccines

Prophylactic vaccines rely on the production of antibodies that bind to free virus particles thereby preventing viral entry into host cells (defined as neutralization) and thus block infection. Vaccines are designed to mimic natural infections, by using live-attenuated virus (measles, mumps), chemically inactivated virus (polio) or recombinant subunits of the virus (Hepatitis B). There is circumstantial evidence that neutralizing antibodies could play a role in the protection against HIV. HIV-neutralizing IgA antibodies have been isolated in frequently exposed individuals, who remained uninfected [62, 79]. In addition, passive immunization with several HIV neutralizing IgG monoclonal antibodies protects macaques against infectious SHIV (simian immune deficiency virus with an HIV envelope) [80, 81]. Although attenuated SIV vaccines provided some level of protection against super-infection in macaques, attenuated HIV is considered too risky to be ever tried in humans [82]. Therefore, much effort has been invested in the development of subunit vaccines that could elicit production of neutralizing antibodies. It should be taken into account that broadly neutralizing antibodies (bNAbs) that inhibit also heterologous viruses *in vitro*, can be detected in 20% of naturally infected individuals. This implies that the production of these

antibodies is not sufficient to provide full protection against established HIV, but could still be efficient in prevention of cell-free transmission. The most important reason why it is difficult to induce bNAbs is the extreme variability of HIV Env antigenic epitopes. Moreover, the virus is shielded by non-immunogenic glycans, which hinder binding of antibodies to the envelope proteins [83, 84]. Due to these problems, prophylactic vaccine trials in humans have failed to elicit protection. The only exception till now is the recent Thai vaccine trial (RV144).

Nevertheless neutralizing antibodies with activity against easy-to-neutralize so-called “Tier 1” viruses have been induced in a number of animal trials, but these antibodies failed to broaden and faded rapidly, even upon repeated heterologous boosts [85]. The failure to induce high titers of NAbs moved the field towards strategies aiming at stimulating polyfunctional and sustained CD4⁺ T help responses [69] to support high quality cytotoxic T cells (both central memory and effector memory). These cells would be necessary to rapidly eliminate infected cells, if antibodies fail to prevent cellular infection [86, 87]. This “second line prevention” hypothesis was further supported by the observations that HIV-specific CD4⁺ and/or CD8⁺ T cells as well as particular human leukocyte antigen (HLA) class I markers, and not antibodies, correlate with resistance to HIV in some highly exposed seronegative children (potential vertical transmission) [88] or women (potential heterosexual transmission) [89-91].

In this connection, current HIV vaccines are also aiming at the induction (prophylactic field) or enhancing (therapeutic field) of HIV specific T cell responses. Such vaccines would elicit or boost HIV specific cytotoxic T cells (CTLs) to eliminate infected cells and CD4⁺ T cells, which can help to induce and maintain B cell and CD8⁺ T cells responses [92]. Several strategies are currently under investigation to establish effective T cell responses in either a preventive or therapeutic setting either based on protein [93, 94] or peptide [95] vaccinations, virus like particles (VLPs) [96], DNA vaccination using viral vectors [97, 98], prime-boost vaccinations [99, 100] or DC-based vaccines [101-109].

4.2. Viral vaccine delivery

Whereas the use of live attenuated HIV is considered to be unsafe for the use in humans, the development of vaccines based on HIV-inactivated with formalin is compromised by the fact that the antigenicity of the envelope gets lost. Milder formalin treatment of the virus, followed by heat-inactivation has been shown to circumvent this hurdle and induce modest neutralizing antibodies titer in non-human primates [110].

During the last decade, a variety of vaccines was designed using (plasmid) DNA/RNA vaccine candidates for priming followed by live vectored recombinant vaccines for boosting, some of which have already been tested in advanced stages of clinical trials [111, 112]. We will highlight here some of the specific characteristics of viral vectors, which have been used in preclinical and early clinical preventive vaccinations against SIV and HIV, respectively.

Adenoviruses, poxviruses and lentiviruses are the most frequently used viral vector systems. The major advantages of these vectors are the high transduction efficiency resulting in high level expression of the encoded protein and the possibility to target specific cells achieved by altering the viral tropism (e.g. by pseudotyping with envelope or counter receptors of another virus) [113, 114]. Major drawbacks are the high risk of insertional mutagenesis, high production cost of large amounts high-titered viral stocks and a limited size of nucleic acids that can be packed [113, 114]. The first trial of a preventive HIV vaccine that was designed to elicit a strong cellular immune response was the STEP trial done by Merck. It involved immunization of almost 3000 healthy uninfected volunteers with three recombinant adenovirus serotype-5 (rAD5) vectors, *Ad5-gag*, *Ad5-pol* and *Ad5-nef*. Unfortunately, it failed to induce protection against infection [115, 116]. Moreover, the vaccine increased the rate of HIV infection in individuals with pre-existing immunity to adenoviruses [117]. The exact mechanism that underlies this phenomenon remains to be elucidated, but it has been suggested that the activation of pre-existing vector specific T cells may have increased numbers of HIV target cells. This might be avoided by the use of less prevalent adenovirus serotypes (e.g. Ad11, Ad24 or Ad35) instead of the Ad5 vector in designing future HIV vaccines [118, 119].

Alternatively, poxvirus-based vectors should be taken into account since they do not pose any problems with pre-existing immunity. In addition, they are used as highly attenuated vaccinia virus strains. Three of the best characterized highly attenuated pox vectors are the recombinant viral canary pox vectors such as the highly attenuated vaccinia virus strain ALVAC [120], the recombinant modified vaccinia Ankara (MVA) vectors [121-123] and canarypox-derived NYVAC [120, 124, 125]. Recombinant pox vectors, encoding HIV antigens, have been shown to be safe in humans and to induce HIV specific immune responses. No protection against HIV infection has been achieved with the exception of the preventive RV144 phase III clinical trial. In this clinical trial, involving 16 000 uninfected individuals, a canary pox vector coding HIV Gag and Env was used as prime immunization followed by a recombinant Env gp120 protein boost (RV144). A 31% efficacy of protection against HIV infection was demonstrated after three years [126]. Very recently, Barouch *et al.* reported that rhesus monkeys, receiving heterologous vector regimes (adeno with MVA), were protected with an efficacy of 80% after SIV challenges [127].

Another type of vectors that could avoid the pre-existing immunity issue is based on lentiviruses. These vectors have been explored extensively in the field of gene therapy since they efficiently transduce non-dividing cells, such as DCs [128, 129], and promote long term antigen expression [130]. Lentiviral vector vaccines have been shown to induce both high short term and long-term anti-HIV immune responses in mice [131, 132]. Even in the absence of circulating CD4⁺ T cells, induction of specific CTLs was obtained [133]. Despite reassuring safety and tolerability results in a phase I clinical trial [134], the major concern remains the risk of insertional mutagenesis [135]. Attempts to overcome this risk, have led to the design of self-inactivating vectors, vectors with targeted integration and non-integrating vectors [135].

Replicating and persistent recombinant cytomegalovirus (CMV) vectors have recently been shown to be a promising system in rhesus macaques [136]. Prophylactically vaccinated animals maintained CD4⁺ and CD8⁺ T effector memory (T_{EM}) cell responses, regardless to pre-existing CMV immunity, and were more resistant to challenge than the control group even in the absence of neutralizing antibodies [137]. The authors suggest that T_{EM} responses are crucial in the protection against HIV infection after sexual exposure. This is obviously also the scope of HIV immunotherapy where sustained effector and memory T cells can eliminate infected cells.

Virus-like-particles (VLPs) have recently emerged as novel delivery systems. They contain envelope and core proteins from SIV/HIV in their native structure. These pseudo-virions are produced in baculovirus or vaccinia virus expression systems where Gag and Env proteins from HIV or SIV are co-expressed and spontaneously assembled. The immunogenicity of these vaccines was only modest in non-human primates [96], however, efficiency was greatly improved when combined with a HIV DNA vaccine prime [138].

Safety concerns and difficulties related to repeated administrations of viral vectors that may evoke dangerous immune reactions are the most important bottlenecks in regard to clinical application in humans. To improve the general safety profile and circumventing the drawbacks inherited to viral delivery, well-defined particulate vaccines have emerged as promising candidates in the field of vaccine development.

5. HIV Immunotherapy with DC-based vaccines

5.1. Therapeutic vaccines

Since the introduction of HAART, HIV-1 infection has evolved into a chronic but treatable disease. Although HAART suppresses viral replication and partially reconstitutes both CD4⁺ T cell numbers [139] and T cell immune responses to opportunistic infections, it cannot restore effective HIV specific T cell responses [140], resulting in a rapid rebound of HIV-1 replication upon treatment interruption [141]. This implies that infected individuals are bound to lifelong treatment, imposing a high burden in terms of adherence, costs and the risk of drug related metabolic disorders [142]. Therefore, therapeutic vaccination has emerged as an option to boost and improve the cellular immune responses in infected individuals [143]. This concept is supported by increasing evidence that strong HIV-1 specific CD4⁺ helper T cells and CD8⁺ CTLs are responsible for viral control both in macaques [144] and humans [145]. During acute infection CD8⁺ T cell responses contribute to control of the initial viraemia peak. However, in most infected subjects, this CD8⁺ T cell response loses its efficacy during chronic infection. This is due to a high mutation rate of the virus, the absence of proliferative and highly functional CD4⁺ T cells [145, 146] and the appearance of impaired or apoptotic HIV-specific CD8 T cells [147]. Clearly, the major challenge for therapeutic vaccination is to elicit strong CD4⁺ and CD8⁺ responses that would allow stopping of HAART. *In vitro* modulation of DCs to efficiently present target antigens to elicit cellular immune responses has been shown to be a promising strategy in cancer

immunotherapy [148], which constituted a model for development of similar HIV immunotherapies.

5.2. Dendritic cells

DCs are the sentinels of the immune system, bridging innate and adaptive immunity, in response to pathogens crossing the mucosal or dermal barrier. Immature DCs (iDCs) continuously sample their environment and take up autologous and foreign antigens [149]. They undergo maturation in response to signals that originate from pathogen-associated molecular patterns (PAMPs). These PAMPs activate a set of pattern recognition receptors (PRRs) such as Toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-lectin receptors (CLRs) and retinoic acid-inducible gene protein (RIG)- like receptors (RLRs). Triggering of PPR results in an increased expression of major histocompatibility complex I and II (MHC I and MHC II), co-stimulatory molecules (CD80 and CD86) as well as secretion of T cell stimulatory cytokines (e.g. IL-12). During this maturation process DCs lose their ability to take up antigens and chemokine receptors (e.g. CCR7) are up-regulated in order to promote their migration to lymph nodes. Mature DCs process endogenous antigens via proteasome into 8-9 amino-acid peptides which are then loaded on MHC I and presented to CD8⁺ T cells. Exogenous antigens are processed via the endolysosome into longer peptides to be load onto MHC II for presentation to CD4⁺ T cells. The capacity of DCs to present exogenous antigens also via MHC I pathway (i.e. cross presentation), distinguishes them from other APCs, such as macrophages and B cells. To stimulate effective T cell responses, peptide-MHC complex on DCs should interact with T cell receptors (TCR). This is accompanied by binding of co-stimulatory molecules on DCs with CD28 present on T cells (**figure 3**). Finally, produced cytokines determine the differentiation of the effector cells into Th1, Th2 or CTL [150]. The latter is achieved after DCs' licensing by the interaction of CD40 on the mature DCs with CD40L expressed on CD4⁺ T cells. IL-4 secretion promotes CD4⁺ Th2 cells, stimulating the production of antibody producing B cells. IL-12 promotes CD4⁺ Th1 cells, providing help to CTL to kill infected cells. Secretion of IL-10 has a negative impact on Th1 or Th2 cells and induces immune tolerance. Licensed DCs also induce differentiation of CD8⁺ T cells into CTL via peptide-MHC I complex and promote survival of CD8⁺ T cells via co-stimulation through CD137L (4-1BBL) [151].

Roughly five DC subsets can be distinguished [152]. Classical or tissue resident DCs are located in lymphoid organs such as spleen and lymph nodes. Migratory DCs, found in non-lymphoid organs such as skin, intestines and lungs, sample their environment and migrate to lymph nodes to present tissue derived antigen to T cells. Langerhans cells reside in the multi-layered epithelium of the skin, oral and genital surfaces. Plasmacytoid DCs (pDCs) and myeloid or monocyte-derived DCs may be present in various tissues, yet they mainly circulate in the blood. pDCs are known as major producers of type I interferons (IFNs) in response to virus-associated molecules such as single-stranded (ss) RNA and unmethylated cytosine-phosphate-guanine (CpG)-rich DNA that trigger TLR7 and TLR9, respectively [153]. Myeloid DCs represent the major fraction of APCs in the blood that responds to TLR ligation by producing IL-12 [154]. Noteworthy, the APC function of DCs is impaired by

HIV, which could contribute to the dysfunction of HIV specific T cell responses since maturation of DCs and IL-12 secretion are diminished, thereby suppressing T cell responses [155]. HIV-infected DCs preferably secrete IL-10 thus limiting T cell proliferation and activation and rather induce tolerance [156]. HIV-1 infected DCs also act as a Trojan horse to infect new T cells by promoting tolerance of T cells to HIV-1 [157].

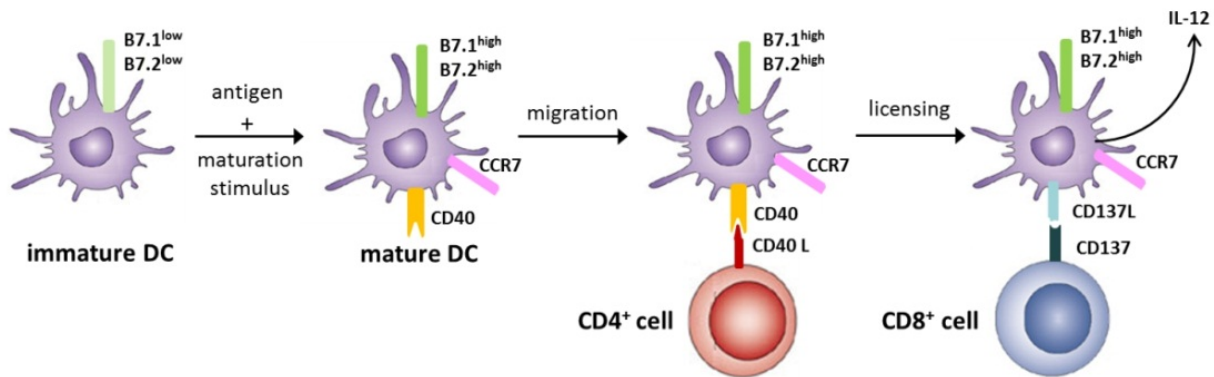


Figure 3. Differentiation process of dendritic cells. Immature DCs take up antigens. Activation of PPRs results in the differentiation towards mature DCs. The co-stimulatory molecules (B7-1, and B7-2) are overexpressed and the expression of the lymph node homing receptor CCR7 is induced. Mature DCs migrate to the draining lymph node, where they present antigens to cognate CD4⁺ T cells. Cross-linking CD40 on the DCs by CD40L expressed on the antigen-activated CD4⁺ T cell, induces the mature DCs to differentiate further, a process known as licensing. Licensed DCs up-regulate additional cell surface proteins, such as CD137L. The licensed DCs now present antigens to cognate CD8⁺ T cells. CD137L-mediated co-stimulation through CD137 on the antigen-activated CD8⁺ T cells enhances the survival and proliferative capacity of the activated CD8⁺ T cells.

5.3. HIV-1 antigen loaded dendritic cells tested in clinical trials

To obtain a large population of DCs to be used in immunotherapy, DCs are derived from blood monocytes that are cultured in the presence of granulocyte and macrophage colony stimulation factor (GM-CSF) and IL-4 [158, 159]. Various approaches for loading DCs with antigens have been applied. They include the use of inactivated virus [104, 160], recombinant viral proteins [107, 161-163], peptides [101, 164], DNA [165-167] and mRNA [168-172]. After loading, DCs are matured using various maturation cocktails composed of cytokines (such as type 1 (α, β) or type 2 (γ) interferons, TNF- α , IL-6) prostaglandins (PG), TLR ligands, T cell derived products (CD40L) or small interfering RNA (siRNA) against suppressors of cytokine signaling (SOCS)-1 [98, 151].

To this date at least ten DC-based immunotherapeutic vaccine trials (**table 2**) have been tested in infected individuals (recently reviewed by García and Routy [173]. Kundu *et al.* were the first to perform a human clinical trial, in which treatment-naïve HIV infected individuals were vaccinated with protein or peptides pulsed autologous DCs [107]. The vaccine was well tolerated and resulted in an increase of HIV specific CD8⁺ T cell response in 3 out of 6 individuals, but no effect on viral load was observed. Three other clinical trials tested peptides pulsed DCs [101, 105, 106]. In two of these trials an increase in HIV-specific

T cell responses in the absence of virological responses was shown [101, 105], whereas in the third study a transient decrease in viral load was found in 50% of vaccinated individuals [106]. Lu *et al.* were the first to demonstrate *in vivo* that a vaccine made of autologous monocyte-derived DCs, pulsed with autologous, aldithriol-2-inactivated HIV-1 was capable of inducing HIV-1-specific T cell responses. Moreover, there was an 80% decrease in plasma viral load levels over the first 112 days after vaccination. A prolonged suppression of viral load (of more than 90%) was seen for at least 1 year after vaccination in 8 out of 19 vaccinated patients. This suppression of viral load correlated with HIV-1-specific IL-2 and IFN- γ expressing CD4⁺ T cells and with HIV-1 Gag-specific perforin-expressing CD8⁺ effector cells [108]. Unfortunately, the lack of a control group does not allow a proper evaluation of the results obtained. The group of García *et al.* performed two clinical trials with autologous DCs pulsed with heat-inactivated virus [103, 104]. In a double blind placebo controlled study with a similar setup as Lu *et al.*, García *et al.* observed a small but significant decrease in viral loads for at least 48 weeks, concomitant with a weak increase in HIV-1 specific T cell responses in therapy naïve HIV infected individuals [103]. Another study performed by the same group in HAART treated patients showed a lengthening of the rebound after therapy interruption and a moderate increase in HIV specific T cell responses [104].

Form of antigen	Vaccine strategy	Subjects	Clinical trial outcome
Inactivated virus	DCs pulsed with autologous AT2 inactivated virus (10 ⁹)	Untreated patients (n= 18)	Suppression of viral load was correlated with HIV specific IL-2 or IFN- γ producing CD4 ⁺ T cells and perforin producing CD8 ⁺ effector T cells. [108]
	DCs pulsed with autologous heat inactivated virus (10 ⁶)	HAART treated patients followed by therapy interruption (n=18)	Lengthening of viral rebound correlated with numbers of HIV-specific proliferative CD4 ⁺ and CD8 ⁺ T cells. [104]
	DCs pulsed with autologous heat inactivated virus (10 ⁹)	Untreated patients (n= 18)	Inverse correlation between decrease in viral load and HIV-specific T cell responses. [103]
Peptides	DCs pulsed with Gag, Pol and Env peptides	Untreated patients (n= 6)	In 3/6 cases Env-specific and proliferative immune responses were observed. [107]
	DCs pulsed with HLA A*0201 binding epitopes in Gag, Nef and Env	HAART treated patients followed by therapy interruption (n=4)	In 2/4 patients moderate CD8 ⁺ T cell responses were observed. No lower viral setpoints after therapy interruption. [105]

Form of antigen	Vaccine strategy	Subjects	Clinical trial outcome
	DCs pulsed with HLA A*0201 binding epitopes in Gag, Pol and Env	HAART treated patients (n=18)	Increase in CTL responses against vaccine epitopes. [101]
	DCs pulsed with HLA A*0201 binding epitopes in Gag, Pol, Env, Vpu and Vif and Th epitopes in Gag and Env	Untreated patients (n= 12)	Generation of new T cell responses despite high viral loads. [106]
DNA	ALVAC pulsed DCs vs AIVAC alone	HAART treated patients followed by therapy interruption (n=29)	No differences in T cell responses against HIV antigens. Did not lower VL setpoint after therapy interruption. [102]
	ALVAC-Remune	HAART treated patients followed by therapy interruption (n=48)	No lowering of viral setpoint after vaccination, but VL rebound was delayed. [177]
mRNA	<i>Ex vivo</i> electroporated DCs with mRNA encoding CD40L and autologous HIV proteins (Gag, Ref, Nef, Vpr)	HAART treated patients (n=10)	HIV specific proliferative immune responses preferentially targeted to CD8 ⁺ T cells. Correlation with viral control during therapy interruption. [109]
	<i>Ex vivo</i> electroporated DCs with mRNA and autologous HIV proteins (Gag, Tat, Rev, Nef)	HAART treated patients (n=6)	Breadth of IFN- γ response and T-cell proliferation were correlated with CD4 ⁺ and CD8 ⁺ polyfunctional T-cell responses. Autologous CD8 ⁺ T cells inhibited HIV superinfection <i>in vitro</i> . [176]
	<i>Ex vivo</i> electroporated DCs with mRNA and autologous HIV proteins (Tat, Rev, Nef)	HAART treated patients followed by therapy interruption (n=17)	Induced or enhanced CD4 ⁺ and CD8 ⁺ T cell responses specific for the vaccine antigens. [175]

Table 2. Overview of therapeutic DC-based clinical trials.

Nucleic acids are also used as a source of antigens in DC-based immunotherapies. Phase I and II clinical trials were conducted to evaluate the potential of a canarypox HIV-vaccine (ALVAC), containing DNA encoding Env, Gag as well as parts of Nef and Pol to either

directly vaccinate HAART-treated patient or *ex vivo* load autologous DCs. The results obtained did not demonstrate clear differences in the immunological or virological outcome and a control group was lacking [102]. In a very recent study, DNA encoding HIV proteins was complexed with polymers and topically administered with a patch method named DermaPrep [174]. The results showed that vaccination induced new HIV-specific immune responses in HAART treated patients. Moreover, a better control of viral replication during treatment interruption was observed [167]. DCs loaded with mRNA have also been tested in clinical trials. Routy *et al.* reported the results of a trial in which HAART-treated patients received autologous DCs *ex vivo* electroporated with mRNA encoding autologous HIV sequences and CD40L [173]. This pilot study showed CD8⁺ T cells specific for the presented HIV antigen were preferentially targeted. Two other clinical trials employing autologous DCs electroporated *ex vivo* with mRNA encoding Tat, Rev, Nef and Gag were recently concluded [175, 176]. Both studies showed that vaccine-specific CD4⁺ and CD8⁺ T cell responses were enhanced. In one study vaccinated individuals interrupted treatment. Six out of 17 individuals remained off therapy 96 weeks after cessation [175].

One should keep in mind, however, that *ex vivo* manipulation of DCs is a complex personalized vaccination procedure, which prevents its accessibility to large numbers of patients. Therefore, there is a constant need for new approaches that could target DCs directly *in vivo*. In the following paragraphs we will discuss various non-viral delivery approaches which promote antigen uptake by DCs and induce potent immune responses.

6. The way forward: Antigen delivery by non-viral carriers

Even though viral vectors are generally considered more efficient, non-viral delivery vehicles receive increasing attention since they are safer, more versatile, easier to prepare and hence more accessible for up-scaling [178]. In addition, they allow delivery of larger quantities of antigens. Importantly, encapsulation protects antigens from their environment and therefore permits a prolonged release of antigens in tissues or particular cells [179]. The usual size of particulate vaccines, ranging from a few hundred nanometers to a few microns, is ideal for uptake by DCs [180]. Moreover, the nature of non-viral carriers allows their functionalization with moieties permitting specific targeting to DCs. Additionally they allow co-delivery of immunostimulatory molecules which can direct the immune system toward the humoral or cellular arm [181]. The simultaneous delivery of antigens and immune-stimulators to the same DC is a feature which has been reported to significantly augment the strength of the induced adaptive immune responses [182-184]. In the following paragraphs we will discuss different lipid- and polymer-based carrier systems employed to deliver proteins and nucleic acids relevant for HIV-specific immunotherapy (table 3).

6.1. Choice of antigen

Peptides, proteins or nucleic acids (DNA or RNA) have been used as a source of antigen in the majority of therapeutic vaccination strategies. Each of them comes with specific

characteristics in terms of safety, stability, potential to cover antigenic variability, requirements for delivery and nature of the immune response induced [185]. Recombinant proteins or peptides utilized as subunit vaccines are safe and simple forms of antigens. However, their production at clinical grade quality is very expensive. As a consequence, only one or a few antigenic variants can be produced at an affordable price. Moreover, they are susceptible to pre-mature proteolytic degradation. Since DCs recognize them as exogenous antigens, they are preferentially presented in a MHC class II context [143] and to a lesser extent onto MHC I molecules via cross-presentation [180]. Therefore, they will not induce strong and broad CD8⁺ T cell responses, which are considered as a prerequisite for a therapeutic vaccine. These limitations can partially be resolved by their encapsulation. Encapsulation protects proteins and peptides from being prematurely degraded by proteolytic enzymes. Additionally, particulate antigen delivery favors cross-presentation thereby enhancing CD8⁺ T cell responses [186, 187]. In the context of HIV immunotherapy, however, the use of proteins as antigens is not ideal since HIV generates escape variants during the course of infection which remain present as a latent reservoir in cells [188]. With the technology available, it is not feasible to produce hundreds of variants of the same protein and include them in a therapeutic vaccine.

Delivery vehicle	Form of antigen	Advantages	Disadvantages
PLA-PLGA particles	Proteins	FDA approved, induction of specific antibodies and Th1 cellular responses	Harsh preparation process, expensive to upscale.
Polyelectrolyte capsules	Proteins, peptides	Induction of both Th1 and Th2 responses. Easy to tailor with immunostimulators. Stability and release kinetics correlate with a number of bilayers.	Not (yet) FDA-approved
Polyplexes	Nucleic acids	Protect nucleic acids, facilitate escape from the endosomal compartment.	Strong electrostatic interactions may hamper release of nucleic acids from the carrier
Liposomes	Proteins, peptides, nucleic acids	Good protection of antigens, facilitate intracellular uptake. Induction of both Th1 and Th2 responses.	Poorly immunogenic
Lipoplexes	Nucleic acids	Protect nucleic acids, facilitate escape from the endosomal compartment.	Might aggregation in the presence of serum

Table 3. Advantages and disadvantages of different non-viral delivery systems.

Nucleic acids, such a plasmid DNA (pDNA) or messenger RNA (mRNA), encoding viral proteins, can more easily cover the wide range of viral quasi species [189, 190]. As compared

to pDNA, mRNA-based delivery may hold several advantages. First of all, mRNA is easier to engineer; there is no need for specific promoters and terminators to be present in the construct. Secondly, the synthesis of proteins encoded by mRNA is transient, which ensures a controlled antigen exposure [191]. Thirdly, in contrast to pDNA, mRNA does not need to cross the nuclear membrane to be effective and therefore offers the possibility to produce proteins in slow or non-dividing cells [192]. Furthermore, the use of mRNA excludes the risk of integration into the cell genome, eliminating possible insertional mutagenesis [189, 193]. For years, the application of mRNA has been hampered by a general belief that it is too labile to guarantee sufficient protein expression. Nowadays, however, mRNA vaccination strategies are being thoroughly investigated in the field of allergy [194, 195], cancer [193, 196-198] and HIV [109, 170, 171] immunotherapy. These studies mostly rely on the *ex vivo* electroporation of dendritic cells. We and others focus on complexing mRNA with cationic lipids and polymers (**figure 4**), which could protect mRNA. Moreover, we believe that complexing mRNA with cationic carriers might hold potential for their application *in vivo*. This approach could serve as an alternative for laborious *ex vivo* loading of DCs.

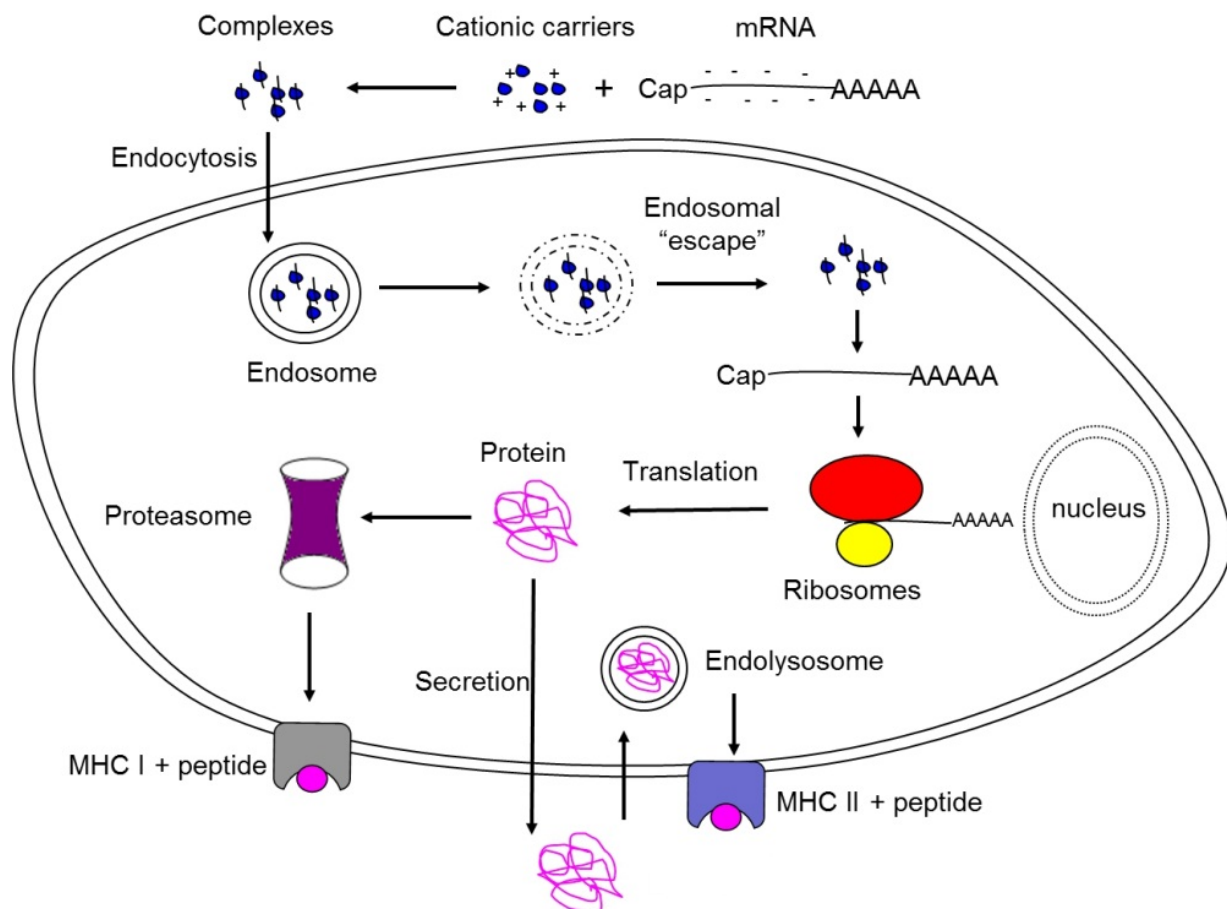


Figure 4. Internalisation and intracellular trafficking of mRNA/cationic carrier complexes. Negatively charged mRNA is complexed with cationic carriers (lipid or polymer based). After endocytosis, complexes end up in endosomes, from where they should release their cargo mRNA into the cytoplasm to enable translation by ribosomes. Translated protein is either secreted or can directly be processed via proteasome to be presented with MHC class I. Secreted protein can be taken up and promote presentation in a MHC class II way after cleavage in the endolysosomes.

6.2. Polymer-based antigen delivery systems

Various polymers have been used to prepare nano- and micro- particles for antigen delivery. Here, we will focus on polymers that have shown to be promising in terms of immunotherapeutic vaccination.

6.2.1. Polymers based on lactic acid (PLA) and glycolic acid (PLGA)

The most studied polymers for antigen delivery in the context of vaccination are the biodegradable poly(D,L-lactide) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA), which have been FDA approved for human use. These particles have mainly been evaluated for their potential to deliver proteins and peptides to DCs or macrophages with the aim to induce CD8⁺ cytotoxic T cell immune responses in the context of prophylactic vaccination. It has been demonstrated that PLGA particles are phagocytosed by APCs [199] and ensure prolonged antigen presentation by DCs [200]. Whereas empty PLGA particles do not influence the maturation status of DCs [201, 202], DC activation can be induced, if the PLGA polymer is employed to encapsulate TLR ligands (poly I:C, MPLA) or surface loaded with anti-CD40 antibodies clear activation of DCs was observed [203-205]. PLA particles carrying p24 protein have been shown to induce both mucosal antibody production as well as CTL responses [206, 207].

The main drawback of these polymers is that some antigens tend to aggregate during the encapsulation process. Moreover, the exposure of proteins to organic solvents, required to dissolve the polymer, makes them highly susceptible to denaturation leading to the loss of antigenic epitope recognition [208]. This can partially be overcome by adsorption of proteins on the particle surface [209, 210]. Additionally, a rather expensive up-scaling process and clean-up procedure to ensure sterile production constitute difficult obstacles [211]. Due to the harsh preparation process and the hydrophobic nature of PLGA and PLA, these carriers are not suitable for nucleic acids delivery [212-214].

6.2.2. Polyelectrolyte microcapsules

Polyelectrolyte microcapsules (PeMCs) fabricated using a so-called layer-by-layer (LbL) technology are a relatively novel class of particles [215]. The process of their preparation is less harsh as compared to that of PLGA/PLA particles. A template containing an antigen and colloid nanoparticles is used as a sacrificial core. This core is coated with several bilayers of polymers of opposite charges. At the end of the procedure the template core is dissolved (**figure 5**). Since the encapsulation process is performed in a purely aqueous environment, minimal stress for a protein antigen is ensured. With appropriate choice of polymers, these particles can be fully biodegradable. De Koker *et al.* used ovalbumin as a model antigen and showed the benefit of antigen encapsulation in PeMCs to stimulate antigen presentation to T cells by murine bone marrow-derived DCs [186, 216].

PeMCs containing p24 and poly I:C (TLR-3 ligand) have been shown to be promising as an HIV-1 immunotherapeutic vaccine. Both antigen and maturation stimulus could be

delivered in the same particle to DCs inducing maturation and stimulation of HIV-specific responses both *in vitro* and *in vivo* [184]. PeMCs were also used for the delivery of Gag-peptides to APCs. These APCs could activate SIV-specific T cells in an *ex vivo* non-human primate model [217]. Immune responses could be further improved by sensitizing the PeMCs to enzymatic or reductive degradation upon cellular uptake, thereby assuring release of its cargo [218, 219].

PeMCs have been also employed to deliver pDNA [213, 220]. When considering their use for the delivery of mRNA, however, one should make sure that the preparation process is rigorously RNase-free, which might present a challenge.

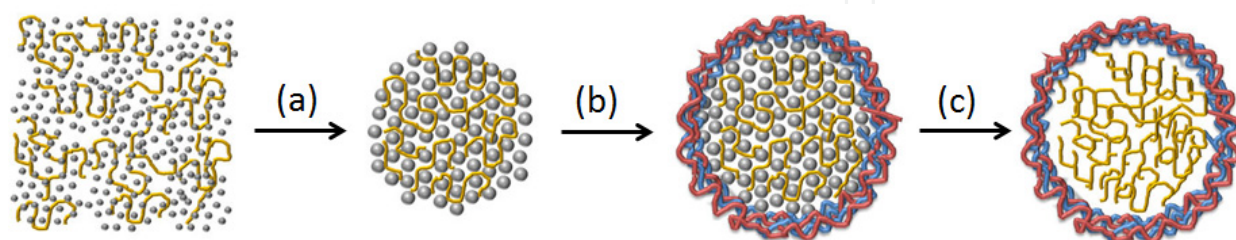


Figure 5. Preparation of polyelectrolyte microcapsule [186]. (a) In the first step an antigen (yellow) of interest is mixed with colloid nanoparticles (grey) to form a core template. (b) In the next step a layer of negatively charged polymer is deposited (blue). The non-adsorbed polymer is removed by washing. This is followed by applying a positively charged polymer (red) and another washing step. (c) When the desired number of polyelectrolyte layers is obtained, the core is dissolved.

6.2.3. Polyethyleneimine-based polyplexes

Polyethyleneimine (PEI) has been extensively used to deliver pDNA and siRNA to cells [221-223]. PEI consists of repeating units that contain two carbon atoms and a protonatable nitrogen atom. It exists in a linear or branched conformation (**figure 6**), both appearing in a broad range of molecular weights. PEI can efficiently bind pDNA to form so-called polyplexes. The linear form has been shown to release complexed pDNA more easily than the more stable branched form and thus results in higher transfection efficiencies [224]. The net positive charge of the polyplexes promotes their adhesion to the overall negative charge of the cellular membrane and facilitates their uptake. The protonatable units of PEI have a buffering capacity resulting in an influx of hydrogen ions into the endosomes upon polyplex uptake. Due to osmotic pressure thus building up, the endosomes are disrupted resulting in complex release into the cytosol [222, 225]. This mechanism of escape from the endosomes is called the proton sponge mechanism [226].

The group of Lisziewicz developed a therapeutic HIV vaccine, DermaVir, consisting of PEI-mannose complexed with pDNA encoding several HIV-1 antigens [227]. The vaccine was administered using a patch (DermaVir Patch) and was meant to target Langerhans cells (LCs) [174]. The vaccine induced specific and long lasting immune responses resulting in reduced viral load in SIV-infected macaques [228]. The safety of the vaccine formulation and of the delivery method was demonstrated in a phase I clinical trial in humans. A phase II

clinical trial started in 2009 and aims at evaluating immunogenicity and efficacy of the vaccine in treatment-naïve and HAART-treated patients [229, 230].

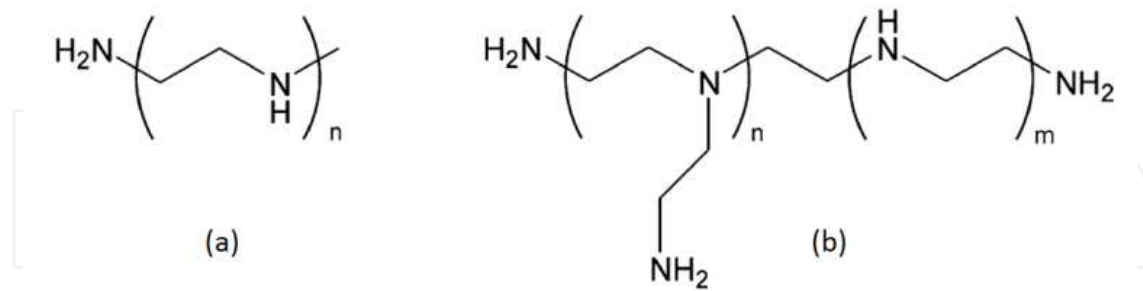


Figure 6. Structures of polyethylenimine: (a) Linear backbone. (b) Branched backbone.

The main drawback of using PEI is its toxicity and non-specific interactions with cellular compartments. Another challenge is the aggregation of polyplexes in the presence of serum.

Rejman *et al.* reported that complexes made of mRNA and cationic lipids are much more efficient in transfecting different cell lines than PEI polyplexes. The authors suggest that this was likely due to the strong affinity of the polymer to mRNA impeding the release of the nucleic acid from the complexes. [191]. Bettinger *et al.* tested the potency of different protein derivatives for the delivery of mRNA. They demonstrated that complexing mRNA with short peptides results in better transfection efficiencies, likely due to a weaker electrostatic interaction with the nucleic acid. It should be noted, however, that the expression levels obtained were in general very low [192].

6.3. Lipid-based antigen delivery systems

6.3.1. Liposomes

Liposomes are spherical entities consisting of a phospholipid bilayer and an aqueous inner compartment. They have been used for drug delivery to treat cancer and infectious diseases. Until now, few liposomal formulations reached the pharmaceutical market of the U.S.A. Liposomes have been also employed to deliver antigens [231]. Given the liposome structure, the antigen can be encapsulated in its core (hydrophilic molecules) or accommodated within the lipid bilayer (hydrophobic molecules) [232]. It has been demonstrated that the lipid composition determines the immunogenicity of liposomes. For example, the incorporation of cationic lipids has been shown to elicit elevated CTL responses compared to neutral or anionic lipids [233]. Liposomal particles can be modified with specific ligands or antibodies to improve the uptake or to enhance/skew the immune response. Virosomes or virus like particles that consist of functional viral envelope proteins, anchored in a lipid membrane, have proven to be promising vaccine candidates [234]. Importantly, antigenic proteins encapsulated in liposomes can elicit both MHC I and II mediated immune responses as demonstrated by Zheng *et al.* [235]. These authors showed that HIV-1 Gag, Pol and Env proteins delivered to DCs by cationic liposomes induced stimulation of HIV-1 specific CD8⁺ T cell responses. This was not observed when DCs were pulsed with the soluble proteins. It

has been reported recently that mice immunized with Gag protein encapsulated in liposomes functionalized with lipid A induced both humoral and cellular immunity [236]. Another strategy to increase immunogenic potential of liposomal formulations involves the use of so-called lipopeptides. These structures consist of lipids linked to peptides that contain potential CD4⁺ and CD8⁺ T cell epitopes. These vaccines aim at the induction of strong T cell responses in HIV-1 infected patients [237-239]. Currently, a lipopeptide-pulsed DC vaccine is under evaluation in a phase I/II clinical trial in HAART-treated patients chronically infected with HIV-1 [240].

6.3.2 Cationic lipid based lipoplexes

The combination of positively charged lipids and negatively charged nucleic acids results in spontaneous formation of complexes called lipoplexes (**figure 7c**). These systems typically consist of two lipid species: a cationic lipid (such as DOTAP - 1,2-dioleoyl-3-trimethylammonium-propane) and a helper lipid with long unsaturated fatty acid chains (such as DOPE - dioleoylphosphatidylethanolamine) (**figure 7**). New cationic lipids are being synthesized almost daily aiming at introducing additional positive charges and reducing toxicity. Helper lipids are introduced to facilitate endosomal escape of these complexes [241].

The positive charge of lipoplexes promotes their cellular uptake, which was found to occur via clathrin-dependent and independent endocytosis [243-245]. The route of lipoplex uptake is determined by their size, chemical nature of the lipids and the cell line. After being internalised, the lipoplexes are located in endosomes. Escape from these compartments probably occurs via a mechanism proposed by Xu and Szoka [246]. It implies formation of neutral molecular pairs of lipoplex-derived cationic lipids and negatively charged phospholipids present in the endosomal membrane, eventually leading to release of the nucleic acid into the cytoplasm.

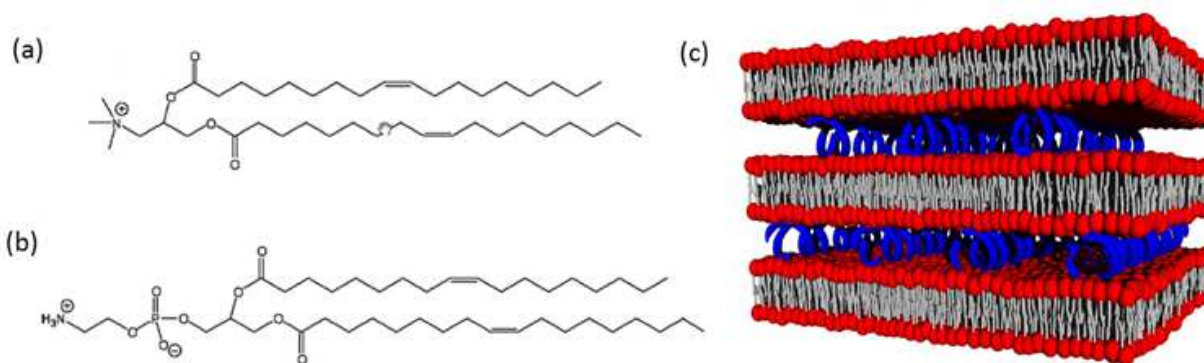


Figure 7. Chemical structures of DOTAP, cationic lipid, and DOPE, neutral helper lipid, used to form lipoplexes [213, 242]. DOTAP (a) and DOPE (b) contain a hydrophilic head group, a glycerol linker and two hydrophobic fatty acid chains. (c) Schematic representation of a lipoplex containing nucleic acids (blue) stabilized by bilayer of lipids: hydrophilic groups (red), hydrophobic fatty acid chain (grey).

Lipoplexes are mainly used to transfect primary cells or cell lines *in vitro*. However, since the rise of DNA vaccines, cationic lipids are considered as a versatile method to replace the hazardous viral vectors. In mice DNA lipoplexes have been shown to induce antitumor activity [247] and both antibody and Th1 cellular responses against hepatitis C virus [248, 249]. In the late eighties the first transfection with mRNA-lipoplexes was performed [250]. Generally it is established that mRNA transfection using non-viral cationic carriers is more efficient with cationic lipids than with cationic polymers [191, 192, 251, 252]. For both types of mRNA complexes the onset of protein expression was faster, but also lasted shorter than that produced by pDNA-complexes [191, 252]. The potential of mRNA-lipoplexes as an anti-cancer or influenza vaccine dates back to the nineties [253, 254]. Since then most anti-cancer and anti-virus immunotherapies have been focused on the *ex vivo* loading of DCs mainly by means of electroporation [109, 196, 198, 255, 256].

One of the most serious challenges of the use of lipoplexes is the extrapolation from the *in vitro* to the *in vivo* situation. Optimal transfection conditions are maintained *in vitro*, while *in vivo* lipoplexes encounter serum proteins causing their aggregation [257]. Depending on the target cells or tissue, this aggregation can either negatively or positively affect the uptake of the lipoplexes [258, 259]. Additional coating of the lipoplexes with polyethyleneglycol (PEG) can prevent the formation of aggregates [260], yet brings along other problems. Concerns have been raised that PEGylation of lipoplexes may be unfavorable in the context of vaccination as this could inhibit uptake of nanoparticles by APCs and negatively interfere with the release of complexes from the endosomal compartment [213]. However, a study performed by Singh and colleagues demonstrated that subcutaneous immunization of rabbits with PEGylated lipoplexes carrying a synthetic gp41 epitope of HIV-1 induced two times higher immune responses and prolonged persistence of antibodies than liposomes carrying epitopes without PEG moieties [261].

6.4. Targeting of DCs

Conceptually, particulate vaccines mimic the particulate nature of pathogens, including the size (nano- to micro-meter range), which facilitates their uptake by APCs. The actual size of the particles influences the uptake mechanism by APCs and the way of antigen presentation. Generally, larger particles are predominantly internalised via phagocytosis or macropinocytosis, while smaller particles are taken up by other endocytic mechanisms [262-264]. Internalisation through phagocytosis is known to lead to antigen cross-presentation in DCs [265, 266]. This emphasizes the enhanced potential of particulate antigen delivery to induce cellular immune responses as compared to soluble antigens, which are poorly cross-presented and preferentially presented via the MHC class II pathway.

Most nano- or microparticles can be functionalized with ligands or antibodies directed against cell surface receptors to target specific tissues or cells [267]. DCs express lectin-like receptors such as mannose receptor, DEC-205 and DC-SIGN which are believed to be involved in the phagocytosis of pathogens [268]. One way of increasing or promoting particle uptake by DCs is the attachment of mannose groups recognized by the mannose

receptors present on DCs and macrophages. This has been shown to increase transfection efficiency of DNA-based vaccines [269, 270], antigen presentation following protein delivery [271] and the induction of cellular T cell responses in cancer and HIV immunotherapeutic strategies using lipoplexes and polyplexes [228, 270]. Another way of targeting is the use of ligands or antibodies directed against DEC-205 or DC-SIGN, which have shown potential to improve antigen uptake by immature and mature DCs [272-275]. It should be kept in mind, however, that attaching the desired ligand or protein is not always a straightforward process. The engraftment of chelator lipids on the surface of liposomes or lipoplexes, such as histidine tags, is crucial to ensure the functionality of the coupled target molecule [276-278]. In this context, the use of nanobodies directed against DCs could be of particular interest as they are mostly generated with a histidine tag for purification purposes. Alternatively, ligands could also be linked to liposomes via palmitoylation of the ligand [279].

In summary, targeting of particles may not only enhance efficacy but also the specificity of interaction with the surface receptors on DCs [179]. Moreover, coupling of specific adjuvants (e.g. TLR ligands), with the aim to promote the desired immune response, can also positively influence the uptake by DCs [280].

6.5. Co-delivery of antigens and adjuvants to improve immunogenicity of DCs

Although particulate antigen delivery improves antigen uptake and presentation by DCs, most particles are not immunogenic by themselves. This offers a possibility to use a specific adjuvant that can skew towards the desired type of immune responses [187]. Despite some positive results in animal models, one should be aware of the toxicity of potential immunomodulators in humans, that jeopardizes their clinical use [281]. Aluminum salts (referred to as alum) are the oldest and most widely used adjuvants for human vaccines. It has been shown that antigens can be precipitated with alum to form colloid particles, creating a depot effect after vaccination, that elicits strong humoral immune responses [282]. These results, together with further observations clearly showed that the physical linkage of antigen and immunomodulator is crucial to induce strong immune responses [182, 183, 283].

Immunostimulating complexes (ISCOMs) are another interesting delivery system. It is a sort of liposomal delivery vehicle with a built-in adjuvant. They are composed of a protein antigen, phospholipids and the saponin Quil A adjuvant, derived from the bark of the *Quillaja saponaria*, a South American tree. It has been demonstrated that the use of ISCOMs improves CTL responses of influenza virus based vaccines [284, 285]. ISCOM-based vaccines have been tested in animal models and in clinical trials against cancer [286] and viral infections [287-289] [290, 291]. In all studies satisfactory safety and tolerance were shown and both humoral and cellular responses were induced.

Several studies have reported the use of ISCOMs as a system to deliver HIV or SIV antigens. In non-human primate models, incorporation of HIV or SIV peptides into ISCOMs has been shown to induce protective immunity [288, 292, 293]. Moreover, studies in mice demonstrated that ISCOMs can be used to elicit immune responses against HIV-1 antigens [287]. To generate mucosal immunity, Koopman and colleagues immunized rhesus

maquages intranasally or via lymph nodes with HIV-1 peptides formulated into PR8-Flu ISCOMs [294]. Intranodal injection of these ISCOMs induced strong systemic and mucosal immune responses. In contrast, intranasal application resulted in very weak responses. Currently, ISCOM-based vaccines have been approved for veterinary use and are undergoing clinical trials for human use [295].

As described before, for an optimal immune response it is crucial that DCs, besides actively taking up the antigen, undergo activation and maturation to stimulate effective T cell responses. Ligands mimicking PAMPs that can target PPRs are therefore of potential interest. Extracellular and intracellular PPRs are divided into four groups: TLRs, NLRs, CLRs and RLRs [181]. Depending on the receptor that is triggered, DCs produce and secrete various sets of cytokines, determining the type of immune response [296]. Moreover, the incorporation of TLR ligands promotes phagocytosis of APCs [280]. TLRs represent the majority of PPRs studied in the development of effective adjuvants [281]. TLRs can be divided into two groups: the surface bound receptors (TLR 1, 2, 4, 5, 6) and the intracellular receptors present in the endosomes (TLR 3, 7, 8, 9) (**table 4**).

TLR	TLR ligand	DC subset	Effect of activation
1:2	Triacyl lipoproteins	Mo-DCs, myeloid DCs	Upregulation of CCR7, IL-6, IL-10, IL-12p70, TNF- α
2	Lipoproteins	Mo-DCs, myeloid DCs	Upregulation of CCR7, IL-6, IL-10, IL-12p70, TNF- α
3	Double stranded RNA	Mo-DCs, myeloid DCs	IFN- α/β activation and up-regulation IL-12p70
4	Lipopolysaccharide	Mo-DCs, myeloid DCs	Upregulation of CD80, CD86, CD83, CCR7. Secretion of IL-6, IL-8, IL-10, IL-12p70, IFN- β
5	Flagellin	Mo-DCs, myeloid DCs	Upregulation of CD80, CD86, CD83, CCR7. Secretion IFN- α/β , IL-1, TNF- α , IL-8, IL-12p40
6:2	Triacyl lipoproteins	Mo-DCs, myeloid DCs	Upregulation of CCR7, IL-6, IL-10, IL-12p70, TNF- α
7	Single stranded mRNA	pDC, myeloid DCs	Upregulation of CCR7, CD40, CD80 and CD86. Secretion of IL-12p70 (myeloid DCs). Secretion of IFN- α (pDCs).
8	Single stranded mRNA	Mo-DCs	Increased TNF, IL-8, IL-12p40, MCP-1, CCL2, CCL3, CCL4, CCL5
9	Double stranded DNA	pDC	Upregulation of CD40, CD80, CD86, CD83, HLA-DR, CCR7. Upregulation of IFN- α (very high), IFN- β (lower), IL-6, TNF- α (low), IL-8

Table 4. Overview of Toll like receptors (TLRs) with their ligands and effect of activation [297].

The extracellular TLRs mainly recognize bacterial invaders, but also fungi and some enveloped viruses. Bacterial lipoproteins are recognized by the heterodimers TLR1:2 (triacyl lipoproteins) and TLR2:6 (diacyl lipoproteins). Liposomes engrafted with palmitoyl chain lipopeptides have been shown to up-regulate DC maturation markers *in vitro* [298]. Lipopolysaccharide (LPS), a gram negative bacterial carbohydrate, is recognized by TLR4. Recently, a phase I clinical trial has started evaluating the potential of autologous dendritic cells, *ex vivo* loaded with lipopeptides and activated with LPS, as an immunotherapy in HIV infected patients (NCT00796770). Lipopolysaccharide (LPS), a gram negative bacterial carbohydrate, is recognized by TLR4. Since the *in vivo* use of LPS for human purposes is not possible due to the risk of septic shocks, a derivative, monophosphoryl lipid A from *Salmonella Minnesota* referred to as MPLA, was proposed as a substitute. It has been shown to be less toxic than LPS and thus preferable for human vaccine applications. MPLA has been incorporated in PLGA particles and liposomes [299, 300]. Furthermore, MPLA is already used in combination with alum (AS04) as an adjuvant in Cervarix®, a human papilloma virus vaccine from GlaxoSmithKline. Another bacterial component, flagellin, is recognized by TLR5 and is also used as a potential adjuvant. Flagellin-related peptides containing a His-tag and incorporated in liposomes containing a tumor antigen, induced antitumor responses resulting in complete tumor regression in mice [278]. The advantage of flagellin is that it can be co-integrated as a protein into particulate vehicles or integrated as His-tagged peptides into liposomes.

The intracellular TLRs specifically recognize nucleic acids and are of major importance to the recognition of double stranded DNA (dsDNA) from viral or bacterial intruders. TLR-9, only expressed in pDCs, recognizes unmethylated cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODNs). CpG motifs were demonstrated to induce cell-mediated responses *in vivo* when incorporated into PLGA particles [301, 302], liposomes [303] and other microparticles [304]. All these studies reported enhanced Th1 biased immune responses. Viral single stranded RNA (ssRNA) is recognized by TLR7 (expressed by all DC subsets and B-cells) and TLR8 (expressed by macrophages, monocytes and myeloid DCs) [181]. Agonists used to trigger these receptors are the synthetic imidazoquinoline drug compounds imiquimod and resiquimod (R848) or polyuridylic acid (polyU). Only few studies so far have used TLR7/8 ligands for particulate antigen delivery. Johnston *et al.* demonstrated that the combination of TLR 7 ligand with liposomes increased the cellular immune responses against OVA protein in a mice model [305]. Another study reported that co-encapsulation of OVA antigen and polyU in PLA-DOTAP microparticles increased the antibody titers and numbers of IFN- γ secreting T cells [306]. Finally, the endosomal TLR3 recognizes dsRNA, which can be mimicked by the synthetic polyinosinic:polycytidylic acid (poly I:C). This dsRNA analog is widely evaluated as a potential adjuvant since it strongly promotes the maturation of DCs [184, 204]. Furthermore, the combination of poly I:C with protein antigens encapsulated in either polymeric [183, 184, 307] or liposomal [308] particles favors enhanced Th1 immune responses. With regard to mRNA vaccination, the use of endosomal TLR ligands is problematic, since upon recognition by their receptor, high levels of type I interferon are produced leading to the activation of RNases [309, 310]. However, mRNA by itself can also trigger endosomal TLRs, implying the crucial importance of stabilizing the antigen encoding mRNA (reviewed by Tavernier *et al.*) [189].

Another possibility to improve immunogenicity is the addition of co-stimulatory molecules. The addition of CD40L to DCs loaded with liposome-complexed HIV-1 proteins could prime HIV-1 specific CD8⁺ T cells *in vitro* [162]. Within the scope of mRNA transfection, mRNA encoding co-stimulatory molecules can be co-transfected with mRNA encoding an antigen, which has been shown to strongly enhance the capacity of electroporated DCs to stimulate HIV-specific T cell responses [311].

7. Concluding remarks

Ex vivo loading of DCs shows promising results as an immunotherapy for HIV and cancer. However, the currently applied strategy is not applicable on a large scale, especially in Africa where the largest incidence of HIV infections is observed. The use of particulate vehicles able to directly deliver the antigen of interest, be it proteins, peptides or nucleic acids, into DCs *in vivo* is a very attractive concept. Different antigen formulations can be further tailored with targeting antibodies or immunomodulatory ligands to promote uptake by DCs and to trigger the desired type of immune response. In the context of HIV immunotherapy a large number of epitopes needs to cover a broad panel of quasi species. Therefore, mRNA-based vaccination strategies present an attractive option. In contrast to pDNA, mRNA needs to be delivered only into the cytoplasm and induces transient antigen expression without the risk of genomic insertion. Further research will be required for designing an optimal carrier system preferably comprising an adjuvant, necessary to achieve strong HIV-specific CTL responses with the ultimate goal to control viral replication in the absence of additional HAART therapy.

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