Published in final edited form as:

Eur J Immunol. 2012 August ; 42(8): 2019–2030. doi:10.1002/eji.201242478.

CD34-derived dendritic cells transfected ex vivo with HIV-Gag mRNA induce polyfunctional T-cell responses in nonhuman primates

Gabrielle Romain^{1,2}, Ellen van Gulck³, Olivier Epaulard^{1,2}, SangKon Oh⁴, Dapeng Li⁴, Gerard Zurawski⁴, Sandra Zurawski⁴, Antonio Cosma^{1,2}, Lucille Adam^{1,2}, Catherine Chapon^{1,2}, Biliana Todorova^{1,2}, Jacques Banchereau⁴, Nathalie Dereuddre-Bosquet^{1,2}, Guido Vanham^{3,5}, Roger Le Grand^{1,2}, and Frédéric Martinon^{1,2,6}

¹CEA, Division of Immuno-Virology, Institute for Emerging Diseases and Innovative Therapies (iMETI), DSV, Fontenay-aux-Roses, France

²Université Paris-Sud, UMR E1, Orsay, France

³Virology Unit, Division of Microbiology, Department of Biomedical Sciences (HIV and Retrovirology Research Unit, Department of Microbiology), Department of Microbiology, Institute of Tropical Medicine of Antwerp (ITMA), Belgium

⁴Baylor Institute for Immunology Research and INSERM U899, Dallas, TX 75204, USA

⁵Faculty of Medicine and Pharmacy, Free University of Brussels (VUB), Brussels, Belgium

⁶Inserm, Paris, France

Abstract

The pivotal role of DCs in initiating the immune responses led to their use as vaccine vectors. However, the relationship between DC subsets involved in antigen presentation and the type of elicited immune responses underlined the need for the characterization of the DCs generated in vitro. The phenotypes of tissue-derived APCs from a cynomolgus macaque model for human vaccine development were compared with ex vivo-derived DCs. Monocyte/macrophages predominated in bone marrow (BM) and blood. Myeloid DCs (mDCs) were present in all tested tissues and were more highly represented than plasmacytoid DCs (pDCs). As in human skin, Langerhans cells (LCs) resided exclusively in the macaque epidermis, expressing CD11c, high levels of CD1a and Langerin (CD207). Most DC subsets were endowed with tissue-specific combinations of PRRs. DCs generated from CD34⁺ BM cells (CD34-DCs) were heterogeneous in phenotype. CD34-DCs shared properties (differentiation and PRR) of dermal and epidermal DCs. After injection into macaques, CD34-DCs expressing HIV-Gag induced Gag-specific CD4⁺ and CD8⁺ T cells producing IFN- γ , TNF- α , MIP-1 β or IL-2. In high responding animals, the numbers of polyfunctional CD8⁺ T cells increased with the number of booster injections. This DC-based vaccine strategy elicited immune responses relevant to the DC subsets generated in vitro.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Corresponding author: Dr Frédéric Martinon, Service d'Immuno-Virologie, iMETI, CEA, 18 route du Panorama, 92265 Fontenay-aux-Roses, France; frederic.martinon@cea.fr; Phone: +33 1 4654 8757; Fax: +33 1 4654 7726.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Keywords

Dendritic cells; Vaccination; Immune responses; Antigen presenting cells

1. Introduction

DCs are a heterogeneous population of migratory and tissue-resident immune cells with the capacity to prime specific immune responses. As part of their function, DCs are able to detect PAMPs through their PRRs [1]. Several types of tissue-resident DCs can be distinguished by the expression of particular PRRs, such as C-type lectin receptors (CLRs), which act as anchors for a large number of microbes. CLRs at the surface of DCs allow antigen internalization and initiate the process of antigen presentation to T and B lymphocytes [2]. The signaling motifs in the cytoplasmic domains of some CLRs lead to their activation or suppression upon triggering, and thereby modulate both innate and adaptive immune responses [1, 2]. Altogether, these important properties of DCs can be utilized for the design of new vaccine strategies.

DC-based therapeutic vaccines have been considered for the treatment of cancers [3, 4] and the control of chronic infections, such as those caused by HCV and HIV [5] because of their unique ability to prime naïve T cells against specific antigens. Patients have been infused with autologous DCs derived ex vivo from blood monocytes or from CD34⁺ hematopoietic progenitor cells (HPCs) [6–8] loaded with various antigens, including proteins, peptides, inactivated viruses, tumor lysates and recombinant viral vectors. The use of antigen-encoding mRNA to transfect DCs ex vivo may have multiple advantages [9]. Specifically, the cost of antigen production may be significantly reduced and nucleic acids offer multiple possibilities for immunogen engineering and design. It is also possible to develop “personalized” immunogens adapted to each immunized individual. These personalized immunogens, used to transfect the DCs, can be derived directly from patient tumor cells or from viral variants replicating in the host [10, 11]. The use of mRNA offers the additional advantage over DNA and retroviral vectors that it cannot possibly be integrated into the human DNA and hence avoids the potential risk of insertional genetic damage [12].

In the present study, we extensively characterized the phenotype of different DC populations of the macaques used as animal models for immunogenicity and safety tests of human vaccines. We demonstrate that DCs derived from macaque BM CD34⁺ cells and transfected ex vivo with HIV Gag-encoding mRNA can efficiently prime a specific and polyfunctional T-cell response.

2. Results

2.1. Characterization of DC subsets in blood and lymphoid tissues of cynomolgus macaques

DCs represent a heterogeneous APC population expressing diverse differentiation and maturation markers according to their localization and contribution to the host immune response [3]. We first analyzed the different DC subsets in the BM, blood, spleen, LNs and skin of cynomolgus macaques with the aim of comparing their phenotypes with the phenotype of the DCs we generated ex vivo for our vaccine purposes. HLA-DR⁺ cells that were negative for the lymphocyte lineage (CD3[−], CD8[−], CD20[−]) were analyzed for expression of CD1a, CD14, CD11c and CD123 (Fig. 1 and Table 1). As reported for humans, BM contained the highest proportion of HLA-DR⁺Lin[−] cells in all tissues examined, the majority of which were CD1a[−]CD14⁺ monocytes/macrophages (subset 1 in Fig. 1). These cells were also represented in blood. In contrast, LNs contained mainly

CD1a⁺CD14⁻ APCs whereas spleen contained a mixed population of CD1a⁺CD14⁻ APCs and monocytes/macrophages. In all tissues, CD11c⁺CD123⁻ mDCs (subset 2 in Fig. 1) were more highly represented than CD11c⁻CD123⁺ pDCs (subset 3 in Fig. 1). In accordance with previously published results, including those of our group for macaque cells [13–16], CD11c⁻CD123⁺ pDCs were in fewer proportions in blood than in the spleen and LNs (Table 1). The skin is populated with a wide variety of APCs [17], providing a very attractive route for vaccine delivery. Similar to human skin, the macaque epidermis contains a single DC subset composed of CD14⁻ cells expressing CD11c and high levels of CD1a. These cells also express langerin (Fig. 2) and thus correspond to LCs [17]. Altogether, these results and our previously published data indicate that cynomolgus macaque tissues exhibited APC subset distributions that were very similar to those reported for the same tissues in humans.

In BM, blood and spleen, the monocytes/macrophages were mainly activated based on the level of expression of CD40 and CD86 (Fig. 1). In LNs and at steady state, this subset was almost undetectable by flow cytometry. Moderately activated mDCs (HLA-DR⁺Lin⁻CD14⁻CD11c⁺CD123⁻) were observed in BM and blood whereas in spleen and LNs this subset was highly activated, as previously reported [13]. In spleen, two populations of mDCs could be distinguished based on CD40 expression levels. However, as illustrated by epidermal DCs, CD1a⁺ DCs also expressed CD11c. In the spleen, these CD1a⁺ CD11c⁺ DCs formed a subpopulation within subset 2, which could be relevant with the CD40 expression profile. This indicated that subset 2, designated as mDC, was peculiar to each tissue. As expected from previous studies in NHPs [13, 18], a similar activation profile for pDCs (HLA-DR⁺Lin⁻CD14⁻CD11c⁻CD123⁺) was observed in these same four tissues and was characterized by slightly higher CD40 and CD86 expression levels and low-level of CD83 expression. CD83 expression on mDCs was low in BM, blood and LNs, but higher in spleen, whereas CD1d expression seemed negative. In all four tissues, pDCs expressed neither CD83 nor CD1d and low-level of CD86. With regard to skin DCs, both epidermal and dermal DCs expressed relatively high levels of CD86 and CD1d, whereas CD40 and CD83 were restricted to epidermal DCs.

To further evaluate the capacity of these APC subsets to react to PAMPs, we analyzed the expression of 12 APC-specific markers, including CLRs (Fig. 2). Monocytes/macrophages expressed most of the markers studied with the exception of langerin (Lang), which, as expected, was expressed only on LCs. These epidermal cells also expressed moderate levels of DEC205, in accordance with previous studies [19], DC-ASGPR, and DC-SIGN. In contrast to what was reported for human LCs obtained after migration ex vivo from skin biopsies [20], macaque LCs expressed low levels of DC-SIGN. As expected, the CD163 scavenger receptor was expressed at high levels only on monocyte/macrophages [21]. In BM, blood and spleen, moderate expression levels of DC-ASGPR, CLEC6, MARCO, CD163 and CD1d and high levels of DEC205 expression characterized mDCs. Interestingly, DEC-205 was the only lectin expressed by all APC subsets in all tested tissues. In spleen, DEC-205 expression levels, like those of CD40, allowed to distinguish two cell populations (Fig. 1 & 2). Expression levels for all these markers, except CD163, were high in LN-derived cells, probably in relation to the high level of mDC activation in this tissue (Fig. 1). Plasmacytoid DCs exhibited similar phenotypes in all tissues and were characterized by high levels of DEC-205 expression and moderate levels of MR, DC-ASGPR, CLEC-6 and CD1d expression.

2.2. Characterization of cynomolgus macaque CD34-DCs

We derived DCs from CD34⁺ HPC from BM to generate sufficient numbers of DCs for vaccine purposes. Culture conditions for proliferation, differentiation and maturation, were adapted from human studies [22, 23] and allowed us to obtain a mean of $67.5 \times 10^6 \pm 10.5 \times 10^6$ DCs from a 14-day culture starting from a mean of $2.5 \times 10^6 \pm 0.6 \times 10^6$ CD34⁺ HPCs

(n=19 experiments). Cell numbers increased by a factor of 21.3 ± 3.7 (n=22) between culture days 0 and 7 in presence of SCF, Flt3-L, IL-3 and IL-6, and by a factor of 3.08 ± 0.49 (n=20) between days 7 and 14 (Supporting information Fig. 2A) in presence of GM-CSF and IL-4. These increases are consistent with previous studies of macaque [24] and human HPCs. Maturation was induced by a 24 h incubation with TNF- α , IFN- γ , TLR7/8-L (R848) and PGE2 and resulted in a mean loss of $31\% \pm 6\%$ of the cells (n=13). Overall, the macaque CD34-DC cultures displayed typical irregular round shaped cells emitting long dendrites (Supporting information Fig. 2B), and were similar in appearance to human CD34-DCs.

As expected, most of the cells were HLA-DR⁺Lin⁻ ($84.7\% \pm 2.2\%$, n=34) and had down regulated CD34 expression ($4.0 \pm 0.8\%$) after 15 days of culture (Fig. 3A). Few cells expressed the PMN markers CD66abce ($17.3\% \pm 4.2\%$, n=11), which was probably due to the presence of GM-CSF during the culture. However, CD66abce expression has also been reported for immature DCs. Very few CD41a⁺ megakaryocytes were generated ($0.6\% \pm 0.2\%$, n=9) in our culture system.

At day 15, most of the HLA-DR⁺ cells (Fig. 3B) were predominantly of a mDC-like CD11c⁺CD123⁻ phenotype. As previously reported [25], in vitro-generated CD34-DCs matured towards either the CD14⁺ or the CD1a⁺ pathway of differentiation, in different proportions (Fig. 3B). After 24 h in the presence of maturation cocktail, the cell population became heterogeneous with more immature DCs (CD83⁻CD86^{low}, $60.4\% \pm 4.3\%$, n=17) than mature DCs (CD83⁺CD86^{high}, $29.5 \pm 3.5\%$, n = 17) (Fig. 4). Mature DCs were characterized by increased expression levels of HLA-DR, CD1a, CCR7, CD40, DEC205 and, to a lesser extent, langerin. In contrast, high levels of CD14, CD1d, DC-SIGN, DCIR, Dectin-1 and Lox-1 expression characterized immature DCs (Fig. 4). Similar to the in vivo DC subsets, the level of DEC-205 expression matched the level of CD40 expression. Mature DCs expressing high levels of CD1a also expressed moderate levels of langerin (Fig. 4), as it has been previously reported for human CD34-DCs [20]. Altogether, these results indicated that mature DCs resembled in vivo mDCs whereas immature DCs shared similarities with CD14⁺ APCs.

In order to assess the ability of macaque BM-derived DCs to stimulate T lymphocytes, allogeneic PBMCs were mixed in vitro with various ratios of CD34-DCs (Fig. 5A). Maximum CD4⁺ and CD8⁺ T cell proliferation was induced by a PBMC:CD34-DC ratio of 1:0.1 ($59.9\% \pm 6.2\%$ of CD4⁺ T cells and $40.1\% \pm 9.3\%$ of CD8⁺ T cells). In addition, IL-12/IL-23p40, IL-6, IL-8, IL-5 and IL-15, as well as MIP-1 β and IL-1 β chemokines were induced within the 24 h maturation step (Fig. 5B). Therefore, the macaque CD34-DCs that we generated in vitro were harnessed with lymphocyte antigen presentation and co-stimulation functions. To prepare DCs for the purpose of vaccination, CD34-DCs were transfected with codon-optimized HIV-*gag* encoding mRNA after the maturation step. Gag protein production was analyzed by flow cytometry (Fig. 5C) and was significantly higher ($p=0.014$, n=4) in mature DCs ($78.0 \pm 2.7\%$) than in immature DCs ($44.1 \pm 6.0\%$). Interestingly, and according to previously reported studies using ex vivo-derived DCs for immunization in primates [26], Q-dot loaded CD34-DCs injected into the skin (s.c. and intradermally (i.d.)) of cynomolgus macaques did not appear to massively migrate from the skin to LNs. High levels of fluorescent signal persisted at the injection site as evidenced by a near infrared in vivo imaging (Fig. 5D). By contrast, fluorescent signal due the free Q-dots injected as controls rapidly diminished at the injection site over the 44 h of follow up, suggesting rapid diffusion from the injection site and draining of the particles by regional LNs.

2.3. Immune response induced in macaques by Gag-expressing CD34-DCs

Autologous CD34-DCs expressing Gag were injected into the four cynomolgus macaques from which DCs were derived. The T-cell responses induced in the macaques immunized with the transfected CD34-DCs were compared with those obtained in other macaques immunized with recombinant soluble Gag protein (Fig. 6A). As expected, repeated injections of Gag protein alone (63 µg) without any adjuvant, were insufficient to induce any detectable specific IFN- γ secreting T cells upon ex vivo stimulation with a pool of 15-mer Gag peptides (Fig. 6A). This confirms our previous observations and reported results [27]. In contrast, a single injection of the ex vivo-transfected CD34-DCs induced significant IFN- γ (Fig. 6B) and IL-2 (Fig. 6C) T cell responses in four out of four animals. One animal (#OBBT5) showed a very high IFN- γ T cell response, which was boosted by successive injections of autologous Gag-expressing CD34-DC. One other animal (#OBEA5) responded moderately and booster injections had little effect on the T cell responses. Interestingly, *gag*-mRNA transfected CD34-DCs inoculated into the high responder include both, CD1a⁺CD14⁻ (16.0%) and CD1a⁻CD14⁺ (64.1%) cells whereas almost no CD1a⁺CD14⁻ (1.2%) cells have been injected into the low responder macaque (Fig. 6D and Supporting information Table 1). Although observation in only two animals is too limited to reach clear conclusions, it is worth noting that this is in accordance with studies demonstrating the capability of LCs to generate antigen-specific T cell responses.

A high frequency of Gag-specific T cells producing IFN- γ in the high responder (#OBBT5) was confirmed by flow cytometry (Fig. 7). CD4⁺ and CD8⁺ T cells were both involved. The frequencies of polyfunctional CD8⁺ T cells that produced IFN- γ and at least one of the two cytokines MIP-1 β and TNF- α were clearly increased by booster injections (Fig. 7A). The second Gag-DC injection generated CD8⁺ T cells endowed with 3 or 4 functions. After the fourth vaccine injection, the main increase concerned CD8⁺ T cells that produced IFN- γ , MIP-1 β and TNF- α . In contrast, the frequency of Gag-specific, IL-2-producing CD8⁺ T cells remained unchanged after the third and fourth Gag-DC injections.

Specific CD4⁺ T cells, as identified by the appearance of the activation marker CD154 (CD40L) following ex vivo stimulation with Gag peptide pools, mainly produced IL-2 and very few of them produced MIP-1 β (Fig. 7B). The frequencies of polyfunctional Gag-specific CD4⁺ T cells showing three or four functions also increased as the number of vaccine injections increased. There was also an increase in the frequency of cells expressing only the CD154 marker, indicating that the vaccine may elicit other CD4⁺ T cell functions. Interestingly, no anti-Gag antibody response was induced in any of the CD34-DC injected macaques (not shown).

3. Discussion

To our knowledge, the present study is the first to extensively characterize the DC subsets in the various tissues of macaques. We observe a distribution of CD14 (macrophage-like), CD1a (LCs), CD11c (mDCs) and CD123 (pDCs) in macaque tissues, which is similar to humans. In addition, we also provide evidence that mixed DCs can be generated from CD34⁺ HPCs and that these CD34-DCs can successfully be applied for vaccination purposes against HIV Gag, resulting in the induction of polyfunctional CD4 and CD8 T cells. These observations provide a strong basis for the use of cynomolgous macaques as a reliable model for immunization studies.

CD11c has been used to discriminate mDCs from pDCs amongst HLA-DR⁺Lin⁻ cells in blood [28, 29]. CD14 is a classical marker of monocytes/macrophages in blood [28, 30], which is also expressed by some immature DCs in other tissues [29, 31, 32] such as the skin. We can therefore hypothesize that the CD14⁺ APCs that we have identified *in vivo* in

macaques include both monocytes/macrophages and CD14⁺ DCs. Overall, CD14⁺ APCs are distinct from APCs expressing CD11c, and the respective proportions of both subsets differ in different tissues. These CD14⁺ APCs express higher levels of C-type lectin receptors and scavenger molecules able to recognize and/or capture pathogens through their PAMPs than do CD11c⁺ APCs. Since we used the skin as the vaccine injection site, we also characterized APCs from this tissue [33–35]. In the steady state, the only mDCs present in the skin epidermis are the LCs, expressing Langerin and high levels of CD1a. Human LCs also expresses intermediate levels of DEC205, as observed in macaque skin.

Skin DCs migrate to LNs upon activation by contact with antigen and/or after immunostimulation [33–35]. However very little information is available for these DCs in human LNs, thus studies in NHPs may provide insight into these populations. For vaccine development, identification of these cells is critical. As observed with the free Q-dot particles, some vaccines may be drained directly into the proximal LNs through the lymphatics, and thus have the potential to directly interact with LN-resident DCs and macrophages. In the steady state, specific DC subsets present in the skin and lymphoid tissues have unique CLR and TLR signatures. Targeting multiple CLRs of DCs residing in different locations may trigger distinct types of immune responses [36].

The CD34-DCs generated ex vivo gave rise to a CD1a⁺ population known to be phenotypically and functionally linked to LCs (LC-like DCs). In addition, CD34-DCs also generated CD14⁺ cells, which may be phenotypically interstitial-dermal-like DCs [25]. For these reasons, we wanted to determine whether these two distinct phenotype patterns could be found in vivo in macaques. We demonstrated that CD1a and CD14 expression were mutually exclusive and that the proportions of the corresponding cell populations varied according to the compartment considered. Macaque epidermal LCs are characterized by strong expression of Langerin and CD1a, and as expected, CD1a⁺ APCs fell into the CD11c⁺ subset [26]. In mesenteric and axillary LNs, CD1a⁺ APCs significantly outnumbered CD14⁺ APCs, which were scarcely present in these tissues.

Plasmacytoid DCs are key actors of innate immunity and may contribute to shape the subsequent adaptive T and B-cell antigen-specific responses. We showed that macaque pDCs express significant levels of several lectin receptors, such as DEC-205 and CLEC-6. Other CLRs were expressed more weakly (MR, DC-ASGPR, DC-SIGN, MARCO and CD163). Expression of these PRRs is consistent with the capacity of pDCs to capture and subsequently present antigens to T cells, and such an extensive comparison has not been previously reported [37].

For over 15 years, due to their unique role in antigen presentation and in directing immune response, DCs have been considered for immunotherapeutic protocols aiming at enhancing patients' responses against cancer and several infectious diseases, such as HIV infection [5]. In line with this type of approach, the second goal of this study was to demonstrate the immunogenicity and feasibility of preparing macaque CD34-DCs loaded with Gag mRNA. Generating NHP DCs in vitro either from CD34⁺ HPCs or monocytes [24, 26, 38], has been previously described and given the limitations of monocyte-DC culture, we favored generating DCs from BM CD34⁺ HPCs. Our culture system allowed variable proportions of cells to differentiate into CD1a⁺ DCs and CD14⁺ DCs, with the former displaying a more mature phenotype (higher levels of HLA-DR and costimulatory molecules, together with lower levels of CLRs and scavenger receptors). These distinct patterns of expression for the CD1a⁺ and CD14⁺ DC subsets are consistent with other studies focusing on TLR expression and cytokine secretion patterns [39, 40], and are in accordance with their respective functions. In addition, ex vivo-generated CD34-DCs may possess similar [7], if not better, immunostimulatory capacities than monocyte-derived DCs [8, 41]. Matured CD34-DCs are

strong stimulators in mixed lymphocyte reactions and have been observed to secrete a large spectrum of proinflammatory cytokines [23]. We surmise that the CD34-DC heterogeneity we observed can be beneficial, for instance by extending the range of cytokines secreted by the mixed population, including IL-6, IL-8, IL-13, IL-12 and IL-15 [20, 39, 41, 42].

Our study demonstrated that in cynomolgus macaques, CD34-DCs, loaded with mRNA encoding a viral antigen (HIV Gag), induced virus-specific T cell immunity although we could not detect their migration to LNs by using in vivo imaging, probably due to the low number of migrating injected DCs. A single CD34-DC vaccine injection produced potent HIV Gag-specific CD8⁺ T cell responses in naïve animals with a high frequency of T cells with functional effector phenotype. Boosting injections were also able to increase the polyfunctionality of CD4⁺ and CD8⁺ Gag-specific T cells, while using viral mRNA-loaded DCs to generate strong antiviral T cell responses. It is interesting to note that the humoral response remained undetectable in our vaccinated macaques, which may be related to the low frequency of specific CD4⁺ T cells induced and to the immune properties of injected CD34-DCs [20, 25]. Our work also confirms that mRNA transfection is an efficient mean of loading DCs with a specific viral antigen [9, 43, 44]. This strategy allows the use of HIV sequences derived from the patient's own virus quasispecies [11, 43, 44], thus the patient's immune responses can be theoretically focused and stimulated through the preparation of tailored vaccines [5, 45, 46]. Further studies have been considered to address the use of DCs transfected ex vivo with viral antigen-encoding mRNA and to assess efficacy of these vaccines at protecting against high viral loads and disease progression.

4. Materials and methods

4.1. Animals

Adult male cynomolgus macaques (*Macaca fascicularis*) imported from Mauritius and weighing 4–8 kg, were housed in CEA facilities (accreditation no.: B 92-032-02) and handled (investigator accreditation no.: NDB, 92-171; RLG, B 92-073; FM, C 92-241) in accordance with European guidelines for NHP care (EEC Directive N 86-609). This study was approved by the regional animal care and use committee (Comité Régional d'Ethique Ile de France Sud).

4.2. Phenotypic characterization of cynomolgus macaque tissue specific DCs

DCs were obtained from blood, BM, axillary and mesenteric LNs, spleen, and skin punches collected after euthanasia. LNs were dilacerated and incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 1.5 µg/mL collagenase D (Roche Diagnostics, Basel, Switzerland) for one hour at 37°C. Skin punches were prepared as previously reported [47]. Cells were prepared for flow cytometry by using mAbs (Supporting information Table 2) after incubation with LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen) according to manufacturer's instructions, acquired on a LSR II (BD Bioscience) and analyzed with FlowJo software 7 (Tree Star, Ashland, OR, USA).

4.3. In vitro generation of CD34-DCs

BM samples were collected in 10% sterile acid citrate dextrose (ACD-A, Baxter, Deerfield, IL, USA), and mononuclear cells were isolated on Lymphocytes Separation Medium (Eurobio, Les Ulis, France). CD34⁺ cells were sorted on magnetic beads coated with antibodies to CD34 (Dynal Progenitor Cell Selection, Invitrogen), following the manufacturer's instructions. CD34⁺ HPCs at 1×10^6 cells/mL were cultured in IMDM Glutamax (Invitrogen) supplemented with 1% penicillin and streptomycin (Invitrogen) and 5% heat-inactivated macaque serum for seven days in the presence of 100 ng/mL of

recombinant human SCF, 100 ng/mL Flt3-L, 20 ng/mL IL-3 and 20 ng/mL IL-6 (all from PeproTech, Rocky Hill, NJ, USA). After seven days of culture in the presence of 50 ng/mL of recombinant human GM-CSF and 50 ng/mL rhesus IL-4 (R&D Systems, Minneapolis, MN, USA) and a 24-hour maturation in 2.5 ng/mL TNF- α , 35 ng/mL PGE2 (Sigma-Aldrich), 25 ng/mL IFN- γ (R&D Systems) and 2 μ g/mL TLR7/8-L (CL097, InvivoGen San Diego, CA, USA), the CD34-DC were gently harvested for using as vaccine vectors and stained for phenotyping.

4.4. Transfection of CD34-DCs by electroporation

The codon-optimized HIV-1 HxB-2 *gag* mRNA, prepared as previously described [48] was used to transfect CD34-DCs [49]. Electroporation was performed with the Gene Pulser II (Bio-Rad, Marnes-la-Coquette, France), set at 300 μ F and 300V. Cells were cryopreserved in a serum-free freezing medium (ProFreeze-CDM Freezing Medium, Lonza, Italy) supplemented with 7.5% DMSO and stored at -135°C .

4.5. Cytokine assay in CD34-DC supernatants

At day 14, immature CD34-DCs were harvested, washed twice in RPMI-1640, and incubated at a density 1×10^6 cells/mL in fresh culture medium in the presence or absence of the maturation cocktail. After 24 h of incubation, supernatants were harvested and titrated for the concentrations of cytokines by using the Milliplex MAP Non-Human Primate Immunoassay kit (Millipore, Billerica, MA, USA).

4.6. Allogeneic mixed lymphocyte reaction

The allogeneic mixed lymphocyte reaction was performed by mixing 1×10^5 PBMCs that had been previously stained with the CFSE Celltrace kit (Invitrogen) following the manufacturer's instructions, with graded doses of allogeneic CD34-DCs in order to obtain the indicated PBMC:CD34-DC ratios. At day seven, cells were harvested and prepared for flow cytometry.

4.7. Immunizations

Gag-transfected autologous CD34-DCs were administered 24 h after thawing to four cynomolgus macaques at weeks 0 and 4. Two animals received two additional injections at weeks 9 and 13. Each immunization consisted in an injection of a total number of 1×10^7 live Gag mRNA-transfected CD34-DCs. Cells were injected in the proximal region of the inguinal LNs: one-half was injected s.c. in a volume of 300 μ L and the other half was injected i.d. at 3 other sites (100 μ L each). Consensus clade B HIV-1 Gag protein was used to immunize control animals. The amount of protein we used (63 μ g) for each immunization time is highly immunogenic when injected in presence of Montanide and R848 [27]. Here, HIV-Gag was injected i.d. without adjuvant.

4.8. Imaging

Gag-transfected autologous CD34-DCs were labeled with Qtracker[®] 800 (QD800) cell labeling kit (Invitrogen). One macaque received 1×10^7 labeled DCs in the proximal region of the left inguinal LN and free QD800 non-targeted Q-Dots (20 pmol in PBS solution) in the proximal region of the right inguinal LN. In vivo fluorescence imaging was performed by using a Fluobeam[®] (Fluoptics, Grenoble, France) near-infrared imaging system. All images were acquired under the same conditions and were comparable from day to day.

4.9. IFN- γ and IL-2 ELISPOT

ELISPOT assays were performed on fresh PBMCs as described previously [50] by using anti-monkey IFN- γ mAb (Mabtech AB, Sophia Antipolis, France) or anti-monkey IL-2

mAb (U-CyTech, Utrecht, The Netherlands). PBMCs (2×10^5 cells/well) were stimulated with a pool of 15-mer overlapping peptides (85 peptides, 11 amino acid overlap, 1 μ M per peptide), corresponding to the HIV-1 clade B Gag p17 and p24 consensus sequence. Spots were counted with the automated ELISPOT Reader System (AID, Strassberg, Germany). The results were expressed as the mean numbers of IFN- γ or IL-2 spot-forming cells per 10^6 PBMCs (IFN- γ or IL-2 SFC/million PBMCs) from triplicate wells. Background measured in negative control was deducted.

4.10. Intracellular cytokine staining

PBMCs from immunized animals were stimulated *in vitro* in the presence of Gag peptide pool, medium alone (negative control) or PMA/ionomycin (positive control). After one hour, brefeldin A (5 μ g/mL, Sigma-Aldrich) and monensin (6:10,000 dilution, BD Golgi Stop) were added. Cells were incubated for an additional five hours, then washed twice before staining with LIVE/DEAD Fixable Blue Dead Cell Stain Kit. Cells were then incubated in BD Cytotfix/Cytoperm buffer for 20 min, washed, and stained for an additional 30 min at 4°C in BD Perm/Wash buffer containing mAbs specific for the following markers and cytokines: CD45RA, CD3, CD4, CD8, CD154, IFN- γ , IL-2, MIP-1 β , TNF- α (all purchased from BD Biosciences) to detect surface and intracellular immunoreactivity (Supporting information Table 2). After washes, cells were suspended in PBS and analyzed with an LSR II cytometer. A minimum of 1×10^5 living CD3⁺ T cells was acquired for each sample. Data were analyzed with FlowJo (Tree Star) using a previously described gating strategy [51] (Supporting Information Fig. 5). Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <<http://exon.niaid.nih.gov/spice>> [52].

4.11. Statistical analysis

Data are depicted as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed with Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA) software by using non-parametric Wilcoxon paired or Mann-Whitney unpaired tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work benefits from the strong technical support of the core laboratory (TIPIV) of the division of immunovirology and the ANRS NHP working group. We thank C. Joubert and J.M. Helies, veterinarians, for the supervision of the animal care and help for animal studies. This work was supported by the Agence Nationale de Recherche sur le SIDA et les Hépatites Virales (ANRS, Paris, France) and by the National Institutes of Health (NIH) prime award N° 2U19AI057234-06. It was also part of the ANRS HIV vaccine network (AHVN) and programs of the Vaccine Research Institute (VRI, Créteil, France) directed by Pr Y. Lévy. G.R. received fellowships from the ANRS and Sidaction (Paris, France). O.E. received a fellowship from the ANRS. EVG and GV were also supported by grants from SOFI (Secundaire Onderzoeksfinanciering ITG) and IAP (Inter-University Attraction Poles).

List of abbreviations

BM	bone marrow
mDC	myeloid DC
pDC	plasmacytoid DC
LC	Langerhans cell

CLR	C-type lectin receptor
HPC	hematopoietic progenitor cell
i.d	intradermally

References

1. van Vliet SJ, den Dunnen J, Gringhuis SI, Geijtenbeek TB, van Kooyk Y. Innate signaling and regulation of Dendritic cell immunity. *Curr Opin Immunol.* 2007; 19:435–440. [PubMed: 17629469]
2. Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol.* 2009; 9:465–479. [PubMed: 19521399]
3. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature.* 2007; 449:419–426. [PubMed: 17898760]
4. Schuler G. Dendritic cells in cancer immunotherapy. *Eur J Immunol.* 2010; 40:2123–2130. [PubMed: 20853498]
5. Rinaldo CR. Dendritic cell-based human immunodeficiency virus vaccine. *J Intern Med.* 2009; 265:138–158. [PubMed: 19093966]
6. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 1994; 179:1109–1118. [PubMed: 8145033]
7. Syme R, Bajwa R, Robertson L, Stewart D, Gluck S. Comparison of CD34 and monocyte-derived dendritic cells from mobilized peripheral blood from cancer patients. *Stem Cells.* 2005; 23:74–81. [PubMed: 15625124]
8. Bai L, Feuerer M, Beckhove P, Umansky V, Schirmacher V. Generation of dendritic cells from human bone marrow mononuclear cells: advantages for clinical application in comparison to peripheral blood monocyte derived cells. *Int J Oncol.* 2002; 20:247–253. [PubMed: 11788884]
9. Gilboa E, Vieweg J. Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev.* 2004; 199:251–263. [PubMed: 15233739]
10. Boczkowski D, Nair SK, Nam JH, Lyster HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. *Cancer Res.* 2000; 60:1028–1034. [PubMed: 10706120]
11. Tcherepanova I, Harris J, Starr A, Cleveland J, Ketteringham H, Calderhead D, Horvatinovich J, et al. Multiplex RT-PCR amplification of HIV genes to create a completely autologous DC-based immunotherapy for the treatment of HIV infection. *PLoS One.* 2008; 3:e1489. [PubMed: 18231576]
12. Kustikova O, Brugman M, Baum C. The genomic risk of somatic gene therapy. *Semin Cancer Biol.* 2010; 20:269–278. [PubMed: 20600920]
13. Brown KN, Trichel A, Barratt-Boyes SM. Parallel loss of myeloid and plasmacytoid dendritic cells from blood and lymphoid tissue in simian AIDS. *J Immunol.* 2007; 178:6958–6967. [PubMed: 17513745]
14. Malleret B, Karlsson I, Maneglier B, Brochard P, Delache B, Andrieu T, Muller-Trutwin M, et al. Effect of SIVmac infection on plasmacytoid and CD11c+ myeloid dendritic cells in cynomolgus macaques. *Immunology.* 2008; 124:223–233. [PubMed: 18217951]
15. Szabolcs P, Park KD, Reese M, Marti L, Broadwater G, Kurtzberg J. Absolute values of dendritic cell subsets in bone marrow, cord blood, and peripheral blood enumerated by a novel method. *Stem Cells.* 2003; 21:296–303. [PubMed: 12743324]
16. Malleret B, Maneglier B, Karlsson I, Lebon P, Nascimbeni M, Perie L, Brochard P, et al. Primary infection with simian immunodeficiency virus: plasmacytoid dendritic cell homing to lymph nodes, type I interferon, and immune suppression. *Blood.* 2008; 112:4598–4608. [PubMed: 18787223]

17. Valladeau J, Saeland S. Cutaneous dendritic cells. *Semin Immunol.* 2005; 17:273–283. [PubMed: 15953735]
18. Reeves RK, Fultz PN. Characterization of plasmacytoid dendritic cells in bone marrow of pig-tailed macaques. *Clin Vaccine Immunol.* 2008; 15:35–41. [PubMed: 17989338]
19. Ebner S, Ehammer Z, Holzmann S, Schwingshackl P, Forstner M, Stoitzner P, Huemer GM, et al. Expression of C-type lectin receptors by subsets of dendritic cells in human skin. *Int Immunol.* 2004; 16:877–887. [PubMed: 15113774]
20. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, Briere F, et al. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity.* 2008; 29:497–510. [PubMed: 18789730]
21. Van Gorp H, Delputte PL, Nauwynck HJ. Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. *Mol Immunol.* 2010; 47:1650–1660. [PubMed: 20299103]
22. Bontkes HJ, De Gruijl TD, Schuurhuis GJ, Scheper RJ, Meijer CJ, Hooijberg E. Expansion of dendritic cell precursors from human CD34(+) progenitor cells isolated from healthy donor blood; growth factor combination determines proliferation rate and functional outcome. *J Leukoc Biol.* 2002; 72:321–329. [PubMed: 12149423]
23. Jonuleit H, Kuhn U, Muller G, Steinbrink K, Paragnik L, Schmitt E, Knop J, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol.* 1997; 27:3135–3142. [PubMed: 9464798]
24. Pinchuk LM, Grouard-Vogel G, Magaletti DM, Doty RT, Andrews RG, Clark EA. Isolation and characterization of macaque dendritic cells from CD34(+) bone marrow progenitors. *Cell Immunol.* 1999; 196:34–40. [PubMed: 10486153]
25. Caux C, Massacrier C, Vanbervliet B, Dubois B, Durand I, Cella M, Lanzavecchia A, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. *Blood.* 1997; 90:1458–1470. [PubMed: 9269763]
26. Barratt-Boyes SM, Henderson RA, Finn OJ. Chimpanzee dendritic cells with potent immunostimulatory function can be propagated from peripheral blood. *Immunology.* 1996; 87:528–534. [PubMed: 8675205]
27. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Kedl RM, Mattapallil JJ, Weiss WR, et al. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc Natl Acad Sci U S A.* 2005; 102:15190–15194. [PubMed: 16219698]
28. Haniffa M, Ginhoux F, Wang XN, Bigley V, Abel M, Dimmick I, Bullock S, et al. Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *J Exp Med.* 2009; 206:371–385. [PubMed: 19171766]
29. MacDonald KP, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DN. Characterization of human blood dendritic cell subsets. *Blood.* 2002; 100:4512–4520. [PubMed: 12393628]
30. Wang K, Nishimoto KP, Mehta RS, Nelson EL. An alternative flow cytometry strategy for peripheral blood dendritic cell enumeration in the setting of repetitive GM-CSF dosing. *J Transl Med.* 2006; 4:18. [PubMed: 16635268]
31. Geissmann F, Gordon S, Hume DA, Mowat AM, Randolph GJ. Unravelling mononuclear phagocyte heterogeneity. *Nat Rev Immunol.* 2010; 10:453–460. [PubMed: 20467425]
32. Angel CE, Lala A, Chen CJ, Edgar SG, Ostrovsky LL, Dunbar PR. CD14+ antigen-presenting cells in human dermis are less mature than their CD1a+ counterparts. *Int Immunol.* 2007; 19:1271–1279. [PubMed: 17804688]
33. Sparber F, Tripp CH, Hermann M, Romani N, Stoitzner P. Langerhans cells and dermal dendritic cells capture protein antigens in the skin: possible targets for vaccination through the skin. *Immunobiology.* 2010; 215:770–779. [PubMed: 20599290]
34. Unger WW, van Kooyk Y. ‘Dressed for success’ C-type lectin receptors for the delivery of glyco-vaccines to dendritic cells. *Curr Opin Immunol.* 2011; 23:131–137. [PubMed: 21169001]

35. Romani N, Thurnher M, Idoyaga J, Steinman RM, Flacher V. Targeting of antigens to skin dendritic cells: possibilities to enhance vaccine efficacy. *Immunol Cell Biol.* 2010; 88:424–430. [PubMed: 20368713]
36. Flacher V, Tripp CH, Stoitzner P, Haid B, Ebner S, Del Frari B, Koch F, et al. Epidermal Langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis. *J Invest Dermatol.* 2010; 130:755–762. [PubMed: 19890348]
37. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity.* 2008; 29:352–361. [PubMed: 18799143]
38. O'Doherty U, Ignatius R, Bhardwaj N, Pope M. Generation of monocyte-derived dendritic cells from precursors in rhesus macaque blood. *J Immunol Methods.* 1997; 207:185–194. [PubMed: 9368645]
39. Rozis G, Benlahrech A, Duraisingham S, Gotch F, Patterson S. Human Langerhans' cells and dermal-type dendritic cells generated from CD34 stem cells express different toll-like receptors and secrete different cytokines in response to toll-like receptor ligands. *Immunology.* 2008; 124:329–338. [PubMed: 18194273]
40. Ketloy C, Engering A, Srichairatanakul U, Limsalakpetch A, Yongvanitchit K, Pichyangkul S, Ruxrungtham K. Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. *Vet Immunol Immunopathol.* 2008; 125:18–30. [PubMed: 18571243]
41. Ratzinger G, Baggers J, de Cos MA, Yuan J, Dao T, Reagan JL, Munz C, et al. Mature human Langerhans cells derived from CD34+ hematopoietic progenitors stimulate greater cytolytic T lymphocyte activity in the absence of bioactive IL-12p70, by either single peptide presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells. *J Immunol.* 2004; 173:2780–2791. [PubMed: 15294997]
42. de Saint-Vis B, Fugier-Vivier I, Massacrier C, Gaillard C, Vanbervliet B, Ait-Yahia S, Banchereau J, et al. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J Immunol.* 1998; 160:1666–1676. [PubMed: 9469423]
43. Melhem NM, Liu XD, Boczkowski D, Gilboa E, Barratt-Boyes SM. Robust CD4+ and CD8+ T cell responses to SIV using mRNA-transfected DC expressing autologous viral Ag. *Eur J Immunol.* 2007; 37:2164–2173. [PubMed: 17615585]
44. Van Gulck ER, Vanham G, Heyndrickx L, Coppens S, Vereecken K, Atkinson D, Florence E, et al. Efficient in vitro expansion of human immunodeficiency virus (HIV)-specific T-cell responses by gag mRNA-electroporated dendritic cells from treated and untreated HIV type 1-infected individuals. *J Virol.* 2008; 82:3561–3573. [PubMed: 18234800]
45. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. *Nat Med.* 2004; 10:475–480. [PubMed: 15122249]
46. Routy JP, Nicolette C, Arcelis AGS-004 dendritic cell-based immunotherapy for HIV infection. *Immunotherapy.* 2010; 2:467–476. [PubMed: 20636001]
47. McLellan AD, Heiser A, Sorg RV, Fearnley DB, Hart DN. Dermal dendritic cells associated with T lymphocytes in normal human skin display an activated phenotype. *J Invest Dermatol.* 1998; 111:841–849. [PubMed: 9804348]
48. Van Gulck ER, Ponsaerts P, Heyndrickx L, Vereecken K, Moerman F, De Roo A, Colebunders R, et al. Efficient stimulation of HIV-1-specific T cells using dendritic cells electroporated with mRNA encoding autologous HIV-1 Gag and Env proteins. *Blood.* 2006; 107:1818–1827. [PubMed: 16263796]
49. Ponsaerts P, Van Tendeloo VF, Cools N, Van Driessche A, Lardon F, Nijs G, Lenjou M, et al. mRNA-electroporated mature dendritic cells retain transgene expression, phenotypical properties and stimulatory capacity after cryopreservation. *Leukemia.* 2002; 16:1324–1330. [PubMed: 12094257]
50. Martinon F, Kaldma K, Sikut R, Culina S, Romain G, Tuomela M, Adojaan M, et al. Persistent immune responses induced by a human immunodeficiency virus DNA vaccine delivered in association with electroporation in the skin of nonhuman primates. *Hum Gene Ther.* 2009; 20:1291–1307. [PubMed: 19627235]

51. Kutscher S, Dembek CJ, Allgayer S, Heltai S, Stadlbauer B, Biswas P, Nozza S, et al. The intracellular detection of MIP-1beta enhances the capacity to detect IFN-gamma mediated HIV-1-specific CD8 T-cell responses in a flow cytometric setting providing a sensitive alternative to the ELISPOT. *AIDS research and therapy*. 2008; 5:22. [PubMed: 18837993]
52. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry Part A: the journal of the International Society for Analytical Cytology*. 2011; 79:167–174. [PubMed: 21265010]

Figure 1.

Phenotype of APC subsets in different tissues of cynomolgus macaques. Six compartments were studied by flow cytometry: BM, peripheral blood, spleen (Spl), LN, epidermis (Epi), and dermis (Der). Cell suspensions were first gated for their morphology and viability (Supporting Information Fig. 1). Dot plots on the left column show Lineage (Lin: CD3, CD8 and CD20) and HLA-DR expression. APCs are gated as Lin⁻/HLA-DR⁺. Dot plots in the second and third columns show CD14/CD1a and CD123/CD11c expression in Lin⁻HLA-DR⁺ cells respectively. This gating strategy allows three populations of interest to be distinguished, referred to hereafter as subset 1 (CD14⁺CD11c⁻CD123⁻), subset 2 (CD14⁻CD11c⁺CD123⁻) and subset 3 (CD14⁻CD11c⁻CD123⁺). Histogram plots on the right show the expression of the maturation markers CD40, CD83 and CD86 (black line histograms) in the three populations defined in the dot plots on the left. Isotype-matched staining overlays are shown as gray-filled histograms. Data shown are from one macaque representative of four analyzed.

Figure 2.

Profiles of PRR expression in APC subsets from different tissues of cynomolgus macaques. Histogram plots display the expression of different receptors as analyzed by flow cytometry in subsets 1, 2 and 3 (defined in Figure 1). Cells stained with antibodies targeting APC-specific receptors are represented by black line histograms whereas those stained with isotype-matched controls are represented by gray-filled histograms. BM: Bone marrow; Spl: Spleen; LN: Lymph node; Epi: Epidermis; Der: Dermis. Data shown are from one macaque representative of four analyzed.

Figure 3.

Phenotype of CD34-DCs. BM CD34⁺ HPCs were cultured in vitro as described in the *Materials and methods*. Flow cytometry analyses were performed in order to identify the main cell subtypes derived under our culture conditions. Cells were first gated for their morphology and viability (Supporting Information Fig. 3). (A) Analysis of the CD34⁺ fraction at day 0 (top) and after 15 days of culture (bottom): DCs (Lin⁻HLA-DR⁺), granulocytes (HLA-DR⁻CD66abce⁺) and megakaryocytes/proplatelets (HLA-DR⁻CD41a⁺). Quadrant gates are positioned after non-specific isotype-matched staining analysis. (B) Analysis of CD34-DC HLA-DR⁺ cells after 15 days of in vitro culture. One representative experiment out of 13 performed is presented.

Figure 4.

Activated CD34-DCs express different patterns of APC functional markers. Immature DCs (imDCs) and mature DCs (matDCs) were defined according to the expression of CD83 and CD86. The expression of CD1a, CD14, CCR7, HLA-DR, CD40, CD1d and various C-type lectin receptors (DEC-205, MR, DC-ASGPR, DC-SIGN, langerin, DCIR, CLEC-6, dectin-1, LOX-1) or scavenger receptors (MARCO, CD163) is shown according to the mature (top) or immature (bottom) phenotype of CD34-DCs. Staining of each molecule is shown in the black line histograms while the isotype-matched controls are shown in gray-filled histograms. One experiment out of two with 3 replicates each is presented.

Figure 5.

Functional characterization of mature CD34-DCs. (A) Increasing ratios of matured CD34-DCs were cocultured with allogeneic CFSE-stained PBMCs for 7 days and analyzed by flow cytometry. Histogram plots illustrate the gating performed to measure the percentage of proliferating cells among live CD3⁺/CD4⁺ (top) or CD3⁺/CD8⁺ T cells (bottom) (see Supporting Information Fig. 4 for T lymphocyte gating strategy), with CD34-DCs (left) or without CD34-DCs (middle) as a negative control. Stimulation with Con A (right) was used as a positive control. The graph represents mean values $\pm$ SEM of data obtained with PBMCs from four macaques cocultured with CD34-DC from four other animals. Data are representative of 4 experiments. (B) The cytokines in the culture supernatants from unstimulated CD34-DCs (–; grey bars) and from CD34-DCs stimulated with the maturation cocktail for 24 h (+; black bars) were titrated by Multiplex. The data shown are the mean + SEM of four macaques; each assay was performed in duplicate. (C) CD34-DCs were

efficiently transfected by electroporation with *gag* mRNA. Cells were either mock-electroporated (gray-filled histogram) or electroporated in the presence of 20 µg of *gag* mRNA (black line histogram). Histogram plots on the right show the gating according to intracellular Gag content of the viable immature DC (bottom) and mature DC (top) gated according to the left dot plot. The percentage of Gag-positive cells is indicated according to the Overton statistic function available in FlowJo software. Data shown are from one macaque representative of four macaques tested. (D) In vivo fluorescence imaging 5 min, 2 h, 20 h and 44 h after i.d. and s.c. injections of labeled Gag-DC (top) and free non targeted Q-Dots (Qdot800, bottom) in the proximal region of the left and right inguinal LN respectively (exposure time: 50 ms). Left column corresponds to in vivo optical images performed after injections. Images are from one macaque representative of 2 analyzed.

Figure 6.

gag mRNA-loaded autologous CD34-DCs generate Gag-specific T-cell responses after injection into macaques. (A) Peak of the Gag-specific IFN- γ response, measured by ELISPOT, in PBMCs of macaques injected with *gag* mRNA-loaded CD34-DCs and or Gag protein alone as control after one (V1) or two immunizations (V2). * $p < 0.05$, Mann-Whitney non-parametric test. Four naïve macaques received two immunizations (week 0 and 4) of *gag* mRNA-loaded autologous CD34-DC ($5 - 15 \times 10^6$ cells) injected in the proximal zone of the inguinal draining lymph node, (half i.d., half s.c.). Two macaques (OBBT5 and OBEA5) received two additional injections of the same batches of *gag* mRNA-loaded autologous CD34-DCs. The kinetics of Gag-specific (B) IFN- γ responses and (C) IL-2 responses were assessed by ELISPOT in freshly isolated PBMCs. Means of triplicates were plotted for each animal. (D) Phenotype of Gag-mRNA transfected CD34-DC injected for vaccination. Comparison of the cells injected to the high responder animal OBBT5 and to the low responder animal OBEA5.

A CD8⁺ T cells **B CD4⁺ T cells** **Figure 7.**

gag mRNA-loaded CD34-DCs generate Gag-specific polyfunctional T-cell responses in vivo. The Gag-specific response at the cellular level was assessed by the intracellular staining of PBMCs derived from the high responder macaque #OBBT5 stimulated with a pool of overlapping peptides. Five functions were analyzed simultaneously (IFN- γ , IL-2, MIP1- β , TNF- γ , and CD154) in (A) CD8⁺ and (B) CD4⁺ T cells. Dot plots illustrate specific T-cell responses after the first (V1, week 2), second (V2, week 6) and last (V4, week 15) immunizations. Histograms show the breakdown of the Gag-specific CD8⁺ (A) and CD4⁺ (B) T-cell responses according to Spice analysis. Pie chart diagrams represent the proportion of polyfunctionality of the Gag-specific CD8⁺ (A) and CD4⁺ (B) T cells. Data are shown only for the high responder animal OBBT5.