Role of Dendritic Cells in HIV-Immunotherapy

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Abstract: HIV remains one of the most important deadly infections today, due to the lack of a preventive vaccine and limited access to medical care in developing countries. In developed countries, antiretroviral therapy is available, but it can not eliminate the virus, implying that life-long therapy is necessary. Therefore, it is important that other strategies such as therapeutic vaccination will be developed to control virus replication or even eliminate the virus. The major obstacles towards such a strategy are the huge variability of the virus and the profound HIV-induced immune dysfunction. In this review we focus on dendritic cell based immunotherapies against HIV.

To develop an efficient immunotherapy, several elements should be taken into account such as which antigen and loading strategy to use, how to deliver the immunogen, how to optimize the interaction between antigenic peptide and T cells and avoid tolerance. Clearly, to develop an immunotherapy to complement the effect of HAART, it is not sufficient to enhance T cell responses against a consensus sequence or against the prevailing plasma virus. Broad and potent immune responses are needed to suppress the entire quasispecies, including the latent reservoir, and to prevent any escape.

Keywords: dendritic cells, immunotherapy, HIV, mRNA.

RATIONALE FOR HIV-1 IMMUNOTHERAPY

Human immunodeficiency virus (HIV)-1 is widespread and has been the cause of millions of deaths. The current standard treatment, highly active antiretroviral therapy (HAART), has reduced the morbidity and mortality in the developed world [1]. However HAART has also some limitations. First and foremost, patients in developing countries still have only limited access to treatment. Second, even in properly treated subjects, important side effects result in poor quality of life and/or suboptimal adherence, often leading to resistance development or even discontinuation of treatment [2, 3]. Finally, HAART can completely suppress viral replication, but fails to eradicate the virus reservoirs and is unable to completely restore CD4+ T cell function. Altogether there is an urgent need for alternative therapies that are complementary to HAART (i.e. able to eradicate the virus) or could be substituted for HAART (i.e. able to keep the virus suppressed after stopping HAART). In this review we will focus on dendritic cell (DC)-based immunotherapies. The goal of therapeutic vaccination is to "re-educate" the immune system in order to maintain potent and effective immune responses against the virus both systemically and mucosally [4]. Therefore, an efficient immunotherapy requires the use of an appropriate target antigen and loading strategy, optimization of interaction between antigenic peptide and the T cell, and prevention of negative regulatory elements [5].

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CORRELATES OF PROTECTION: CLUES FOR EVALUATION OF THERAPEUTIC VACCINES

To develop an effective immunotherapy, it has become urgent to know which factors confer protection against HIV and disease progression. However, the precise correlates of protection remain unknown, but important lessons can be drawn from the study of elite controllers (EC, HIV-infected people who present with undetectable viral load), long-term non-progressors (LTNP, who show no clinical evolution after more than 10 years of infection), and most persons infected with HIV-2 [6-8] (Table **1**).

First, it is possible that the virus itself and not the immune system of LTNP is responsible for non-progression. This is probably the case for HIV-2, since it is well known that the majority of subjects infected solely with HIV-2 have an "LTNP" or even "EC" profile, while those infected with HIV-1 or with both HIV-1 and HIV-2 in the same population almost invariably have high viral load and show progression [9]. Our group and others have convincingly shown that all HIV-2 strains tested were indeed less replication-competent ("less fit"), as compared to all HIV-1 subtypes tested [10]. A similar case was shown in the LTNP cohort of hemophiliacs in Sydney, who were infected with blood products from a single HIV-1 (+) donor. This particular virus had a deletion in *nef*, which profoundly reduced its replication capacity [11]. Furthermore, Blankson and colleagues reported isolation and culture of replication–competent HIV virus from a large number of elite controllers [12]. However, in all these cases of EC/LTNP, protection is not complete, because some patients develop AIDS after varying amounts of time

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Table 1. Potential Mechanisms for Virus Control in HIV "Controllers"

Factor	Cohort	Factors Associated with Protection
Viral factors	LTNP	Deletion in nef [11]
Host factors	LTNP	HLA-B57 and HLA-B27 [14-16, 149]
Innate Immunity	EC	HLA-B57+ loss of NK inhibitory signal KIR3DL1, lysis of infected cells could ensue [16, 34, 150]
Adaptive immunity	LTNP/EC	Polyfunctional and proliferative HIV-specific CD8+ T cells [21-23]
	LTNP	Avidity of HIV-specific CD8+ T cells [24]
	LTNP/EC	Strong and polyfunctional CD4+ T cells (IFN-γ and IL-2) [151]
	EC	Low immune activation [26, 38]
	HIV-2	Proliferative polyfunctional CD4+ T cells (IFN-γ and IL-2) [29, 30]

[13]. Altogether, these observations provide evidence that infection with an attenuated virus may be the main reason for life-long control during HIV-2 infection, but only exceptionally in HIV-1 infection.

It has been well established that host genetics, including human leucocyte antigen (HLA) polymorphisms, have a role in controlling the virus. In HIV-1 elite controllers, people with HLA-B57 and –B27 are overrepresented [14-16]. This observation suggests a causal role for the adaptive immune system because HLA molecules are involved in CD8+ T cell-mediated recognition of virus-infected cells [17]. Moreover, several studies have examined correlations between the magnitude and/or number of HIV-specific CD8+ T cells and viral control [18, 19]. Until now, no clear associations were found between these parameters and the elite controllers or LTNP status. Besides the quantitative aspect, also the functionality of HIV-specific CD8+ T cells can be correlated with virus control. HIV-specific CD8+ T cells from elite controllers and LTNP are polyfunctional, meaning that they proliferate vigorously upon encounter with antigen and that they simultaneously produce multiple cytokines [20-23]. In contrast, HIV-1-specific CD8+ T cells from progressors show a more restricted functional capacity presumably because they are exhausted due to persistent immune activation [24]. These data suggest that polyfunctional HIV-specific CD8+ T cells with proliferative capacity are related with HIV control. Recently, it was shown that also the lytic capacity of HIV-specific CD8+ T cells is important for viral control. Migueles *et al.* have demonstrated that HIV-specific CD8+ T-cells of LTNP efficiently eliminate primary autologous HIV-infected CD4+ T cells. This function required lytic granule loading of effectors and delivery of granzyme B to target cells [25]. Moreover, Saez-Cirion *et al.* demonstrated that HIV-specific CD8+ T cells from elite controllers strongly suppress *ex vivo* HIV-infection of autologous CD4+ T cells [26].

Another important host factor that modulates control is the functional state of the CD4+ T cells. Rosenberg and colleagues demonstrated that the overall functional CD8+ T cell activity was in fact dependent on the presence of proliferative CD4+ T cell responses [27]. Later on, studies from Harari [28] and Rowland-Jones [29, 30] on LTNP and HIV-2- infected individuals indicated that polyfunctional CD4+ T cells (defined as proliferative interferon (IFN)-γ- and interleukin (IL)-2-producing) were important for HIV control.

The possibility that HIV neutralizing antibodies have a role in viral control has been suggested [31, 32]. However, in most studies, the level of neutralizing antibodies in the plasma was more correlated with duration of infection than with clinical stage. In fact, sometimes elite controllers even showed the lowest level of neutralizing antibodies. These studies argue against humoral immunity as a major mechanism for control during an established infection [33]. Clearly, whereas induction of neutralizing antibodies remains a goal for preventive vaccination, it is probably not a useful aim or correlate for protection upon therapeutic vaccination.

Next, there is some evidence that innate immunity plays a role in viral control and more specifically the natural killer cells. As already mentioned above, HLA-B57 plays a role in viral control by the adaptive (CD8+ T cell-mediated) immune system but it also plays a role in innate immune protective mechanisms. HLA-B57 is one of the Bw4 alleles, which are natural ligands of KIRDL1, an inhibitory killer immunoglobulin-like receptor (KIR). HIV-infected cells downregulate the expression of HLA-B57, which results in a loss of the NK inhibitory signal mediated through KIR3DL1 because HLA-B57 cannot bind to its ligand [34]. As a consequence, lysis of infected cells ensues, and therefore this genetic constitution is associated with natural virus control.

Finally, also the level of immune activation in HIV-infected individuals is important for pathogenesis. HIV induces a strong inflammatory response, part of which may be anti-virally oriented and thus beneficial. However, the system seems to go in "overdrive", with ill-directed activation, which has a rather deleterious effect. Activated CD4+ T cells serve as the primary target of HIV and thus accelerate HIV disease progression simply by enhancing the ability of the virus to replicate. Chronic non-specific T cell activation leads to T cell exhaustion, which is characterized by the upregulation of programmed death receptor (PD)-1. This cell surface molecule is generally upregulated in both CD4+ and CD8+ T cells in HIV-infected progressors [35-37] but not in controllers [38]. Another factor that can predict disease outcome independent of CD4+ T-cell counts is the level of urokinase-type plasminogen activator receptor in serum (suPAR). It was shown that high levels of suPAR correlate with disease progression [39].

Altogether, none of these factors alone predicts protection, but it seems that several interrelated characteristics of CD4+ and CD8+ T cells play a role. Therefore, the question remains which factors should be measured to evaluate the immune response after vaccination?

A negative lesson can be taken from the unsuccessful STEP HIV vaccine trial. The trial was stopped because the candidate vaccine, based on a recombinant adenovirus serotype 5 (Ad5), not only failed to protect Ad5-seronegative individuals against infection, but even may have enhanced infection in vaccinees with prior exposure to adenoviruses [40, 41]. In this phase II trial, the immunogenicity was measured by IFN-γ ELISPOT and polychromatic flow cytometry (IFN-γ, CD107a, MIP-1β, TNF-α, IL-2) [42]. Although long-lasting polyfunctional (i.e. producing at least 4 of the 5 factors mentioned above) immune responses were induced, they were not considered as broad T cell responses, meaning that only a limited amount of epitopes was targeted.

In contrast, the DNA/NYVAC prime boost regimen was based on vaccinia virus induction of HIV-specific CD4+ and CD8+ T cell responses that are polyfunctional; these cells contain much more IL-2 than those induced in the STEP HIV trial [43]. In addition, well-established protective vaccines such as cowpox, yellow fever and hepatitis A were recently shown to successfully elicit broad integrated responses that encompass all effector arms of the immune system [44-47].

If we can extrapolate these results from the prophylactic field to immunotherapy, not only IFN-γ should be measured but also the quality and the breadth of the immune response. The quality of the immune response should be evaluated by polychromatic flow cytometry, possibly by the avidity (ability to respond to low levels of antigenic peptides) and perhaps even more importantly, by the capacity of the CD8+ T cells to inhibit *in vitro* viral replication in autologous CD4+ T cells. The latter assumption is based on results which pointed out that CD8+ T cells from elite controllers could inhibit viral replication in autologous CD4+ T cells whereas progressors failed to do so [26].

DENDRITIC CELLS AS ANTIGEN-PRESENTING CELL IN IMMUNOTHERAPY

Several immunotherapy strategies have been proposed and attempted, including the systemic injection of cytokines such as IFN-α or IL-2 or of inactivated "standard" HIV strain [48-50], but they have met with limited if any success, maybe because they are not antigen-specific (e.g. cytokines) or not very much patient-specific (e.g. inactivated HIV). To develop a more "personalized" immunotherapy, the principle is to generate *ex vivo* a population of specific HIV antigen-loaded antigen-presenting cells (APC) that, after injection *in vivo*, should induce high quality and long-lasting (i.e. memory) CD4+ and CD8+ T cell responses. The APC used thus far include dendritic cells (DC), CD40-activated B cells or B lymphoblastoid cell lines. These APC can be loaded with viral antigens using several methods, including viral transduction, peptide/protein pulsing, uptake of virally infected apoptotic cells or transfection with non-viral DNA or mRNA [51-54]. In this review the use of DC, regarded as the most potent APC for immunotherapy will be discussed in more detail.

DC constitute a heterogeneous population of cells located in the blood, peripheral tissues and lymphoid organs. There are many DC subsets with distinct phenotypic and functional characteristics (out of the scope of this review), but all are derived from CD34+ hematopoietic progenitor cells and have the capacity to prime antigen-specific T cells (see below). Given the low number of DC available in the circulation for use in *ex vivo* therapy, there is a need for other sources to obtain sufficient DC for immunization purposes. Currently, two methods are available, one starting from bone marrow precursor cells and the other from peripheral blood monocytes. Nowadays, monocytes are most often used as the source of DC, because the differentiation phase is more rapid and they are more readily accessible than bone marrow progenitor cells [55, 56]. Although these *in vitro* generated DC have no exact phenotypic counterpart *in vivo*, they rather faithfully mimic the functional characteristics of "average" DC *in vivo*.

One concern about the use of monocytes as precursor for DC is their susceptibility to HIV-1. Although monocytes can be infected *in vivo*, it was recently shown that circulating monocytes from HAART-treated individuals are non-permissive to HIV infection *in vivo* [57]. If vaccination happens under the coverage of HAART, the risk for transmission of HIV between CD4+ T cells and DC should be minimal. Moreover, we and others have shown that mature DC (mDC) from both HAART-treated and untreated individuals have comparable abilities to process antigens and stimulate HIV-specific T cells [58]. These data support the idea of using monocyte-derived DC from HAART-treated HIV-1-seropositive individuals in immunotherapeutic settings against HIV-1.

In vivo activation of DC occurs in three stages: antigen uptake, maturation and licensing [59]. Immature DC take up antigens in the periphery through endocytosis. Afterwards a maturation process is induced in response to danger signals such as inflammatory stimuli, originating from pathogen-associated molecular patterns (PAMP), from dying cells or from soluble and membrane-bound forms of the tumor necrosis factor (TNF) family [60]. This maturation is characterized by upregulation of MHC class I, II, T cell costimulatory molecules (e.g. CD80/CD86) and loss of the capacity to take up antigens [61]. DC also upregulate the expression of the CC chemokine receptor 7 (CCR7) and become responsive to CCL19, 21 and other chemo-attractants. Due to these signals, DC can home to lymph nodes. In the lymph nodes mature DC present their antigen to naive T cells in the context of MHC class I and II molecules and undergo licensing. This process is characterized by cross-linking of CD40 on the DC with CD40L, which is expressed on antigen-activated CD4+ T cells [62]. Licensed DC upregulate additional cell surface products such as ligands for OX40 and CD137 and secrete cytokines promoting CD4+ T cell differentiation into T helper (Th)1 (Fig. **1**), Th2 or regulatory T (Treg) cells [63]. For the generation of Th1 responses IL-12 production is important, while IL-4 is important for Th2 generation. IL-10 production has a negative influence on both Th1 and Th2 responses [64]. Licensed DC can also present antigen to

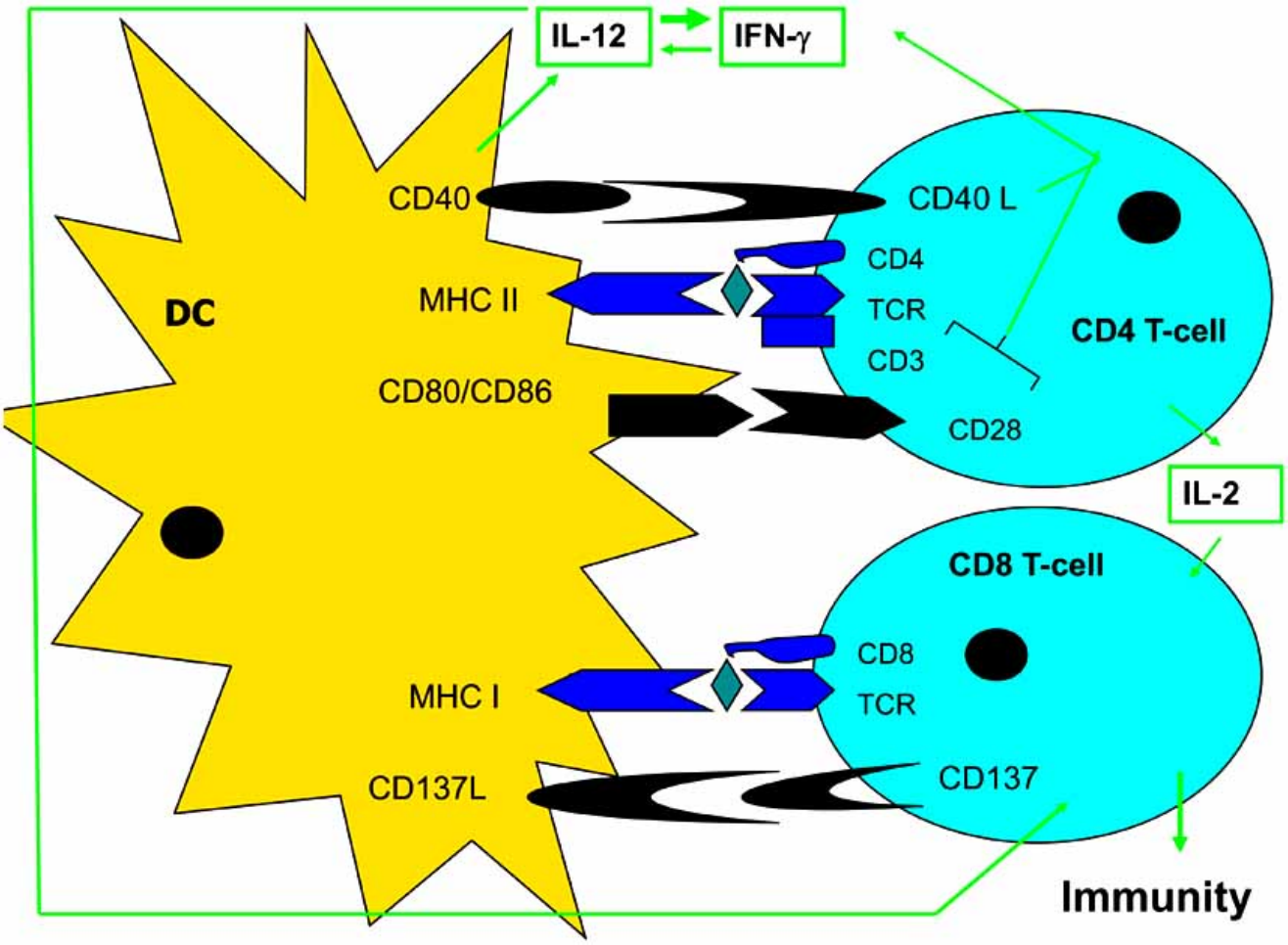

Fig. (1). Interaction of DC with T cells. To generate an effective immune response several steps are necessary. DC take up antigens and present the processed peptides *via* MHCI or MHCII to CD8+ or CD4+ T cells. For efficient activation of CD4+ T cells, the DC expressed CD80/86 have to provide a "second signal" *via* CD28. Properly activated CD4+ T cells upregulate CD40L that will interact with its receptor on DC, inducing the production of IL-12 by DC. As a consequence of all these signals CD4+ T-cells will produce IFN-γ and IL-2. These cytokines are important to induce cytotoxic T-cells. CD137 ligation has a positive effect on the induction, amplification and persistence of the CD8+ T cell immune response.

CD8+ T cells, which differentiate into cytotoxic T lymphocytes (CTL). The survival and proliferative capacity of activated CD8+ T cells can be improved through CD137L costimulation [65, 66]. Likewise, OX40L-mediated costimulation enhances the survival and proliferation of activated CD4+ T cells.

DC MATURATION FOR OPTIMAL INTERACTION BETWEEN DC AND T CELLS

HIV infection is associated with a gradual loss of immune competence in CD4+, CD8+ T cells and APC, leading to increased susceptibility to infection. Primarily, dysfunction of CD4+ T cells is manifested by their low IL-2 production, resulting in poor proliferative capacity. Nevertheless these dysfunctional CD4+ T cells are in an "over-activated" state, which increases their susceptibility to HIV. They are gradually depleted due to direct cytopathic effects of the virus [67, 68], to activation-induced apoptosis [69, 70] and to bystander apoptosis [71-74]. Due to dysfunction and depletion of CD4+ T cells, CD8+ T cells do not receive appropriate T cell help, which is required for their full maturation [3, 75]. As a consequence, CD8+ T cells in HIV patients exhibit dysmature properties. Moreover, they are exhausted due to persistent antigenic stimulation [76]. Finally, the APC are defective. More specifically, the capacity of CD4+ T cells to upregulate CD40L following stimulation through T cell receptor (TCR) becomes progressively impaired, in parallel with the overall immune suppression [77, 78]. As discussed, above engagement of CD40L with CD40 on the APC is pivotal for full activation ("licensing") of APC (Fig. **1**) [79, 80].

Obviously, the immunostimulatory capacity of DC is very important for an efficient immunotherapy, since the *ex vivo* APC should be able to correct HIV-induced T cell dysfunctions *in vivo*. To this end application of an appropriate maturation cocktail and expression levels of costimulatory molecules are of paramount importance.

A variety of products can be used to induce maturation of DC, including type I (α-β) or type 2 (γ) interferon (IFN), Toll-like receptor (TLR) ligands, members of the tumor necrosis factor (TNF) family, siRNA against suppressors of cytokine signaling (SOCS)1 and other mediators such as inflammatory cytokines, chemokines, prostaglandins (PG) and T cell-derived products (e.g. soluble CD40L). All these products act *via* partly different intracellular pathways and therefore any combination could result in additive, synergistic or antagonistic effects [59].

The gold standard, used in most of the clinical trials up to now, is a cytokine cocktail, consisting of IL-1β, IL-6 and TNF-α, which mimic the natural pro-inflammatory environment [81]. PGE_2 is added to this cocktail because it increases the expression of CCR7 which is important for chemotactic migration of DC to the lymph node. Unfortunately, it also inhibits IL-12p70 production which is important for Th1 skewing [82-84]. Type I interferons (IFN-α and β) are used to imitate an antiviral immune response [85]. Although, IFN-α–matured peptide-loaded DC are very immunogenic [86, 87], this maturation pathway is not compatible with mRNA electroporation of DC for antigen loading, most probably because type I interferons stimulate RNases, inhibiting mRNA translation [88]. TLR ligands can be applied to mimic pathogen-induced maturation of DC [59]. For example poly-inosinic-cytidylic acid (poly-I:C), a synthetic double-stranded RNA molecule that acts as a TLR3 ligand, is often used. Another possibility is to add Resiquimod® (R848) as a TLR7/8 ligand. We (unpublished results) and others [89] have shown that DC matured with R848 are very immunogenic in terms of Th1 skewing and CTL stimulation. Lipopolysaccharide (LPS), a TLR4 ligand, induces antigen-specific cytotoxic T cells (CTL) *in vitro* and *in vivo* in animal models [90, 91]. However, clinical use of LPS is limited due to potential cytotoxicity. Therefore, alternatives were used like transfection of DC with mRNA encoding constitutively active TLR-4 (caTLR-4) [92] and monophosphoryl lipid A (MPLA) [93]. Transfection of DC with caTLR4 induced a mature phenotype and enhanced the generation of CTL specific for melanoma antigen recognized by T cells (MART) [92]. DC matured with MPLA and IFN-γ produce high levels of IL-12p70 upon CD40 triggering and they induce mainly Th1 cells *in vitro* [93].

An additional approach is to inhibit negative regulatory pathways that attenuate DC maturation. One example is SOCS1, this molecule inhibits the JAK/STAT pathway and is a negative regulator of signaling of cytokines such as IFN-γ, IL-2, IL-6, IL-7, IL-12 and IL-15 [94, 95].

To induce effective T cell-mediated immune responses, not only maturation is important but also the interaction between costimulatory molecules present on the APC and T cells. The two best known and studied costimulatory molecules are CD40 and CD137 that are cross-linked by ligands expressed on activated T-cells and DC, respectively. In healthy individuals triggering these pathways lead to T cell survival and proliferative signals [65, 66]. Current strategies to enhance costimulation include the systemic administration of agonistic antibodies or exogenous recombinant proteins or transfection with replication-deficient adenoviruses which carry the gene of interest. Previously, it was shown that DC matured with CD40L trimers could enhance the cytotoxic activity of CD8+ T cells in the absence of CD4+ T cell help *in vitro* and *in vivo* [96, 97]. Unfortunately, DC co-electroporated with HIV antigen and CD40L mRNA could not stimulate HIV-specific CD8+ T cells in our hands (unpublished results). Recently, Jonuleit *et al.* have even shown that DC transfected with CD40L are tolerogenic [98]. Recently, a clinical trial in HIV-infected patients was published, where HIV-infected individuals were injected with DC matured by CD40L [99]. Proliferative responses were seen, however it was not compared with DC matured without CD40L. This indicates that, whereas CD40L can be tolerogenic *in vitro*, it might be immunogenic *in vivo*.

Engagement of CD137L with CD137 receptor prolongs the survival of CD8+ T cells following stimulation with anti-CD3/CD28 [100, 101]. A number of studies in mice have shown that CD137L or anti-CD137 antibodies improve antiviral and antitumor CD8+ T cell responses [100, 102, 103]. In humans monocytes, transfected with replication-deficient adenovirus encoding CD137L and CD80, CD137L and CD70 [103, 104] or OX-40L and CD80 [105], all induced an increased expansion of HIV-specific T cells. In contrast, we showed that costimulation with CD137L, expressed by DC after electroporation with mRNA can only enhance direct *ex vivo* triggering of both influenza and HIV-specific effector T cells producing IFN-γ. However, this strategy failed to improve the *in vitro* expansion of HIV- and influenza-specific T cells as compared to DC loaded only with antigen (unpublished data). These differences can be explained by the mode of gene delivery. Transfection of DC with replication-defective adenovirus encoding CD137L results in constitutive expression of CD137L, whereas electroporation with mRNA encoding CD137L results in expression for a maximum of 24 hours. Although the adenovirus-based strategy is very promising *in vitro*, there are some problems *in vivo* [42]. The majority of people have antibodies against adenoviruses which could dampen the effect and, as shown in the STEP trial, adenoviruses may stimulate CD4+ T cells, which are potential targets for HIV infection [42].

Altogether, perfectly conditioned DC for use in immunotherapeutic settings have not yet been developed. Ideally, these DC should be programmed to manifest their maximal migratory capacity immediately after injection and subsequently their maximal T cell stimulatory capacity after arrival in the regional lymph nodes. It is presently unclear through which *ex vivo* manipulations such a fine-tuning can be obtained.

ANTIGEN LOADING STRATEGIES FOR DC

The nature of the antigen, the quantity of antigen to load, the efficiency of loading and duration of antigen persistence and presentation are all important criteria that should be considered in the development of an efficient immunotherapy.

Antigen can be provided to DC in many formats. Primarily, antigens can be delivered exogenously as peptides, whole proteins or apoptotic cells. Secondly, the antigen can be delivered in a genetic format by transfecting DC with antigen-encoding viral vectors, DNA or mRNA.

The use of antigenic peptides is an efficient loading strategy, but is restricted to a limited number of HLA-restricted epitopes. Alternatively, DC can be pulsed with whole recombinant HIV-1 proteins [106]. In principle, this strategy should preferentially induce MHC class II-restricted CD4+ T cells. However, DC are able to "cross-present" proteins into the class I pathway and it has indeed been shown that *in vitro* CD8+ T cell responses can be induced. Including many variants of each antigenic HIV protein seems technically not feasible, however. The use of whole inactivated HIV-1 particles is an approach that has already been successfully translated in clinical trials [107-110]. It was shown that DC pulsed with autologous inactivated virus could stimulate CD8+ T cell responses *in vitro* [111] and *in vivo* [107, 109, 110]. Other approaches included the use of apoptotic or necrotic preparation of HIV-infected T cells to load DC [54, 108]. In this case DC take up exogenous antigens, followed by processing through nonconventional pathways and cross-presentation of antigen in the context of MHC I to CD8+ T cells [54]. Also live HIV-1-infected cells were used in some reports [112, 113]. In all cases both CD4+ and CD8+ T cell responses were induced. However, all these methods are difficult to standardize due to numerous variables including the source of virus, type and activation state of infected cells, mode of apoptosis or necrosis, method of virus inactivation and the amount of antigen in preparation. Moreover, they require meticulous quality control testing on the inactivation procedure in order to eliminate any biological risk of infection.

The use of HIV antigens encoded by nucleic acid, either cDNA or mRNA is more easy to standardize, it does not carry infectious risk and hence it seems straightforward for clinical application. In our opinion, transfection with cDNA is less attractive than with mRNA. After transfection with cDNA, more cell death was observed and expression levels were lower [114]. This is due to the more stringent electrical pulses because plasmid DNA has to pass through the nuclear membrane, which is a very inefficient process in post-mitotic cells such as DC. Moreover, non-specific immune responses were also induced when using plasmid DNA transfection of DC. In contrast, transfection with mRNA encoding antigens requires cytoplasmic penetration only and has turned out to be a very efficient method for loading DC with antigens and subsequent stimulation of HIV-specific T cells [115-118].

mRNA encoding consensus or clade-specific HIV antigens (e.g. Gag) can be used to load DC. Whether it will suffice to induce broadly protective T cell responses remains controversial. Recent data argue against implementing clade-specific vaccinations, because clade-specific antigens used in a lot of clinical trials augmented the immune responses but failed to reduce the viral load [119-121]. Moreover, cross-clade reactivity detected in many *in vitro* studies was overestimated due to the addition of excess of exogenous peptides [122]. We [116] and others [115, 123] have developed new techniques based on autologous viral antigens, which take into account patient-to patient HIV diversity. To this end, we produced mRNA that encoded autologous proviral or viral sequences derived from infected peripheral blood mononuclear cells (PBMC) or plasma, representing archived latent and actively replicating virus, respectively. Recently, Tcherepanova and co-workers published a method to amplify autologous sequences from the plasma of HIV-infected individuals, based on multiplex RT-PCR amplification method [123]. With this strategy they can amplify virus sequences independent of the subtype and the amplified products contain a complex mixture of autologous HIV antigen encoded by the viral quasispecies. However, they exclusively considered the plasma virus at one time point, which, by definition, represents an immune escape variant, while the infected PBMC, representing the archived latent virus, was not taken into account. In addition, they have tested their approach only on plasma with a viral load higher than 7500 copies/ml, whereas the most likely target populations for receiving immunotherapy are HAART-treated individuals, characterized by an undetectable viral load. Using our approach based on five primers, we can amplify virus sequences independent of the subtype and it is significantly less expensive than multiplex RT-PCR proposed by Tcherepanova. Moreover, we propose proviral DNA as the starting material, because it can easily be amplified from HAART patients and it is more likely to represent the archived quasispecies.

This mRNA-based strategy provides a major perspective for the development of patient-specific immunotherapy directed against the entire autologous HIV quasispecies without the biological risk of intact virus in the final vaccine product.

OBSTACLES TO BE OVERCOME FOR EFFECTIVE IMMUNOTHERAPY

Besides improvement of DC-mediated immune responses through methods described above, novel vaccine design will also have to take into account the negative regulatory elements and pathways that can hinder immunotherapy. Specific T cell subsets or mechanisms that can be involved in these inhibitory processes include regulatory T cells (Treg) and activation of programmed death (PD)-1/PD-L1 or L2 pathway, respectively. In HIV infection, it remains controversial whether Treg have a detrimental effect by impairing T cell responses, thus facilitating viral persistence, or rather a protective role by limiting immune activation [124]. Studies in HIV-1-infected individuals showed that "natural" Treg exert a suppressive activity on the proliferation of T cells triggered by p24 protein [125-128]. Banerjee pointed out that cytokine-treated monocyte-derived DC (Mo-DC) are the most effective Treg inducers *in vitro*, and that DC-induced Treg effectively suppress T cell responses in allogeneic, mixed leukocyte reactions both from healthy donors and from myeloma patients [129]. In our study, we confirmed that our cytokine-treated Mo-DC indeed increased the frequency of cells with a Treg phenotype. However, we doubt that those are also functionally active Treg, since we could not show their suppressive activity based on proliferation, blocking and depletion experiments [117].

A second mechanism that could be responsible for impeding immunotherapy is the binding of PD-1 to its ligand PD-L1, which in HIV infection is partially responsible for inhibiting proliferation and cytokine secretion by CD8+ T cells [35-37]. Recently, several studies have shown that PD-1 is upregulated in HIV-specific CD8+ T cells from therapy-naïve individuals, and that interrupting PD-1 signaling using antagonistic antibodies "rejuvenates" the T cell effector

functions [35, 130]. In addition, HAART-treated individuals showed a reduced expression level of PD-1 comparable with the expression found in LTNP [37, 130]. We have shown *in vitro* a significant but limited improvement of effector function when anti-PDL1 was added, underscoring the importance of this pathway [117].

STRATEGIES CURRENTLY IN CLINICAL TRIALS

Both therapy-naive and HAART-treated individuals have participated in various immunotherapeutic trials. Because HAART-treated HIV-1-infected individuals have higher CD4+ T cell counts and lower viral load, they exhibit partially restored cellular immunity, especially against pathogens other than HIV [131, 132]. However, some authors believe it is better to vaccinate patients not on HAART to maximize the endogenous antigen present, to minimize potentially immunosuppressive effects of HAART, and to limit confounding variables in the treatment strategy [133]. In our opinion, in order to test safety and effectiveness of a therapeutic vaccine, it will be better to focus on HIV-1-infected persons under coverage of well-established HAART because their immune system has partly recovered and there is no risk that the immune activation, induced by vaccination, will significantly increase viral replication.

An important question that needs to be addressed is whether it is safe and desirable to interrupt HAART after vaccination. To avoid confusion, we need to distinguish between structured treatment interruption (STI) after a period of HAART only and analytical treatment interruption (ATI) after a period of combined HAART and immunotherapy. The original hypothesis on STI was that it could boost the immune system with HIV antigens after it had recovered under HAART. After a lot of discrepancies in smaller STI trials, the double blind CPCRA SMART study has resolved the debate [134]. This study compared the safety and efficacy of 2 treatment strategies: CD4-guided intermittent therapy and continuous therapy. It was shown that in general STI resulted in more disease progression. The risk of disease progression was greatest in persons who started therapy with very low CD4+ T cell count and in older individuals [135]. In contrast, in people who started therapy with a relatively high CD4+ T cell nadir, stopping therapy was relatively safe. Furthermore, people who were in the STI arm presented an unexpectedly higher rate of cardiovascular, kidney and liver problems, toxicity due to HAART and a higher rate of AIDS-related illness. Clearly, the use of STI as such for immunotherapeutic purposes has no benefit and may be dangerous. However, the safety of ATI (in the context of immunotherapy) remains a matter of debate. It seems to be safe in several clinical trials including ACTG 5068 and the ongoing ACTG 5197 [134]. A prerequisite was that patient started therapy with CD4 count higher than 400/mm³.

In addition, DC half life after injection should be taken into account as well as the consequences of this on the number and frequency of injections. Few studies on this topic are published. Verdijk *et al.* have investigated the fate of DC after intradermal (i.d.) or intranodal (i.n.) injection. After i.d. injection, around 4% of the DC reached the draining lymph nodes. Further on, independently of which route of administration was performed large numbers of DC remained at the injection site, lost viability and were cleared within 48 hours. However, from the DC that survives more than 80% reached the T-cell areas, where they induce antigen-specific immune responses. Furthermore, these studies demonstrated that the immune responses induced with i.d. and i.n. injections were comparable and that even less than 5.10^5 DC was enough to induce an immune response [136, 137]. So in our opinion, it is more attractive to perform i.d. injections because it is easier than i.n. Further on, we suggest to administer around 10 million DC to have enough DC that reach the T-cell areas.

A number of DC-based trials for HIV-1 infection have recently been completed in animals and humans (Table **2**). The first two trials described were performed in human PBL/severe combined immune deficiency (SCID) mice and were prophylactic by design. Two different methods of DC preparation were applied. Yoshida and colleagues used IL-4 DC, which are monocytes differentiated in the presence of IL-4 and GM-CSF and matured with IFN-β, whereas Lapenta and colleagues used IFN-DC, which are monocytes differentiated in the presence of IFN-α and IL-4. In both trials, autologous DC were pulsed with aldothriol (AT)-2-inactivated R5 subtype B viruses: HIV-1 SF162 [138] and HIV-1 JR-CSF [86]. After vaccination with DC, HIV-1-specific CD4+ and CD8+ T cells were induced *in vivo*. Moreover, upon infection with homologous virus, there was also evidence of protection.

Around the same time, Lu *et al.* published their paper on therapeutic vaccination in macaques. They used AT-2-inactivated SIVmac251-pulsed DC (IL-4 DC), matured using the classical Jonuleit cocktail consisting of IL-1β, IL-6, TNF-α and PGE_2, to elicit simian immunodeficiency virus (SIV)-specific immune responses in SIV-infected Chinese rhesus monkeys [109]. All animals displayed a significant decrease in viral load 10 days after vaccination, and an increased CD4+ T cell count with enhanced SIV-specific cellular immunity was observed. However, these promising data should be interpreted with caution because treatment was initiated during the acute phase, during which viral control can be achieved much easier as compared to treatment intervention during the chronic phase. De Rose *et al.* performed a clinical trial in pigtail macaques [139]. Blood from these macaques was exposed to overlapping SIV peptides for 1 hour *ex vivo* and re-injected. Vaccinated animals were randomized to receive Gag peptides alone or peptides spanning all nine SIV proteins. SIV specific CD4+ and CD8+ T-cell responses were induced following immunization. Virus levels were approximately 10-fold lower for 1 year in immunized animals compared to controls.

The first human clinical trial *in vivo* was performed by Kundu [140]. This small study showed that the administration of HIV peptide- or protein-pulsed autologous DC (IL-4 DC) was well tolerated and could enhance the immune response to HIV in therapy-naive individuals with normal CD4+ T cell counts. No effect on viral load was observed. The second human trial was based on the use of DC (IL-4 DC) pulsed with autologous AT-2 inactivated virus [110]. Vaccination of 18 untreated chronically-infected HIV-1 patients by Lu *et al*. resulted in an effective HIV-1-specific T cell response with sustained viral suppression of over 90%

Table 2. Overview of HIV Immunotherapeutic Strategies

***In Vitro* Studies**				
Organism	**Treatment**	**Loading Strategy of DC**	**Antigen**	**Results**
Monkey	naive	Nucleofection	mRNA encoding Gag	Stimulated significant antigen-specific recall T cell responses [152].
Human	HAART and naive	pulsing	AT-2 inactivated virus	Expansion of virus-specific CD8(+) T cells which were capable of killing HIV-1-infected cells and eradicating the virus from cultured patient peripheral blood mononuclear cells independently of the disease stages and HAART response statuses of the patients [111].
Human	naive	Pulsing	Autologous IIIB infected apoptotic T cells	Induction of HIV-specific CD4+ and CD8+ T-cells [54].
Human	HAART	Pulsing	Proteins	Induction of IFN-γ producing CD8+ T cells [106].
Human	HAART	pulsing	Apoptotic or necrotic cells expressing HIV antigens	Activation and expansion of HIV-specific CD4+ and CD8+ T cells [108].
Human	Naive	Electroporation	Nef-DC-LAMP	Induction of HIV-specific CD4+ and CD8+ T-cells [115].
Human	HAART and naive	Electroporation	Autologous mRNA encoding Gag and Env	Triggering [116] and expansion [117] of HIV-specific CD4+ and CD8+ T-cells
Human	HAART	Electroporation	Chimeric mRNA encoding Tat, Rev, Nef	Generate HIV-specific functional T-cell responses [153].
***In Vivo* Studies**				
Organism	**Treatment**	**Loading Strategy**	**Antigen**	**Results**
Chinese rhesus macaques	HAART	Pulsing	AT-2 inactivated virus	Effective and durable SIV-specific cellular and humoral immunity is elicited. At week 34 of the study: 50-fold decrease of SIV DNA and a 1,000-fold decrease of SIV RNA [109].
Pigtail macaque	36 HAART	Pulsing of whole blood	Gag proteins or peptides spanning all 9 SIV proteins	SIV-specific CD4+ and CD8+ T cell responses during antiretroviral cover and without. Virus levels were 10-fold lower in immunized animals for 1 year [139].
Human	6 naive	Pulsing	Recombinant HIV-1 MN gp160 or synthetic peptides corresponding to HLA-A2-restricted cytotoxic epitopes of envelope, Gag, and Pol proteins	Well tolerated and no effect on viral load. In patients with normal or near normal CD4+ T cell counts immune response to HIV were enhanced [140].
Human	18 naive	Pulsing	AT-2-inactivated virus	Plasma viral load levels were decreased by 80% (median) over the first 112 d following immunization. The suppression of viral load was positively correlated with HIV-1-specific interleukin-2 or IFN-γ expressing CD4(+) T cells and with HIV-1 gag-specific perforin-expressing CD8(+) effector cells [111].
Human	12 HAART	Pulsing	Heat-inactivated virus	Safe and well tolerated. Partial viral control 24 weeks after ATI [107].
Human	4 HAART	Pulsing	seven CTL peptides with HLA-A*2402 restriction	Well tolerated, and only mild local and general symptoms were observed during vaccine administration Discontinuation of HAART after vaccination failed to lower viral set points. CD8+ T cell responses induced in 2 out of 4 patients [141].
Human	18 HAART	Pulsing	HLA-A0201 Gag, Env, Pol and influenza A matrix protein peptide	Safe and well tolerated (primary endpoint). Significant increase in frequency of HIV-specific IFN-γ positive cells (secondary endpoint) [142].
Human	13 HAART	Live virus	ALVACvcp1452	Well tolerated, no lowering of viral setpoint during ATI [154].
Human	9 HAART	Electroporation	Autologous mRNA encoding Gag, Vpr, Rev and Nef	Mild adverse events. Full or partial HIV-specific immune responses in 7/9 subjects [99].

in 8 of 18 subjects. Based on statistical correlations, this study also demonstrated that robust virus-specific CD4+ T-helper cells were required to induce and maintain virus-specific CD8+ effector function for virus containment. In another clinical trial heat-inactivated autologous virus was used in 12 HIV-1-infected persons under HAART. Partial viral control could be obtained 24 weeks after analytical treatment interruption (ATI) [107]. However and in contrast to the findings of Lu *et al*, the HIV-specific cellular immune responses observed in this study was weak and transient. Whether this was due to the effect of HAART on the immune system or to differences in the nature of the loaded antigenic preparation (AT-2 versus heat-inactivated virus) warrants further investigation. Later on, Ide *et al.* administered 6 doses of synthetic HIV-1 peptide-pulsed autologous DC (IL-4 DC) to 4 HAART-treated, HLA-A2402 individuals who underwent ATI. Although the vaccination was safe, no significant changes in viral load or CD4+ T cells was observed [141]. In 2008 a second clinical trial was performed based on autologous DC (IL-4 DC) pulsed with three HIV-1 HLA-A0201 Gag, Env and Pol peptides and one influenza A matrix protein peptide [142]. The vaccine was administered to HAART treated HIV-infected individuals. The vaccine was safe and well tolerated, but had no effect on the viral load. There was a significantly increased frequency of HIV-1 peptide-specific IFN-γ-positive T cells two weeks after the second vaccination.

Most recently, a clinical trial was performed using DC (IL-4 DC) electroporated with autologous HIV antigen mRNA for the first time [99]. Nine HAART-treated individuals were vaccinated with autologous DC electroporated with mRNA encoding CD40L and autologous HIV antigens Gag, Vpr, Rev and Nef. Patients received monthly injections in combination with HAART. The primary endpoint of the study was the proliferative capacity of CD8+ T cells in response to four HIV antigens. Seven out of 9 patients showed proliferative responses. Effect on viral load was not evaluated because the patients did not undergo ATI.

GENERAL CONCLUSIONS

In conclusion, to design an efficient cellular immunotherapy for HIV, DC should be maturated using a cocktail of Th1-skewing factors that promotes IL-12 production and chemokine-driven migration capacity. Further on, we advocate the use of an mRNA-based loading strategy to introduce autologous viral antigens. This method might be able to maintain the residual virus at a low level and prevent or dampen the rebound of virus following cessation of HAART. To prevent negative regulatory responses, blocking of the PD-1/PD-L1 pathway can be considered.

FUTURE DIRECTIONS *IN VIVO* TARGETING OF DC

Current immunotherapeutic strategies involve the *ex vivo* manipulation of autologous DC. This vaccination procedure is labor-intensive, logistically complicated, expensive and not useful in developing countries. A possible solution is *in vivo* delivery of suitable antigens and co-stimuli to resident DC. A first attempt, already tried in animal models, was to use protein antigens complexed with antibodies to DC-SIGN or DEC-205 [143, 144], thus directly targeting specific surface receptors on DC. Another strategy could be the delivery of biodegradable nanoparticles that will be taken up by endocytic DC *in vivo* [145, 146]. This principle requires further preclinical and clinical investigation on which format of antigens (proteins, nucleic acids) and which costimuli are suitable to load the nanoparticles, to induce DC maturation and antigen presentation for effective anti-viral T cell responses. One type of nanoparticles already used in humans are dendrimers, which are polymers (reviewed in [147]. Further information about clinical trials can be found at www.clinicaltrial.com.

A hotly debated topic is whether it is still acceptable to perform ATI and which correlate of protection can be used. A recent review on ATI suggested that viral endpoints following ATI can be used as a correlate of immunotherapy efficacy. The condition is that precautions are taken to avoid drug resistance and drops in CD4+ T cell count [148]. To this end, only patients with relatively high CD4+ T cell nadir and restored CD4+ T cell counts under HAART should be considered. The differential half-life of the different antivirals in the regimen should be taken into account to decide on the order of drug interruption. Under those conditions the dynamics of the viral rebound and viral set point reached after ATI can be used as study endpoint. Obviously, therapy should be promptly resumed as soon as the viral load increases above or the CD4+ T cell count drops below a preset safety level.

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ABBREVIATIONS

Ad5	=	Recombinant adenovirus serotype 5
APC	=	Antigen-presenting cells
AT-2	=	Aldothriol-2
ATI	=	Analytical treatment interruption
CCR	=	CC chemokine receptor
CTL	=	Cytotoxic T lymphocytes
DC	=	Dendritic cell
EC	=	Elite controllers
HAART	=	Highly active antiretroviral therapy
HIV	=	Human immunodeficiency virus
HLA	=	Human leukocyte antigen
IFN	=	Interferon
IL	=	Interleukin
LTNP	=	Long-term non progressors
MART	=	Melanoma antigen recognized by T cells
mDC	=	Mature DC

MHC = Major histocompatibility complex

Mo-DC = Monocyte-derived DC

MPLA = Monophosphoryl lipid A

PAMP = Pathogen-associated molecular patterns

PBMC = Peripheral blood mononuclear cells

PD = Programmed death receptor

PG = Prostaglandin

SCID = Severe combined immune deficiency

SIV = Simian immunodeficiency virus

SOCS = Suppressors of cytokine signaling

STI = Structured treatment interruption

TCR = T cell receptor

Th = T helper

TLR = Toll-like receptor ligands

TNF = Tumor necrosis factor

Treg = Regulatory T cells

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