The Journal of Immunology

RESEARCH ARTICLE | AUGUST 01 2004

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J Immunol (2004) 173 (3): 1647–1657.
https://doi.org/10.4049/jimmunol.173.3.1647

CpG-C Immunostimulatory Oligodeoxyribonucleotide Activation of Plasmacytoid Dendritic Cells in Rhesus Macaques to Augment the Activation of IFN-γ-Secreting Simian Immunodeficiency Virus-Specific T Cells[1]

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There are two principle subsets of dendritic cells (DCs); $CD11c^+CD123^-$ myeloid DCs (MDCs) and $CD11c^-CD123^+$ plasmacytoid DCs (PDCs). DC activation via TNF-TNFRs (e.g., CD40L) and TLRs (e.g., immunostimulatory oligodeoxyribonucleotides (ISS-ODNs)) is crucial for maximal stimulation of innate and adaptive immunity. Macaque DC biology is being studied to improve HIV vaccines using the SIV macaque model. Using lineage (Lin) markers to exclude non-DCs, $Lin^-HLA\text{-}DR^+CD11c^+CD123^-$ MDCs and $Lin^-HLA\text{-}DR^+CD11c^-CD123^+$ PDCs were identified in the blood of uninfected macaques and healthy macaques infected with SIV or simian-human immunodeficiency virus. Overnight culture of DC-enriched Lin-depleted cells increased CD80 and CD86 expression. IL-12 production and CD80/CD86 expression by MDC/PDC mixtures was further enhanced by CD40L and ISS-ODN treatment. A CpG-B ISS-ODN increased CD80/CD86 expression by PDCs, but resulted in little IFN-α secretion unless IL-3 was added. In contrast, a CpG-C ISS-ODN and aldrithiol-2-inactivated (AT-2) SIV induced considerable PDC activation and IFN-α release without needing exogenous IL-3. The CpG-C ISS-ODN also stimulated IL-12 release (unlike AT-2 SIV) and augmented DC immunostimulatory activity, increasing SIV-specific T cell IFN-γ production induced by AT-2 SIV-presenting MDC/PDC-enriched mixtures. These data highlight the functional capacities of MDCs and PDCs in naive as well as healthy, infected macaques, revealing a promising CpG-C ISS-ODN-driven DC activation strategy that boosts immune function to augment preventative and therapeutic vaccine efficacy. *The Journal of Immunology,* **2004, 173: 1647–1657.**

Dendritic cells (DCs)[4] are central to innate and adaptive immunity. Myeloid DCs (MDCs, $CD11c^+$) and plasmacytoid DCs (PDCs, $CD123^+$) have been identified in humans (1, 2). PDCs produce high levels of IFN-α on stimulation (3, 4), potentially contributing to innate responses to pathogens. DCs activated or matured by signaling through TNF-TNFRs and TLR family members are crucial in initiating adaptive immune responses by strongly activating Ag-specific naive T cells (5) and also help determine the character of T cell responses (6–8).

Interactions between viruses and DCs are complex, revealing different outcomes that potentially favor Ag-specific immunity (9) or viral spread (10–12). For example, PDCs produce IFN-α in response to HIV infection (13). Type I IFNs are important in innate antiviral defense, inhibiting HIV replication (14–16) and stimulating monocytes, NK cells (17), and T cells (18, 19). IFN-α likely has a critical role in directing the activation of potent Ag-specific Th1 immune responses (1, 20–22). IFN-α produced by PDCs activates and matures MDCs, resulting in enhanced Ag presentation and raising the issue of cross-talk between DC populations in contributing to development of effective immunity (21, 23–26). In vitro, HIV infection of PDCs can lead to increased viability and maturation, potentially facilitating more effective antiviral immune responses (13). However, HIV infection of PDCs (13, 27), resulting in enhanced viability, may also initially maintain infected target cells, promoting virus amplification and spread. Presumably reflecting the net effects of these competing influences, the number of PDCs in blood and levels of IFN-α produced decrease in advanced HIV infection (28, 29). Initial infection of PDCs to promote viral amplification and dissemination, followed by dampening of PDC functions, may represent an aspect of HIV pathogenesis by which the virus facilitates infection through interfering with PDC-MDC interplay critical for the stimulation of optimal antiviral immunity. Because many alterations of DCs (28–33) and other APCs (34, 35) have been reported in HIV disease, and the basis of these alterations and their impact on pathogenesis is only poorly understood, additional studies of the interactions between viruses and these critical cells are clearly warranted.

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Received for publication February 24, 2004. Accepted for publication June 2, 2004.

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[1] This work received significant support from the Elizabeth Glaser Pediatric AIDS Foundation, as well as from National Institutes of Health Grants R21 AI52060 and R01 AI40877 (to M.P.), and the Tulane National Primate Research Center Base Grant RR00164. This work was also funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, Contract NO1-CO-12400. M.P. is an Elizabeth Glaser Scientist.

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[4] Abbreviations used in this paper: DC, dendritic cell; MDC, myeloid DC; PDC, plasmacytoid DC; LN, lymph node; ISS-ODN, immunostimulatory oligodeoxyribonucleotide; AT-2, aldrithiol-2; SHIV, simian-human immunodeficiency virus; MFI, mean fluorescence intensity; wt, wild type; SFC, spot-forming cells; SEB, *Staphylococcal enterotoxin* B; Lin, lineage.

0022-1767/04/$02.00

An effective way to trigger DC activation coincident with IFN-α release is readily achieved by immunostimulatory CpG-containing oligodeoxyribonucleotides (ISS-ODNs), resulting in the induction of strong immune responses (36–38). Recently described CpG-C ISS-ODNs activate both PDCs (sustained IFN-α release) and B cells, whereas CpG-B ISS-ODNs directly activate B cells and upregulate CD80/CD86 on PDCs but induce only low-level IFN-α secretion (39, 40). Hence, CpG-C ISS-ODN activation of PDCs (and B cells) represents an interesting strategy to boost DC activity and augment innate and adaptive antiviral immunity.

The SIV macaque model provides a unique system in which anti-HIV vaccine and therapeutic approaches can be investigated (41). Macaque and human DCs exhibit comparable biology (42–45), with more recent work confirming the presence of MDCs and PDCs in circulation (46–48). Aldrithiol-2 (AT-2)-inactivated SIV that is noninfectious yet retains functional surface proteins, interacts authentically with DCs (49, 50), making it a useful tool to monitor the presentation of DC-captured virions for immune stimulation (44, 51); similar results have been obtained in vitro with AT-2 HIV and human DCs (52, 53). In this study, we examined macaque PDCs and MDCs, to determine whether ISS-ODNs could be used to exploit DCs to bolster innate and adaptive responses against AT-2 SIV. We identified circulating DCs in naive and infected healthy macaques, developed a strategy to enrich DCs from blood, and characterized the functional properties of enriched DCs, such as their responsiveness to CD40L and ISS-ODN stimulation as well as their ability to activate SIV-specific T cells. CpG-C ISS-ODN and AT-2 SIV induced significant activation of and IFN-α release by DCs coincident with activation of IFN-γ-secreting SIV-specific T cells. Our data highlight the functional capacities of macaque MDCs and PDCs, revealing potential DC activation strategies to boost immune function to augment vaccine efficacy.

Materials and Methods

Animals and treatment

Adult male and female Indian rhesus macaques (*Macaca mulatta*) were bred and housed at the Tulane National Primate Research Center (TNPRC; Covington, LA). At the commencement of these studies, all naive animals tested negative by PCR for simian type D retroviruses, simian T cell leukemia virus, and SIV. Animals (AR 76, AR 83, AR 84, AT 62, L964, M284, PO75, PO98) were inoculated via i.v. injection of 2.35×10^4 tissue culture ID_{50} of SIVmac239 Δ*nef* 15 wk before being challenged i.v. with 10^2 tissue culture ID_{50} of SIVmac239. This protocol is known to prime strong immune responses against SIV (54). Two additional animals (L507 and N992) available from a previous study (55) received cell-associated or cell-free Δ*nef* injected s.c. and were i.v. challenged with SIVmac239 11 mo later. Such Δ*nef*-vaccinated and wild-type (wt) challenged animals (Δ*nef*/wt) show strong SIV-specific immune responses (44, 51) and have not shown evidence of SIV-associated disease over at least 2–6 years of post-challenge follow up.

We also studied macaques infected intravaginally (AP85, T820, BD19) or i.v. (T122, T833, T660, T373, T528, CB79, TO27) with simian-human immunodeficiency virus (SHIV, SHIV162P) 2–6 years ago. Comparable to the Δ*nef*/wt macaques, PBMCs from these animals secrete IFN-γ in response to AT-2 SIV in vitro (our unpublished observations). SHIV-infected and Δ*nef*/wt macaques were used as a source of SIV-primed T cells to monitor the presentation of AT-2 SIV by MDC/PDC mixtures in vitro. Animals were anesthetized with ketamine/HCl (10 mg/kg) before all procedures. Heparinized blood samples were taken (10 ml/kg/mo). Superficial lymph nodes (LNs) were surgically removed from two uninfected animals. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the TNPRC. Animal care procedures were in compliance with the regulations detailed under the animal welfare act and in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC).

Cell isolation

PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Total LN cell suspensions were prepared following published protocols (56, 57). PBMCs were used to isolate DCs or split to isolate both DCs and T cells. DCs were enriched by depleting cells expressing CD3, CD8, CD11b, CD14, CD20 lineage markers $(Lin)^+$. We developed a two-step depletion strategy using the Miltenyi AutoMACS System (Miltenyi Biotec, Auburn, CA). PBMCs were incubated with CD20 beads (non-human primate; Miltenyi Biotec) followed by collection of the $CD20^-$ fraction. $CD20^-$ cells were incubated with FITC anti-CD20 (clone L27; BD Immunocytometry Systems, Mountain View, CA), anti-CD3 (clone SP34; BD Pharmingen, San Diego, CA), anti-CD8 (clone SK1; BD Immunocytometry Systems), followed by incubation with a mixture of anti-FITC, anti-CD11b (non-human primate), and anti-CD14 (non-human primate) beads (Miltenyi Biotec), and a collection of the Lin^- fraction (AutoMACS program Depletes, Miltenyi Biotec). T cells were obtained by incubating PBMCs or $CD20^-$ cells with FITC anti-CD16 (clone 3G8; BD Pharmingen) followed by anti-FITC, anti-CD14, anti-CD11b, and anti-HLA-DR (human) beads to remove APCs and NK cells. T cells were typically >95% pure as determined by two-color staining for CD3 vs HLA-DR, CD11b, CD14, and CD20 (data not shown). In a few experiments, $CD14^+$ monocytes and $CD20^+$ B cells were depleted with anti-CD14 and anti-CD20 beads (AutoMACS program Depletes) to enrich DCs and T cells in the mixtures (90–95% $CD14^-CD20^-$ by FACS) with limited manipulation of the cells.

FACS analysis

To characterize DCs, three- or four-color flow cytometry was used. $CD123^-$ (MDC-containing) cells and $CD123^+$ PDCs were identified within $Lin^-HLA\text{-}DR^+$ populations using FITC anti-Lin Abs (anti-CD3, anti-CD8, anti-CD11b (clone F6.2; Exalpha Biologicals, Watertown, MA), anti-CD14 (clone M5E2; BD Pharmingen), anti-CD20 with allophycocyanin anti-HLA-DR (clone G46-6; BD Pharmingen), and PE anti-CD123 (clone 7G3; BD Pharmingen). Because macaque monocyte-derived DCs are $CD16^+CD56^+$ (44) and monkey NK cells are $CD56^-$ (H. Andersen, J. L. Rossio, V. Coalter, B. Poore, M. P. Martin, M. Carrington, and J. D. Lifson, manuscript in preparation), anti-CD11b and anti-CD8 were included to stain NK cells to avoid exclusion of $CD16^+CD56^+$ DCs. Of note, a potential minor subset of $Lin^-HLA\text{-}DR^+CD8^+$ DCs (48) is excluded using this strategy. Initially, $CD11c^+$ MDCs were identified in $Lin^-HLA\text{-}DR^+$ cells stained with PE anti-Lin Abs (same clones as FITC anti-Lin), allophycocyanin anti-HLA-DR, and FITC-CD11c (clone 3.9; Serotec, Raleigh, NC) (see Fig. 1*A*). $Lin^-HLA\text{-}DR^+$ populations looked similar when staining with FITC or PE anti-Lin Abs (see Fig. 1*A* vs Figs. 2*A* and 3*A*) and FITC anti-Lin Abs were used routinely, unless stated otherwise. To simultaneously define $CD11c^-CD123^+$ PDCs and $CD11c^+CD123^-$ MDCs as well $CD11c^-CD123^-$ cells within $Lin^-HLA\text{-}DR^+$ cells, FITC anti-Lin, PerCP-CyChrome anti-HLA-DR (clone G46-6; BD Pharmingen), PE anti-CD123, and allophycocyanin anti-CD11c (clone S-HCL-3; BD Immunocytometry Systems) were used. CyChrome anti-CD80 (clone L307.4; BD Pharmingen) or anti-CD86 (clone 2331 FUN1; BD Pharmingen) were used to monitor CD80 and CD86 expression by $Lin^-HLA\text{-}DR^+CD123^-$ cells and $Lin^-HLA\text{-}DR^+CD123^+$ PDCs. Leukocytes were gated based on forward and side scatter characteristics to exclude neutrophils. $Lin^-HLA\text{-}DR^+$ cells within these gated leukocytes were further analyzed as indicated. Isotype Ig controls for each fluorochrome were included in all experiments and typically gave mean fluorescence intensities (MFIs) <1 log. These isotype controls were used to set the gates for calculating the subset percentages.

Medium and reagents

Cells were cultured at $1–5 \times 10^5$ cells/well in 96-well plates (Microtest; BD Labware, Franklin Lakes, NJ) in 200–250 μl of complete medium (RPMI 1640 (Cellgro; Fisher Scientific, Springfield, NJ) containing 2 mM L-glutamine (Invitrogen Life Technologies, Grand Island, NY), 10 mM HEPES (Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen Life Technologies), and 1% heparinized human plasma). ODNs used in the study were: 1018, 5′-TGACTGTGAACGTTCGAGATGA (CpG-B ISS-ODN); C274, 5′-TCGTCGAACGTTCGAGATGAT (CpG-C ISS-ODN); 1040, 5′-TGACTGTGAACCTTAGAGATGA (CpG-B control); and C661, 5′-TGCTTGCAAGCTTGCAAGCA (CpG-C control) (39). ODNs were provided by Dr. G. Van Nest (Dynavax Technologies, Berkeley, CA).

As seen in human studies, C274 works more effectively and at lower concentrations than 1018 (39). Initial titration studies (2–20 μg/ml) using macaque cells (measuring IFN-α release and CD80/CD86 up-regulation) confirmed that 1018 was optimal at 10 μg/ml (although responses were seen against 2–5 μg/ml in some donors) and C274 was optimal at 2–5 μg/ml (5 μg/ml being more consistent; data not shown). 1018 was typically used at 10 μg/ml and C274 at 5 μg/ml, unless stated otherwise. Human CD40L and IL-3 (R&D Systems, Minneapolis, MN) were used at a final concentrations of 1 μg/ml and 20 ng/ml, respectively. Chemically inactivated, noninfectious SIVmneE11S (AT-2 SIV) was provided by the AIDS Vaccine Program (Science Applications International Corp-Frederick, National Cancer Institute, Frederick, MD). Virus content of purified concentrated preparations was determined with an Ag capture immunoassay for SIV gag p27 (AIDS Vaccine Program) and/or by HPLC analysis (58). Virus stocks were diluted in 1% BSA (Intergen, New York, NY) in PBS and stored as aliquots (3 μg of p27 Eq/ml) at −80°C. Thawed aliquots were kept at 4°C for 1 wk or less and AT-2 SIV used at final concentrations of 3–300 ng of p27/ml.

PBMC and Lin$^-$ cell cultures

PBMCs or Lin$^-$ cells were cultured with the indicated stimuli overnight. After 24 h, cell-free supernatants were collected and stored at −20°C before ELISA analysis. Cell-free supernatants were analyzed for the presence of IL-12 p70 and the free p40 subunit and IFN-α (BioSource International, Camarillo, CA). Cultured cells were collected, viable cells counted by trypan blue exclusion and assayed by flow cytometry.

Loading of Lin$^-$ cells with AT-2 SIV

For assays of immune function, DC-enriched (Lin$^-$) cells (up to 0.5×10^6) were isolated from Δ*nef*/wt or SHIV-infected macaques. Cells were placed into 1.5-ml Eppendorf tubes, pelleted, resuspended with AT-2 SIV (30 ng of p27/10^5 cells) in 50 μl, and incubated for 1 h at 37°C. Control cells were incubated with the equivalent amount of buffer (1% BSA/PBS). The cells were then washed five times to remove cell-free virus (49), and viable cells were recounted by trypan blue exclusion and used in IFN-γ ELISPOT assays.

IFN-γ ELISPOT

DCs pulsed with AT-2 SIV or sham-pulsed with buffer were mixed with autologous T cells and cultured with (or without) C274 or 1040 (5 μg/ml) or CD40L (1 μg/ml). Alternatively, CD14$^-$CD20$^-$ DC-T cell-enriched mixtures were cultured with AT-2 SIV (3–300 ng of p27/ml) in the presence or absence of 5 μg/ml C274 vs C661. IFN-γ ELISPOT assays were performed as previously described (59). Briefly, Lin$^-$ DC-T cell mixtures (4×10^3–10^4 Lin$^-$ cells and 2×10^5 T cells) or CD14$^-$CD20$^-$ cells (2×10^5 cells) were plated in duplicate or triplicate for 24–36 h on Millipore HA membrane 96-well plates coated with anti-IFN-γ Abs (clone MD-1; BioSource International). The cells were lysed and the plates were developed using biotinylated rabbit, anti-rhesus IFN-γ Abs (BioSource International), streptavidin-alkaline phosphatase (Sigma-Aldrich), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Spots were counted using an AID ELISPOT reader (Cell Technology, Columbia, MD) with once optimized settings through all experiments. Background IFN-γ release by cells cultured in medium in the absence of additional Ags varied between the infected donors (contrasting the levels spontaneously released by cells from naive animals; higher levels reflective of circulating activated cells likely as a result of infection). These ranged from 2 to 111 spot-forming cells (SFC)/2 $\times$ 10^5 cells, averaging 25.94 ± 7.6 SFC/2 $\times$ 10^5 cells (mean ± SEM from 11 independent experiments). This includes data from two animals, which consistently gave higher backgrounds (59.39 ± 13.4, mean ± SEM from three experiments). All experiments comparing the ability of C274 with boost SIV-specific IFN-γ responses were performed using other animals giving lower background values (13.4 ± 3.18, mean ± SEM from eight animals). When compared, the ODNs did not alter the background IFN-γ production (14.96 ± 2.79 for control ODNs and 16.83 ± 3.32 for ISS-ODNs, mean ± SEM from nine experiments). In each experiment, one quarter of the cells (2.5×10^3 Lin$^-$ DCs and 5×10^4 T cells) were cultured with 5 ng/ml *Staphylococcal enterotoxin* B (SEB; Toxin Technologies, Sarasota, FL, vs medium) to control for T cell and APC functionality.

Statistical analyses

The nonparametric Kruskal-Wallis rank analysis was used to evaluate percentages of Lin$^-$HLA-DR$^+$ APCs as well as DC subsets in naive, Δ*nef*/wt and SHIV-infected animals.

Results

Identification of Lin$^-$HLA-DR$^+$ subsets in uninfected and healthy, infected macaques

To evaluate strategies for augmenting DC function to improve preventative and therapeutic vaccines, we studied Lin$^-$HLA-DR$^+$ DC subsets in the blood of uninfected macaques or healthy macaques infected with Δ*nef*/wt or SHIV-162P (Fig. 1). Representative FACS plots are provided for naive and Δ*nef*/wt animals demonstrating the small subsets of Lin$^-$HLA-DR$^+$ APCs in blood and LN (Fig. 1*A*). Due to the higher frequency of blood DCs and the greater accessibility of blood compared with LNs, blood DCs were more extensively examined. The percentages of Lin$^-$HLA-DR$^+$ cells among PBMCs were comparable between animal groups (3–4%, Fig. 1*B*). Within Lin$^-$HLA-DR$^+$ cells, CD123$^-$ cells predominated: 91.85, 94.15, and 88.81% in naive, Δ*nef*/wt, and SHIV-infected animals, respectively (Fig. 1*B*). The percentage of CD123$^+$ PDCs was highest in SHIV-infected animals (11.25% vs 8.25 and 5.66% in naive and Δ*nef*/wt-infected animals). Statistical

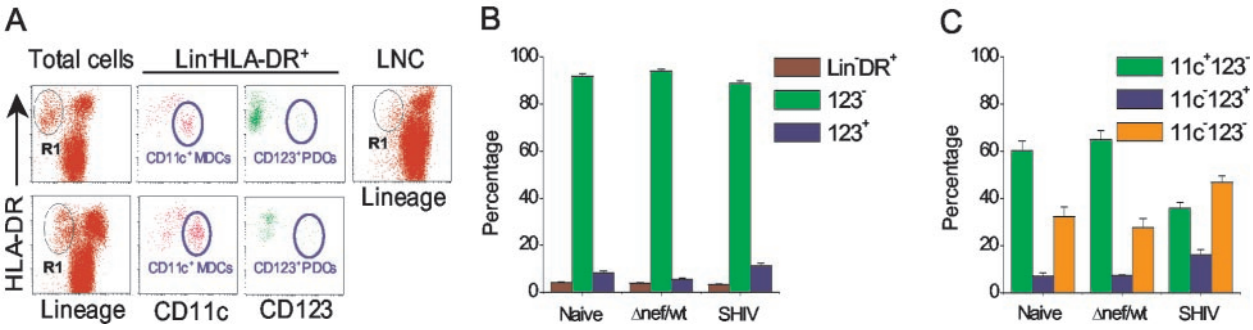

FIGURE 1. DC subsets in the blood and LNs of macaques. *A*, PBMCs (*left* three, *top* and *bottom rows*) and LN cells (LNC) from naive (*right top row*) or Δ*nef*/wt (*bottom row*) animals were stained with PE or FITC anti-Lin, allophycocyanin anti-HLA-DR, and FITC anti-CD11c or PE anti-CD123. Representative Lin$^-$HLA-DR$^+$ cells (R1) in the total cell suspensions (PE Lin vs allophycocyanin HLA-DR) is shown as a representative example. CD11c$^+$ MDCs and CD123$^+$ PDCs (oval gates) within the gated Lin$^-$HLA-DR$^+$ cells (R1) are shown for blood. *B*, PBMCs were stained with FITC anti-Lin, allophycocyanin anti-HLA-DR, and PE anti-CD123. The percentages (mean ± SEM) of Lin$^-$HLA-DR$^+$ cells in PBMCs (Lin$^-$DR$^+$, burgundy) and the HLA-DR$^+$CD123$^-$ (green) vs HLA-DR$^+$CD123$^+$ (blue) cells within the DC subset from 15 naive (31 experiments), 10 Δ*nef*/wt (28 experiments), and 10 SHIV-infected (20 experiments) animals are shown. *C*, PBMCs stained with FITC anti-Lin, PerCP-CyChrome anti-HLA-DR, allophycocyanin anti-CD11c, and PE anti-CD123 were used to better define the APCs within the Lin$^-$HLA-DR$^+$ cells. The percentages (mean ± SEM) of CD11c$^+$CD123$^-$ (green), CD11c$^-$CD123$^+$ (blue), and double negative CD11c$^-$CD123$^-$ (orange) cells within the Lin$^-$HLA-DR$^+$ subset from 6 naive (8 experiments), 7 Δ*nef*/wt (11 experiments), 10 SHIV-infected (20 experiments) animals are shown.

analysis revealed that percentages of Lin$^-$HLA-DR$^+$ cells did not differ between the groups. Although there was no significance difference between the CD123$^-$ and CD123$^+$ subsets in naive animals and either infected animal group, there was a significant difference between the frequencies of both subsets in SHIV- vs Δ*nef*/wt-infected animals (CD123$^-$, $p < 0.0009$ and CD123$^+$, $p < 0.0005$).

To identify CD11c$^+$CD123$^-$ MDCs, CD11c$^-$CD123$^+$ PDCs, and double negative CD11c$^-$CD123$^-$ cells (comprising CD11c$^-$CD123$^-$ DCs or precursors and/or HLA-DR$^+$CD11c$^-$CD20$^-$ B cells not stained by the Lin mixture), PBMCs were stained with FITC anti-Lin, PerCP-CyChrome anti-HLA-DR, allophycocyanin anti-CD11c and PE anti-CD123. Similar to the three-color analyses shown in Fig. 1*B*, CD11c$^-$CD123$^+$ PDCs comprised the smallest subset in all three groups of animals (Fig. 1*C*). Approximately 60% of the Lin$^-$HLA-DR$^+$ cells were CD11c$^+$CD123$^-$ MDCs in naive and Δ*nef*/wt animals, whereas only ~35% of the Lin$^-$HLA-DR$^+$ cells in the blood of SHIV-infected animals were CD11c$^+$CD123$^-$ MDCs (Fig. 1*C*). Conversely, the SHIV-infected animals possessed the highest proportion of CD123$^+$CD11c$^-$ PDCs (15.97 vs ~7% in the naive and Δ*nef*/wt animals) (Fig. 1*C*). The difference in CD11c$^+$CD123$^-$ MDC percentages was significant between naive and SHIV-infected animals ($p < 0.0002$) as well as between SHIV- and Δ*nef*/wt-infected animals ($p < 0.0002$). The percentages of CD11c$^-$CD123$^+$ PDCs and CD11c$^-$CD123$^-$ cells in SHIV-infected animals differed significantly from Δ*nef*/wt animals ($p < 0.026$ and $p < 0.002$, respectively).

To further characterize the Lin$^-$HLA-DR$^+$ cells, we monitored CD80 and CD86 expression. Circulating Lin$^-$HLA-DR$^+$ cells in naive and healthy, infected animals exhibit an immature phenotype expressing low levels of CD80 and CD86 (Fig. 2). The expression of both molecules was comparable among the groups with CD86 being slightly higher in infected animals (especially on the CD123$^+$ PDCs), although CD86 expression was lowest on CD123$^+$ PDCs in each group. There were no statistically significant differences in CD80/CD86 expression by freshly isolated DCs.

Enrichment of DCs from macaque blood

For more accurate monitoring of the Lin$^-$HLA-DR$^+$ cells, Lin$^+$ cells were depleted from PBMCs, enriching the Lin$^-$HLA-DR$^+$ cells to 33–48% in all three groups of animals (Fig. 3). The yield of isolated Lin$^-$ cells relative to starting PBMCs was 0.7% (averaged from 51 experiments using samples from naive and infected animals). The remaining Lin$^+$HLA-DR$^+$ cells in the enriched fractions (e.g., Fig. 3*A*) were mostly CD20$^+$ B cells (data not shown). As in PBMCs (Fig. 1, *B* and *C*), the majority of DC-enriched cells were CD123$^-$; 89.51, 98.09, and 94.51% CD123$^-$ cells in Lin$^-$HLA-DR$^+$ cells from naive, Δ*nef*/wt, and SHIV-infected animals, respectively (three-color analysis; data not shown).

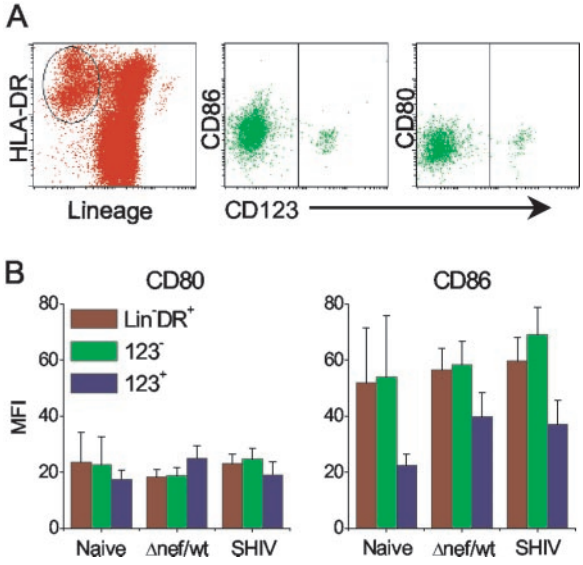

FIGURE 2. Expression of CD86 and CD80 on circulating DCs. *A*, Expression of CD86 and CD80 was documented on CD123$^+$ and CD123$^-$ cells gated first as Lin$^-$HLA-DR$^+$ cells (*left*, oval gate). A representative FACS analysis of naive macaque PBMCs is shown and is illustrative of PBMCs from infected animals. The rectangular gates in the green plots indicate the settings used to delineate the CD123$^+$ and CD123$^-$ fractions within the Lin$^-$HLA-DR$^+$ cells. *B*, The MFI (± SEM) of CD80 and CD86 expression on Lin$^-$HLA-DR$^+$ cells (Lin$^-$DR$^+$, burgundy), CD123$^-$ cells (green), and CD123$^+$ DCs (blue) in freshly isolated PBMCs from naive (23–24 experiments, 10–11 animals), Δ*nef*/wt (27–28 experiments, 10 animals) and SHIV (16 experiments, 10 animals) infected animals are shown.

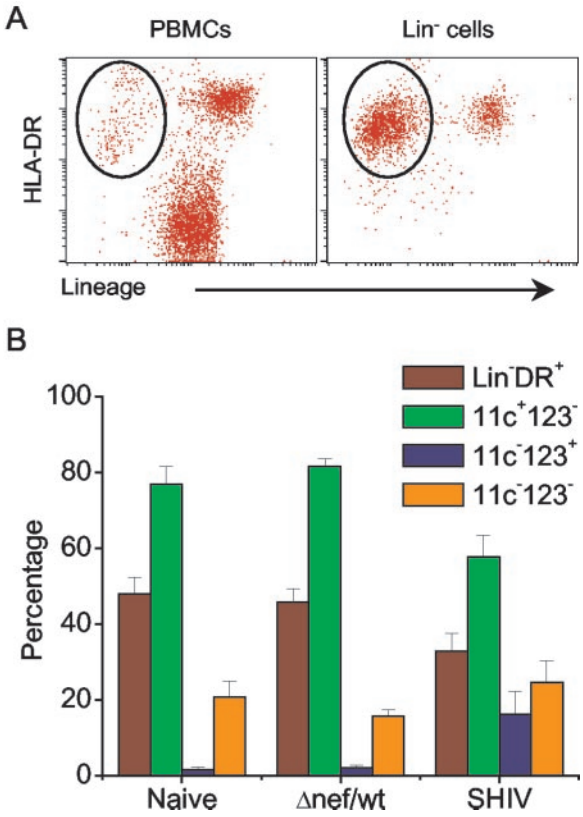

FIGURE 3. DC enrichment following depletion of Lin$^+$ cells. *A*, Macaque PBMCs were isolated and an aliquot kept for FACS. The remainder of the cells was depleted of Lin$^+$ cells. PBMCs and Lin$^-$ cells were stained with FITC anti-Lin and allophycocyanin anti-HLA-DR highlighting the increased Lin$^-$HLA-DR$^+$ DC frequency in Lin$^-$ cells (oval gates). An example (naive animal) is shown that is representative for naive and infected samples. *B*, Lin$^-$ cells were stained with FITC anti-Lin, allophycocyanin anti-HLA-DR, and PE anti-CD123 and the mean percentages ± SEM of enriched Lin$^-$HLA-DR$^+$ cells (Lin$^-$DR$^+$, burgundy) were determined (12 naive animals, 22 experiments; 10 Δ*nef*/wt animals, 22 experiments; 7 SHIV-infected animals, 13 experiments). Staining with FITC anti-Lin, PerCP-CyChrome anti-HLA-DR, allophycocyanin anti-CD11c, and PE anti-CD123 was used to identify the specific APC subsets within the Lin$^-$HLA-DR$^+$ cells. Mean percentages (± SEM) of CD11c$^+$CD123$^-$ (green), CD11c$^-$CD123$^+$ (blue), and CD11c$^-$CD123$^-$ (orange) subsets within the Lin$^-$HLA-DR$^+$ cells are shown for naive (6 experiments, 4 animals), Δ*nef*/wt (12 experiments, 7 animals), and SHIV (11 experiments, 7 animals) infected animals.

More detailed analysis by four-color flow cytometry of Lin-depleted cells confirmed that the proportions of $CD11c^{-}CD123^{+}$ PDCs, $CD11c^{+}CD123^{-}$ MDCs, and $CD11c^{-}CD123^{-}$ APCs were comparable to those seen in the $Lin^{-}HLA\text{-}DR^{+}$ fraction of the PBMCs of each group of animals, with a slight increase in the percentages of $CD11c^{+}CD123^{-}$ MDCs (~58–82%) and a relative decrease in the percentages of $CD123^{+}$ PDCs (~2–16%) and double negative cells (~16–25%) after Lin-depletion (Fig. 3*B*). In all, PDCs represent a minor fraction compared with the MDCs.

CpG-B ISS-ODN 1018 activation of macaque DCs

Having defined characteristic DC subsets in macaque blood, we set out to determine their responsiveness to CD40L and ISS-ODNs and to assess whether there were differences between DCs from naive and healthy, infected animals. Lin^{-} cells were cultured for 24 h with CD40L or ISS-ODNs (vs control ODN). Lin^{-} cells from naive animals (12 experiments) survived better than those from Δ*nef*/wt animals (seven to nine experiments; 63–72% vs 30–34% viable recovery). The higher ends of each range indicate the slightly enhanced viability of cells cultured with ODNs (comparable between control and stimulatory ODNs). Lin^{-} cells from naive animals also survived better than cells from Δ*nef*/wt-infected animals upon culture with CD40L (58%, five experiments vs 31%, seven experiments), further suggesting that SIV infection may interfere with DC functions.

After culture of Lin^{-} cells (initially comprising ~32–48% $Lin^{-}HLA\text{-}DR^{+}$ cells (e.g., Fig. 3*B*), the $Lin^{-}HLA\text{-}DR^{+}$ cell percentage decreased by about one-third coincident with a decrease in the percentage of $CD123^{+}$ PDCs by ~40% and a relative increase of ~6% in the $CD123^{-}$ fraction. An increase in $CD11c^{-}CD123^{-}$ APCs within the $CD123^{-}$ cells was at least in part attributed to down-regulation of CD11c by $CD11c^{+}$ MDCs (data not shown). PDCs are particularly sensitive to in vitro culture (9, 60, 61) and this can be remedied by the inclusion of IL-3 (9, 60). However, the few experiments performed with Lin^{-} DCs obtained from naive or SHIV-infected monkeys showed no dramatic effect of IL-3 and ISS-ODNs on macaque DC survival rate (data not shown), despite some effects on functional capacities (see below).

Similar to data in humans (62, 63), macaque DCs are activated simply during culture at 37°C (Fig. 4*A*). This phenomenon can also account for increased CD80 and CD86 expression on total $Lin^{-}HLA\text{-}DR^{+}$ cells and individual subsets upon culture with 1040 (Fig. 4, *B* and *C*) compared with the expression of both markers on DCs by freshly isolated PBMCs (Fig. 2*B*). These observations were true for cells from naive and infected animals being more pronounced in infected animals (Fig. 4*C*), suggesting that the cells in infected animals possessed heightened activation. Macaque Lin^{-} cells cultured with 1018 (vs 1040), up-regulated CD80 and CD86 on $CD123^{+}$ PDCs (Fig. 4, *A–C*). CD80 was more dramatically increased compared with CD86 on cells from naive (Fig. 4*B*) and infected (Fig. 4*C*) macaques. Slightly elevated CD80 was observed on $Lin^{-}HLA\text{-}DR^{+}CD123^{-}$ cells, probably due to bystander activation (Fig. 4, *B* and *C*). Relative to CD40L, this effect of 1018 on DCs was reproducibly stronger and the CD80 and CD86 expression after CD40L stimulation (on total DCs and individual subsets) was only moderately elevated over that induced by overnight culture (compare medium-cultured cells to those before culture; data not shown).

We also monitored cytokine release by stimulated Lin^{-} cell cultures, focusing on IFN-α and IL-12 responses. Similar to human cells (39, 64), 1018 induced IL-12 and (low-level) IFN-α production by macaque Lin^{-} cells (Fig. 4, *D* and *E*). In agreement with recent findings (46), macaque Lin^{-} cells cultured in medium released IL-12 (indicative of culture-induced activation) and this

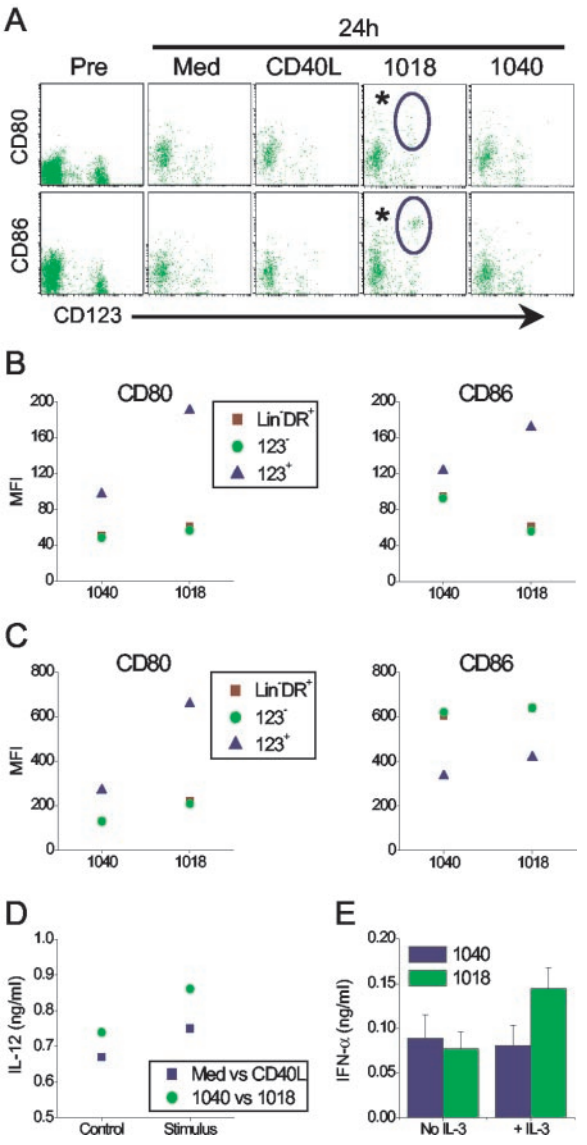

FIGURE 4. Macaque blood PDCs respond to classical PDC stimuli. Lin^{-} cells (Pre) were cultured for 24 h in medium (Med) or in the presence of CD40L (1 μg/ml), 1018 or 1040 control ODN (10 μg/ml) (2×10^{5} cells/well, 96-well U-bottom plate). After culture, the cells were stained with FITC anti-Lin, allophycocyanin anti-HLA-DR, PE anti-CD123, and either CyChrome anti-CD80 or anti-CD86. *A*, provides representative FACS data of the $Lin^{-}HLA\text{-}DR^{+}$ cells before and after culture with the indicated stimuli. $CD123^{+}$ PDCs with elevated CD80 and CD86 levels in response to 1018 are indicated in the oval gates. ∗, moderate bystander activation of $CD123^{-}$ cells is indicated. Increased CD80 (less so CD86) expression by the medium-cultured $CD123^{-}$ cells compared with the preculture cells is evident. Note that CD40L had little additional impact on CD80/CD86 expression. MFIs of CD80 and CD86 on cells after culture with 1040 vs 1018 are summarized for $Lin^{-}HLA\text{-}DR^{+}$ ($Lin^{-}DR^{+}$), $CD123^{-}$ and $CD123^{+}$ cells from 14 to 15 experiments for (9–10) naive animals (*B*) and from six experiments using cells from six infected animals (*C*) (gated as shown in Fig. 2*A*). Cell-free supernatants were collected from Lin^{-} cells cultured for 24 h in 200 μl of medium alone or in the presence of CD40L (1 μg/ml), 1040 or 1018 (2–5 μg/ml; separate assays comparing 10–20 μg/ml 1018 vs 1040 induced comparably low levels of IFN-α release, data not shown). IL-3 (20 ng/ml) was included (+IL-3) or not in the ODN cultures. Samples were stored at −20°C until being assayed for the presence of IL-12 (*D*) or IFN-α (*E*) by ELISA (mean values). *D*, The averaged data from 10 to 11 experiments (3–4 naive animals, 5 Δ*nef*/wt-infected animals) are shown. Similar IL-12 release was observed in the presence of IL-3 (eight experiments, data not shown). *E*, The averaged data from 12 to 16 animals are shown (mean ± SEM).

was marginally (but consistently) increased by CD40L (Fig. 4*D*). 1018 induced comparable increases in IL-12 production over the background 1040-stimulated levels (slightly above those seen in medium-cultured cells) (Fig. 4*D*). Despite the elevated CD80 and CD86 on 1018-activated PDCs, IFN-α production was negligible unless IL-3 was added (Fig. 4*E*).

CpG-C ISS-ODN C274 is a more potent stimulus for macaque DCs

Recent human data indicated that CpG-C ISS-ODNs exhibit very potent activity, enhancing IFN-γ and IFN-α responses significantly higher than CpG-B ISS-ODNs (39, 40). To ascertain whether CpG-C ISS-ODNs would also stimulate macaque cells more effectively, we cultured macaque Lin$^-$ cells with the CpG-C ISS-ODN C274 and monitored DC phenotypes and functions. Similar to 1018, C274 induced an increase of CD80 and (less so) CD86 mainly on CD123$^+$ macaque PDCs (Fig. 5*A*, both uninfected and infected animals). C274 also induced IL-12 production (Fig. 5*B*). Notably, unlike 1018, C274 stimulated detectable levels of IFN-α release by PBMCs (Fig. 5*C*) and Lin$^-$ cells (Fig. 5*D*) even in the absence of IL-3. IL-3 may have had a slight boosting effect on the IFN-α released by PBMCs, but this was not apparent for Lin$^-$ cells (in which DCs are enriched). As expected (39), C274 works more effectively at lower doses than 1018 (2–5 μg/ml vs 10 μg/ml; data not shown). C274 induced an ~8-fold increase in IFN-α production over that induced by the control 1040, compared with the ~2-fold increase in IFN-α release induced by 1018 and only in the presence of IL-3.

Inactivated SIV increases CD80 and CD86 on PDCs and stimulates IFN-α production

HSV, Influenza virus, measles virus, and HIV induce PDC maturation and the release of type I IFNs (9, 65–67). Even AT-2 HIV was recently shown to activate human PDCs (26). Because AT-2 SIV is also being evaluated as a candidate vaccine immunogen in macaques and is being used as the SIV Ag in this model, we thought it important to determine whether AT-2 SIV itself could additionally contribute to DC activation and the subsequent induction of virus-specific T cells.

Culture of macaque cells with AT-2 SIV induced marked increases in CD80 and CD86 expression by PDCs from naive as well as healthy, infected macaques, without significant bystander activation of the MDC-containing fractions (Fig. 6*A*). AT-2 SIV also induced IFN-α production by PBMCs in a dose-dependent manner without requiring additional IL-3 (Fig. 6*B*, *left panel*). This was even more striking in the Lin$^-$ cells that tended to release higher levels in response to AT-2 SIV compared with the other stimuli (Fig. 6*B*, *right panel*). These responses were consistent for naive and healthy, infected macaque cells. Much like the recent report in humans (26), we observed only a subtle increase (if any) in IL-12 production in response to AT-2 SIV (535 pg/ml vs 515 pg/ml for medium-cultured cells; five experiments).

C274 boosts the immunostimulatory capacity of PDC/MDC mixtures

Focusing on the more potent ISS-ODN C274, we explored whether we could augment the APC activity of Lin$^-$ cells by first monitoring their ability to augment autologous T cell responses to SEB.

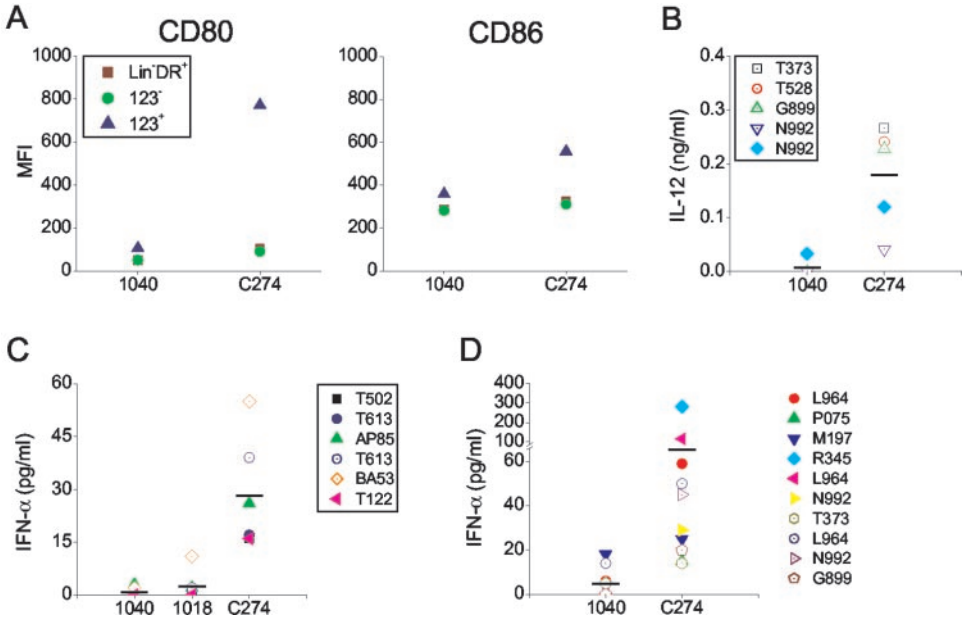

FIGURE 5. CpG-C ISS-ODN activation of Lin$^-$HLA-DR$^+$ cells and induction of stronger IL-12 and IFN-α responses. Lin$^-$ cells (1–2 $\times$ 10^5 cells) (*A*, *B*, and *D*) or PBMCs (5 $\times$ 10^5 cells) (*C*) were cultured in 200–250 μl of medium containing 1040 or C274 (2–5 μg/ml) (per well of a 96-well plate). IL-3 (20 ng/ml) was included (open symbols, two and four experiments with PBMCs and Lin$^-$ cells, respectively) or not (solid symbols, four and seven experiments with PBMCs and Lin$^-$ cells, respectively) as indicated. After culture, the cells were stained with FITC anti-Lin, allophycocyanin anti-HLA-DR, PE anti-CD123, and either CyChrome anti-CD80 or anti-CD86. *A*, MFIs of CD80 and CD86 on the indicated subsets (Lin$^-$HLA-DR$^+$ (Lin$^-$DR$^+$), CD123$^-$, CD123$^+$) within the Lin$^-$ cells after culture (9 experiments for 3 naive, 2 Δ*nef*/wt, and 2 SHIV-infected animals). Cell-free supernatants were collected and stored at −20°C until assayed for the presence of IL-12 or IFN-α by ELISA. The horizontal black bars in each graph indicate the mean values for each. *B*, C274 induced IL-12 production by Lin$^-$ cell cultures. The results are shown for 1 naive monkey (G899), 1 Δ*nef*/wt (N992), and 2 SHIV (T373, T528) infected monkeys. *C*, C274 stimulated higher IFN-α production compared with 1018 in PBMCs. The individual macaques included are three naive monkeys (T502, T613, BA53) and two SHIV-infected (AP85, T122) monkeys. *D*, C274 consistently induced IFN-α production by Lin$^-$ cells in 11 experiments for three naive (M197, R345, G899), three Δ*nef*/wt (L964, PO75, N992), and one SHIV-infected (T373) animals.

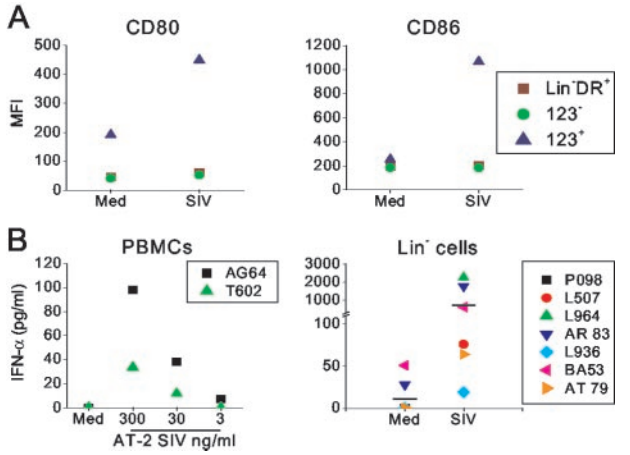

FIGURE 6. AT-2 SIV activates Lin$^-$ cells and induces IFN-α release. PBMCs or Lin$^-$ cells were cultured in the presence of 3–300 ng AT-2 SIV per ml (p27 Eq; 30 ng/ml for the Lin$^-$ cells). Cells were later stained (24 h) with FITC anti-Lin, allophycocyanin anti-HLA-DR, PE anti-CD123, and either CyChrome anti-CD80 or anti-CD86. Cell-free supernatants were collected and assayed for the presence of IFN-α by ELISA. *A*, PDCs up-regulated CD80 and CD86 expression in response to AT-2 SIV. Mean values of CD80 and CD86 MFIs expressed by the Lin$^-$HLA-DR$^+$ (Lin$^-$DR$^+$, burgundy), CD123$^-$ (green), and CD123$^+$ (blue) cells from one Δ*nef*/wt-infected and two naive macaques are shown. *B*, AT-2 SIV induced dose-dependent release of IFN-α by PBMCs derived from two naive (AG64, T602) animals (*left*) and Lin$^-$ cells from two naive (BA53, AT 79, L936) and four Δ*nef*/wt-infected (PO98, L507, AR 83, L964) (*right*). The mean (*right*, bar) IFN-α produced after culture of Lin$^-$ cells with medium (Med) vs AT-2 SIV.

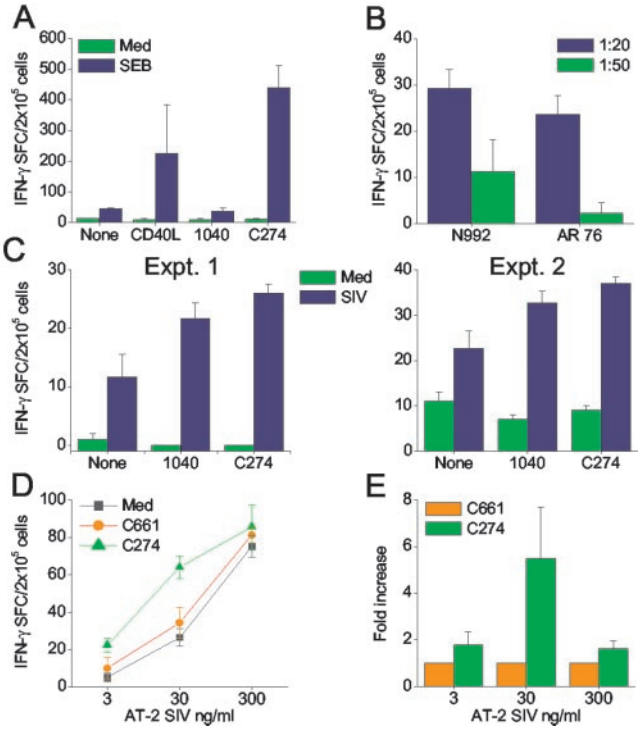

FIGURE 7. C274 augments the immunostimulatory activity of enriched DC-T cell cocultures. *A*, PBMCs from a SHIV-infected animal (T373) were split to obtain the Lin$^-$ MDC/PDC-enriched fraction from one-half and the T cells from the other half (depleted of HLA-DR, CD11b, CD14-expressing cells; >98% pure by FACS). Lin$^-$ cells were mixed with autologous T cells (2.5×10^3 Lin$^-$ cells, 5×10^4 T cells in 100 μl) in the IFN-γ ELISPOT plate in the presence of CD40L (1 μg/ml), C274 (vs 1040, 5 μg/ml), or in medium alone (None). SEB was added (SEB) or not (Med) at a final concentration of 5 ng/ml. Later (~24 h), the plate was developed and counted. The mean SFC per 2×10^5 cells (± SEM, duplicate wells) are shown. These data are representative of results from another four animals (naive and infected). *B*, Lin$^-$ MDC/PDC-enriched cells and T cells were isolated from PBMCs of two Δ*nef*/wt animals (N992 and AR 76; as for Fig. 7*A*). The Lin$^-$ cells were pulsed with AT-2 SIV (30 ng p27/10^5 cells) or medium, washed, recounted and mixed with 2×10^5 autologous T cells at the indicated ratios in the IFN-γ ELISPOT plate. Later (~24 h), the plate was developed and counted. The mean SFC values for cells cultured in medium alone (N992, 87.17 ± 6.69 and AR 76, 42.67 ± 2.97 mean ± SEM SFC/2×10^5 cells) have been subtracted to show SIV-specific responses (mean ± SEM from triplicate wells). Despite the higher background IFN-γ release by these two animals (see *Materials and Methods*), SIV-specific IFN-γ responses could still be measured above this background. *C*, Lin$^-$ MDC/PDC-enriched cells and T cells were isolated from PBMCs of SHIV-infected animals (two representative experiments). The Lin$^-$ cells were pulsed with AT-2 SIV (SIV (blue), 30 ng p27/10^5 cells; vs medium, Med (green)), washed, recounted and 10^4 mixed with 2×10^5 autologous T cells in the presence of 5 μg/ml 1040 or C274 or medium (None) in an IFN-γ ELISPOT plate. ~24 h later, the plate was developed and counted. The mean SFC per 2×10^5 cells (± SEM, duplicate or triplicate wells) are shown. These results reflect data obtained from four infected animals. *D*, CD14$^-$CD20$^-$ cells from a SHIV-infected animal (T122) were cultured (2×10^5 per well in 100 μl) in the presence of medium, control ODN C661 or C274 (5 μg/ml) and titrated doses of AT-2 SIV (3–300 ng p27/ml). One experiment representative of three experiments is shown. Background values (28–33 SFC/2×10^5 cells) have been subtracted to show SIV-specific responses. *E*, The mean fold increases (± SEM) in SIV-specific IFN-γ released by CD14$^-$CD20$^-$ cells cultured with varying doses of AT-2 SIV in the presence of C274 relative to the C661 controls from three different SHIV-infected donors are shown (the mean SFC for the C274/AT-2 SIV and the CC61/AT-2 SIV cultures have been divided by the mean SFC of the C661/AT-2 SIV control cultures).

CD40L and, more dramatically, C274 significantly increased the IFN-γ responses to SEB (Fig. 7*A*). This was true for cells from both naive and healthy, infected animals. This highlights that DCs and contaminating B cells within the Lin$^-$ cells are activated by these stimuli boosting their immunostimulatory abilities.

We also evaluated the impact of C274 on the presentation of AT-2 SIV. AT-2 SIV-loaded mature monocyte-derived DCs activate SIV-specific CD4$^+$ and CD8$^+$ T cells in vitro (51). Because depletion of HLA-DR$^+$ cells reproducibly ablated the IFN-γ response of PBMCs to AT-2 SIV (but B cells were not involved) (51), it is likely that HLA-DR$^+$ MDCs and PDCs are major contributors as APCs. To investigate their contribution directly, we examined the capacity of AT-2 SIV-loaded Lin$^-$ cells to stimulate SIV-specific IFN-γ-secreting T cells. Comparable to our studies using monocyte-derived DCs (44, 51), AT-2 SIV-pulsed Lin$^-$ cells induced SIV-specific IFN-γ release and this was dependent on the number of Lin$^-$ cells added to the cultures (Fig. 7*B*). No IFN-γ release was observed in autologous cultures of AT-2 SIV-loaded Lin$^-$ cells and T cells from naive animals (data not shown).

Both CD4$^+$ and CD8$^+$ T cells respond to AT-2 SIV in PBMCs (51), suggesting that the culture-induced activation of the DCs (as previously described) allows them to present the AT-2 SIV much like the mature monocyte-derived DCs (51). However, we wanted to ascertain whether additional activation via C274 could further boost these responses. Simply adding C274 to PBMCs did not augment the IFN-γ released in response to AT-2 SIV (data not shown). However, culture of AT-2 SIV-pulsed Lin$^-$ cells (significantly enriched for DCs) and autologous SIV-primed T cells with C274 resulted in ~2-fold greater IFN-γ responses compared with the untreated control (Fig. 7*C*). The difference between C274 and 1040 was less apparent, likely due to nonspecific DC activation by 1040 (as seen with IL-12 release, Fig. 4*D*) (39).

Having formally shown that circulating DCs enriched in the Lin^- fraction could capture and present viral Ags and that C274 seemed to bolster this activity, we wanted to examine the enhancing effect of C274 more closely. Because we had seen nonspecific activation of the DCs by the control ODN 1040 (much like the human system (39)), we chose to use the newly defined CpG-C control ODN, C661 (not originally available for the initial studies) that has been shown to elicit less background activation in human cell cultures (39). Moreover, given that Lin^+ cell depletion yields a limiting number of enriched DCs, we depleted $CD14^+$ and $CD20^+$ cells from PBMCs to enrich the DCs and T cells with a minimal amount of manipulation while providing sufficient cell numbers. This allowed us to more accurately study the impact of C274 stimulation on responses to different concentrations of AT-2 SIV. Similar to what was observed after culture of AT-2 SIV-pulsed Lin^- cells with autologous SIV-primed T cells (Fig. 7*C*), C274 increased the SIV-specific IFN-γ release by $CD14^-CD20^-$ cells cultured with AT-2 SIV, especially at lower AT-2 SIV doses (Fig. 7, *D* and *E*). Notably, the C661 control ODN induced less nonspecific activation (similar to cells cultured in the absence of ODNs) compared with the 1040 ODN (as compared with Fig. 7, *C* and *D*). Relative to the cells cultured in the presence of C661, C274 increased the SIV-specific IFN-γ release by 1.6- to 5.5-fold, being most effective at the intermediate AT-2 SIV dose (Fig. 7*E*). C274 was unable to significantly augment the already strong IFN-γ response to the highest Ag dose (300 ng/ml) and had a similarly moderate effect when Ag was limiting (3 ng/ml; e.g., Fig. 7*D*). Interestingly, low-level IFN-α release was detected in these cultures when 30–300 ng/ml AT-2 SIV was added in conjunction with C274 but not C661 (data not shown), suggestive of synergistic effects of CpG-C ISS-ODN and virus, which are dependent on the virus dose. In summary, C274 triggering of PDC activation, coincident with IFN-α and IL-12 release, correlates with increased ability to stimulate SIV-specific IFN-γ release and the whole inactivated virus may synergize with C274 to induce stronger IFN-γ responses.

Discussion

Strategies to enhance DC function represent one way via which vaccine efficiency could be augmented, by ensuring that the vaccine elicits robust innate and adaptive responses to prevent new or control established infections. We are exploring this in the SIV macaque system to advance anti-HIV vaccines. We phenotypically and functionally characterized circulating DCs in naive and healthy, infected macaques. This report is the first to demonstrate that comparable populations of functional DCs can be enriched from the blood of naive and healthy, infected macaques. Although DC numbers may vary slightly between groups, the cells exhibited similar responsiveness to various DC stimuli. Notably, enriched MDC/PDC mixtures were activated with CpG-B and, more effectively, CpG-C ISS-ODN and AT-2 SIV to induce IFN-α secretion and efficient SIV Ag presentation activating IFN-γ-secreting SIV-specific T cells.

Similar to earlier reports (46, 47), we show that macaque blood contains $CD11c^+$ MDCs, $CD123^+$ PDCs, and $CD11c^-CD123^-$ cells. The percentages of DCs in naive macaque PBMCs were slightly higher (4.07 ± 0.4) than the 1% demonstrated by Coates et al. (46). This can probably be attributed to the different staining and analytical strategies used to define the DCs (i.e., due to the variable contamination by neutrophils, neutrophils were not included in the gate for our analyses and anti-CD11b and anti-CD8 (not anti-CD16 or anti-CD56) were included to stain NK cells). SHIV-infected and Δ*nef*/wt animals had similar blood DC frequencies. Analogous to human, the lowest frequency of PDCs in Δ*nef*/wt-infected animals may also represent an important correlate of the course of SIV infection in macaques (29). Although basically healthy, these Δ*nef*/wt-infected animals were challenged with wild-type virus 2–6 years ago and the shift in DC frequency may be suggestive of disease progression despite their CD4 counts remaining relatively stable (data not shown). This supports the idea that the decrease in PDC numbers might reflect a better predictor of disease progression than CD4 counts (29). Additional research is required to determine the reason for the higher percentage of $CD123^+$ PDCs in the SHIV-infected animals. In agreement with human (62, 63, 68, 69) and more recent macaque (46, 48) data, DCs expressed low CD80 and CD86, indicating an immature phenotype unaffected by infection.

To study ex vivo DC functions, Lin^-HLA-DR^+ cells were enriched from PBMCs, allowing MDC-PDC interplay, which probably better reflects the in vivo situation than studies on purified populations. CpG-B and CpG-C ISS-ODNs increase CD80 and CD86 expression and induce IFN-α production by human PDCs, with CpG-C ISS-ODNs being more efficient even at lower doses (39, 40). Similarly, enriched MDC/PDC populations from naive and infected macaques responded to 1018 and C274 by up-regulating CD80 and CD86. The cells were also responsive to CD40L stimulation and increased CD80/CD86, although this increase was modest compared with responses to ISS-ODNs. Despite differences in DC proportions, DC responsiveness to these stimuli was comparable between naive and infected animals. Of note, CD80 and CD86 up-regulation was greatest in PDCs from infected animals suggesting that they possessed increased sensitivity to activation as a result of infection. Interestingly, the increase of CD80 expression in response to ISS-ODNs was more pronounced in all animals (compared with CD86). In DCs, MHC-II colocalizes with costimulatory molecules before reaching the cell surface (70), where MHC class II and CD80 are present in the same clusters before CD40 ligation, providing ligands for the TCR and CD28, respectively. These clusters coaggregate into larger clusters to become a higher avidity target for T cells, which may result in more efficient T cell activation (71). Hence, the up-regulation of CD80 upon DC stimulation bodes well for potent immunostimulatory function and augmentation of vaccine immunogenicity by ISS-ODNs.

Both CD40L and 1018 stimulation induced moderate increase in IL-12 production, which is important for stimulation of Th1 immune responses (72, 73). However, 1018 stimulation alone was not sufficient to induce IFN-α production and required additional IL-3. Even then, the levels were only doubled. Previously, IL-3 alone was demonstrated to maintain PDC viability, induce PDC maturation and increase production of TNF-α but not IFN-α (74). The amount of IFNs produced is tightly regulated by cytokines (75). However, in the absence of stimuli (e.g., viral infection or ISS-ODN activation), cytokines fail to induce IFN-α production. Although the presence of IL-3 did not appear to significantly impact macaque DC viability, it was able to augment IFN-α release upon costimulation with 1018. The former may be a complication of using recombinant human cytokines with macaque cells and/or simply differences between the responsiveness of macaque vs human PDCs to IL-3.

Consistent with data in humans (39), C274 was a more efficient stimulus (not requiring exogenous IL-3) for macaque PDCs inducing ~4-fold more IFN-α production by MDC/PDC-enriched cultures than 1018 (with IL-3). The potency of C274 to induce IFN-α secretion was particularly apparent in PBMC cultures, in which the effect of 1018 was negligible. It is important to note that although the patterns of responsiveness to 1018 vs C274 were comparable between macaque and human cells, the magnitudes of the IFN-α

released by macaque cells were lower. The stronger IFN-α responses induced by C274 coincided with moderate IL-12 release and significant up-regulation of CD80 and (less so) CD86, all attributes important for the activation of Th1 adaptive responses. In agreement with increasing evidence from other systems demonstrating CpG ISS-ODN stimulation of chemokine responses (36, 39, 76–78), preliminary analysis also indicates that macaque cells secrete chemokines in response to ISS-ODN stimulation (i.e., MCP-1/CCL2 and moderate RANTES/CCL5 and MIP-1α/CCL3 detected to date; data not shown).

Interestingly, similar high levels of IFN-α release and PDC activation were observed after culturing cells with AT-2 SIV. This concurs with earlier work that HIV can induce IFN-α production (79) and that this is not dependent on infection (13, 26). Therefore, in addition to being a promising vaccine candidate (80, 81), AT-2 virus may also contribute to directly activating PDCs to boost immunity (26). In fact, synergistic effects were detected when C274 and AT-2 SIV were added to $CD14^-CD20^-$ cultures resulting in greater amounts of IFN-α (and IFN-γ) release (compared with that released in response to AT-2 SIV and the control ODN). Of note, however, AT-2 SIV and HIV (26) alone do not induce strong IL-12 responses reflecting one way via which immunodeficiency viruses can circumvent potent immunity in the absence of additional DC stimuli (82).

Because we hypothesized that C274 can be an effective stimulus for PDCs and subsequently for MDCs (through maturation of MDCs via IFN-α), we tested whether C274 could boost the immunostimulatory activity of MDC/PDC-enriched Lin^- cells and augment responses against AT-2 SIV. We previously showed that AT-2 SIV activates both $CD4^+$ and $CD8^+$ T cells in PBMCs from Δ*nef*/wt (44, 51) and, more recently, SHIV (our unpublished observations) infected macaques. Moreover, mature monocyte-derived DCs stimulate mixed $CD4^+$ and $CD8^+$ T cell responses whereas immature monocyte-derived DCs induce primarily $CD4^+$ T cells (51). Maturation of macaque MDCs and PDCs during overnight culture explains how $CD4^+$ and $CD8^+$ T cells were activated in PBMCs cultured with AT-2 SIV; culture-activated MDCs (and possibly PDCs) presenting AT-2 SIV like mature monocyte-derived DCs. By loading Lin^- cells directly with AT-2 SIV, we provide evidence that macaque PDC/MDC mixtures capture virus and present Ags to activate SIV-specific IFN-γ-secreting T cells, not unlike what was recently reported for AT-2 HIV in the human system (26). Notably, in the Lin^- cell-T cell mixtures and the DC/T cell-enriched $CD14^-CD20^-$ cultures, where higher numbers of DCs are present compared with PBMCs, these responses were augmented up to 5.5-fold by C274 (above those responses induced by the already maturing DCs). It is unlikely that these enhanced responses are simply due to improved DC viability in the presence of the ODNs because DC viability was comparable in the presence of control and stimulatory ODNs, typically $<$1.2-fold more viable than cells cultured in medium. These data represent the first evidence the PDC/MDC mixtures can present AT-2 SIV-derived Ags to boost SIV-specific T cell responses and that these responses are amplified by CpG-C ISS-ODNs.

Although we know B cells are activated by C274 (39) (our unpublished observations), we believe they are playing little role in the presentation of AT-2 SIV, because earlier work confirmed that depletion of $CD20^+$ B cells had no impact on the IFN-γ responses of the SIV-primed PBMCs responding to AT-2 SIV (51). Additionally, the more dramatic effect of C274 on the Lin^- cell triggering of SEB-driven responses, highlights the involvement of the activated B cells and DCs as APCs in this study (compared with the DC-dominated role for SIV-specific responses in which the effect of C274 is more subtle).

Our results suggest that using CpG-C ISS-ODNs to augment the immunogenicity of SIV/HIV vaccines is a useful approach. The boosting effects of C274 documented in this model will likely be more pronounced in vivo (possibly even more so when stimulating primary SIV responses in naive T cells), in the absence of culture-induced DC activation. Preliminary in vivo data has confirmed that C274 effectively activates LN-derived DCs and B cells in vivo and that the synergistic effects of C274 and AT-2 SIV are extremely pronounced upon culturing such in vivo C274-activated cells with AT-2 SIV in vitro (dramatic up-regulation of CD80 and CD86 as well as significant IFN-α production; N. Teleshova and M. Pope, unpublished observations). Despite the subtle differences in the percentages of each DC subset in the naive vs infected animals, the fact that DCs derived from naive and healthy, infected macaques were equally responsive to ISS-ODNs indicates that theses agents could be used for preventative as well as therapeutic vaccines. The ability of C274 to induce IL-12 and IFN-α by PDCs is extremely important to consider for vaccine purposes. Besides its direct antiviral effect and the bystander activation of MDCs, locally produced IFN-α in response to HIV/SIV can protect Ag-specific T cells from Ag-induced apoptosis (83, 84) and inhibit B cell apoptosis (85). Therefore, early production of IFNs in response to HIV/SIV invasion represents an important mechanism regulating consequent events. The most important finding of this study is ability of C274 and AT-2 SIV to activate macaque DCs and synergize to enhance the presentation of AT-2 SIV-derived Ags for the induction of SIV-specific IFN-γ T cell responses. Our data emphasize a potential use in targeting macaque MDCs and PDCs to boost antiviral immunity and augment vaccine efficacy.

Acknowledgment

We thank Irving Sivin for assistance with statistical analysis.

References

1. Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell 106:259*.
2. Liu, Y. J., H. Kanzler, V. Soumelis, and M. Gilliet. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol. 2:585*.
3. Siegal, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science 284:1835*.
4. Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med. 5:919*.
5. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.
6. Kalinski, P., C. M. U. Hilkens, A. Snijders, F. G. M. Snijdewint, and M. L. Kapsenberg. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E_2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol. 159:28*.
7. Rissoan, M. C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt, and Y. J. Liu. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science 283:1183*.
8. Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med. 189:587*.
9. Kadowaki, N., S. Antoneko, J. Y.-N. Lau, and Y.-J. Liu. 2000. Natural interferon-α/β-producing cells link innate and adaptive immunity. *J. Exp. Med. 192:219*.
10. Bosio, C. M., M. J. Aman, C. Grogan, R. Hogan, G. Ruthel, D. Negley, M. Mohamadzadeh, S. Bavari, and A. Schmaljohn. 2003. Ebola and Marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation. *J. Infect. Dis. 188:1630*.
11. Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol. 18:861*.
12. Engelmayer, J., M. Larsson, M. Subklewe, A. Chahroudi, A. Schmaljohn, C. William, R. M. Steinman, and N. Bhardwaj. 1999. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J. Immunol. 163:6762*.
13. Yonezawa, A., R. Morita, A. Takaori-Kondo, N. Kadowaki, T. Kitawaki, T. Hori, and T. Uchiyama. 2003. Natural α-interferon-producing cells respond to human immunodeficiency virus type 1 with α-interferon production and maturation into dendritic cells. *J. Virol. 77:3777*.

14. Yamamoto, J. K., F. Barre-Sinoussi, V. Bolton, N. C. Pedersen, and M. B. Gardner. 1986. Human α- and β-interferon but not γ- suppress the in vitro replication of LAV, HTLV-III, and ARV-2. *J. Interferon Res. 6:143*.
15. Hartshorn, K. L., D. Neumeyer, M. W. Vogt, R. T. Schooley, and M. S. Hirsch. 1987. Activity of interferons α, β, and γ against human immunodeficiency virus replication in vitro. *AIDS Res. Hum. Retroviruses 3:125*.
16. Poli, G., J. M. Orenstein, A. Kinter, T. M. Folks, and A. S. Fauci. 1989. Interferon-α but not AZT suppresses HIV expression in chronically infected cell lines. *Science 244:575*.
17. Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol. 9:24*.
18. Sun, S., X. Zhang, D. F. Tough, and J. Sprent. 1998. Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med. 188:2335*.
19. Noisakran, S., and D. J. Carr. 2000. Plasmid DNA encoding IFN-α 1 antagonizes herpes simplex virus type 1 ocular infection through $CD4^+$ and $CD8^+$ T lymphocytes. *J. Immunol. 164:6435*.
20. Santini, S. M., T. Di Pucchio, C. Lapenta, S. Parlato, M. Logozzi, and F. Belardelli. 2003. A new type I IFN-mediated pathway for the rapid differentiation of monocytes into highly active dendritic cells. *Stem Cells 21:357*.
21. Carbonneil, C., A. Aouba, M. Burgard, S. Cardinaud, C. Rouzioux, P. Langlade-Demoyen, and L. Weiss. 2003. Dendritic cells generated in the presence of granulocyte-macrophage colony-stimulating factor and IFN-α are potent inducers of HIV-specific CD8 T cells. *AIDS 17:1731*.
22. Mattei, F., G. Schiavoni, F. Belardelli, and D. F. Tough. 2001. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J. Immunol. 167:1179*.
23. Luft, T., P. Luetjens, H. Hochrein, T. Toy, K. A. Masterman, M. Rizkalla, C. Maliszewski, K. Shortman, J. Cebon, and E. Maraskovsky. 2002. IFN-α enhances CD40 ligand-mediated activation of immature monocyte-derived dendritic cells. *Int. Immunol. 14:367*.
24. Wiesemann, E., D. Sonmez, F. Heidenreich, and A. Windhagen. 2002. Interferon-β increases the stimulatory capacity of monocyte-derived dendritic cells to induce IL-13, IL-5 and IL-10 in autologous T-cells. *J. Neuroimmunol. 123:160*.
25. Santini, S. M., C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. 2000. Type I Interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J. Exp. Med. 191:1777*.
26. Fonteneau, J. F., M. Larsson, A. S. Beignon, K. McKenna, I. Dasilva, A. Amara, Y. J. Liu, J. D. Lifson, D. R. Littman, and N. Bhardwaj. 2004. Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J. Virol. 78:5223*.
27. Patterson, S., A. Rae, N. Hockey, J. Gilmour, and F. Gotch. 2001. Plasmacytoid dendritic cells are highly susceptible to human immunodeficiency virus type 1 infection and release infectious virus. *J. Virol. 75:6710*.
28. Donaghy, H., A. Pozniak, B. Gazzard, N. Qazi, J. Gilmour, F. Gotch, and S. Patterson. 2001. Loss of blood $CD11c^+$ myeloid and $CD11c^-$ plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. *Blood 98:2574*.
29. Soumelis, V., I. Scott, F. Gheyas, D. Bouhour, G. Cozon, L. Cotte, L. Huang, J. A. Levy, and Y. J. Liu. 2001. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood 98:906*.
30. Macatonia, S. E., R. Lau, S. Patterson, A. J. Pinching, and S. C. Knight. 1990. Dendritic cell infection, depletion and dysfunction in HIV infected individuals. *Immunology 71:38*.
31. Pacanowski, J., S. Kahi, M. Baillet, P. Lebon, C. Deveau, C. Goujard, L. Meyer, E. Oksenhendler, M. Sinet, and A. Hosmalin. 2001. Reduced blood $CD123^+$ (lymphoid) and $CD11c^+$ (myeloid) dendritic cell numbers in primary HIV-1 infection. *Blood 98:3016*.
32. Grassi, F., A. Hosmalin, D. McIlroy, V. Calvez, P. Debre, and B. Autran. 1999. Depletion in blood CD11c-positive dendritic cells from HIV-infected patients. *AIDS 13:759*.
33. Chehimi, J., D. E. Campbell, L. Azzoni, D. Bacheller, E. Papasavvas, G. Jerandi, K. Mounzer, J. Kostman, G. Trinchieri, and L. J. Montaner. 2002. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. *J. Immunol. 168:4796*.
34. Moir, S., A. Malaspina, K. M. Ogwaro, E. T. Donoghue, C. W. Hallahan, L. A. Ehler, S. Liu, J. Adelsberger, R. Lapointe, P. Hwu, et al. 2001. HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. *Proc. Natl. Acad. Sci. USA 98:10362*.
35. Rich, E. A., Z. Toossi, H. Fujiwara, R. Hanigosky, M. M. Lederman, and J. J. Ellner. 1988. Defective accessory function of monocytes in human immunodeficiency virus-related disease syndromes. *J. Lab. Clin. Med. 112:174*.
36. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol. 20:709*.
37. Gursel, M., D. Verthelyi, and D. M. Klinman. 2002. CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells. *Eur. J. Immunol. 32:2617*.
38. Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol. 31:3026*.
39. Marshall, J. D., K. Fearon, C. Abbate, S. Subramanian, P. Yee, J. Gregorio, R. L. Coffman, and G. Van Nest. 2003. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J. Leukocyte Biol. 73:781*.
40. Hartmann, G., J. Battiany, H. Poeck, M. Wagner, M. Kerkmann, N. Lubenow, S. Rothenfusser, and S. Endres. 2003. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-α induction in plasmacytoid dendritic cells. *Eur. J. Immunol. 33:1633*.
41. Desrosiers, R. C. 1990. The simian immunodeficiency viruses. *Annu. Rev. Immunol. 8:557*.
42. O'Doherty, U., R. Ignatius, N. Bhardwaj, and M. Pope. 1997. Generation of monocyte-derived cells from the precursors in rhesus macaque blood. *J. Immunol. Methods 207:185*.
43. Pope, M., D. Elmore, D. Ho, and P. Marx. 1997. Dendritic cell-T cell mixtures, isolated from the skin and mucosae of macaques, support the replication of SIV. *AIDS Res. Hum. Retroviruses 13:819*.
44. Mehlhop, E. R., L. A. Villamide, I. Frank, A. Gettie, C. Santisteban, D. Messmer, R. Ignatius, J. D. Lifson, and M. Pope. 2002. Enhanced in vitro stimulation of rhesus macaque dendritic cells for activation of SIV-specific T cell responses. *J. Immunol. Methods 260:219*.
45. Barratt-Boyes, S. M., M. I. Zimmer, L. A. Harshyne, E. M. Meyer, S. C. Watkins, S. Capuano, III, M. Murphey-Corb, L. D. Falo, Jr., and A. D. Donnenberg. 2000. Maturation and trafficking of monocyte-derived dendritic cells in monkeys: implications for dendritic cell-based vaccines. *J. Immunol. 164:2487*.
46. Coates, P. T., S. M. Barratt-Boyes, L. Zhang, V. S. Donnenberg, P. J. O'Connell, A. J. Logar, F. J. Duncan, M. Murphey-Corb, A. D. Donnenberg, A. E. Morelli, et al. 2003. Dendritic cell subsets in blood and lymphoid tissue of rhesus monkeys and their mobilization with Flt3 ligand. *Blood 102:2513*.
47. Pichyangkul, S., T. P. Endy, S. Kalayanarooj, A. Nisalak, K. Yongvanitchit, S. Green, A. L. Rothman, F. A. Ennis, and D. H. Libraty. 2003. A blunted blood plasmacytoid dendritic cell response to an acute systemic viral infection is associated with increased disease severity. *J. Immunol. 171:5571*.
48. Teleshova, N., J. Jones, J. Kenney, J. Purcell, R. Bohm, A. Gettie, and M. Pope. 2004. Short-term Flt3L treatment effectively mobilizes macaque myeloid dendritic cells. *J. Leukocyte Biol. 75:1102*.
49. Frank, I., M. J. Piatak, H. Stoessel, N. Romani, D. Bonnyay, J. D. Lifson, and M. Pope. 2002. Infectious and whole inactivated simian immunodeficiency viruses interact similarly with primate dendritic cells (DCs): Differential intracellular fate of virions in mature and immature DCs. *J. Virol. 76:2936*.
50. Turville, S. G., J. J. Santos, I. Frank, P. U. Cameron, J. Wilkinson, M. Miranda-Saksena, J. Dable, H. Stoessel, N. Romani, M. Piatak, et al. 2004. Immunodeficiency virus uptake, turnover and two phase transfer in human dendritic cells. *Blood 103:2170*.
51. Frank, I., J. J. Santos, E. Mehlhop, L. Villamide-Herrera, C. Santisteban, A. Gettie, R. Ignatius, J. D. Lifson, and M. Pope. 2003. Presentation of exogenous whole inactivated simian immunodeficiency virus by mature dendritic cells induces $CD4^+$ and $CD8^+$ T cell responses. *J. AIDS 34:7*.
52. Larsson, M., J. F. Fonteneau, M. Lirvall, P. Haslett, J. D. Lifson, and N. Bhardwaj. 2002. Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: roles for cross-presentation and non-infectious HIV-1 virus. *AIDS 16:1319*.
53. Moris, A., C. Nobile, F. Buseyne, F. Porrot, J.-P. Abastado, and O. Schwartz. 2004. DC-SIGN promotes exogenous MHC-I restricted HIV-1 antigen presentation. *Blood 103:2648*.
54. Connor, R. I., D. C. Montefiori, J. M. Binley, J. P. Moore, S. Bonhoeffer, A. Gettie, E. A. Fenamore, K. E. Sheridan, D. D. Ho, P. J. Dailey, and P. A. Marx. 1998. Temporal analyses of virus replication, immune responses, and efficacy in rhesus macaques immunized with a live, attenuated simian immunodeficiency virus vaccine. *J. Virol. 72:7501*.
55. Ignatius, R., K. Tenner-Racz, D. Messmer, A. Gettie, A. Luckay, C. Russo, S. Smith, P. A. Marx, R. M. Steinman, P. Racz, and M. Pope. 2002. Increased macrophage infection upon subcutaneous inoculation of rhesus macaques with simian immunodeficiency virus-loaded dendritic cells or T cells but not with cell-free virus. *J. Virol. 76:9787*.
56. Hu, J., M. Pope, U. O'Doherty, C. Brown, and C. J. Miller. 1998. Immunophenotypic characterization of SIV-infected cells in cervix, vagina and draining lymph nodes of chronically infected rhesus macaques. *Lab. Invest. 78:435*.
57. Hu, J., C. J. Miller, U. O'Doherty, P. A. Marx, and M. Pope. 1999. The dendritic cell-T cell milieu of the lymphoid tissue of the tonsil provides a locale in which SIV can reside and propagate at chronic stages of infection. *AIDS Res. Hum. Retroviruses 15:1305*.
58. Chertova, E., J. W. Bess Jr., B. J. Crise, R. C. Sowder II, T. M. Schaden, J. M. Hilburn, J. A. Hoxie, R. E. Benveniste, J. D. Lifson, L. E. Henderson, and L. O. Arthur. 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol. 76:5315*.
59. Lifson, J. D., J. L. Rossio, M. Piatak, Jr., T. Parks, L. Li, R. Kiser, V. Coalter, B. Fisher, B. M. Flynn, S. Czajak, et al. 2001. Role of $CD8^+$ lymphocytes in control of simian immunodeficiency virus infection and resistance to rechallenge after transient early antiretroviral treatment. *J. Virol. 75:10187*.
60. Grouard, G., M.-C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.-J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with IL-3 and CD40-ligand. *J. Exp. Med. 185:1101*.
61. Olweus, J., A. BitMansour, R. Warnke, P. A. Thompson, J. Carballido, L. J. Picker, and F. Lund-Johansen. 1997. Dendritic cell ontogeny: a human cell lineage of myeloid origin. *Proc. Natl. Acad. Sci. USA 94:12551*.

62. O'Doherty, U., M. Peng, S. Gezelter, W. J. Swiggard, M. Betjes, N. Bhardwaj, and R. M. Steinman. 1994. Human blood contains two subsets of dendritic cells, one immunologically mature, and the other immature. *Immunology 82:487*.
63. O'Doherty, U., R. M. Steinman, M. Peng, P. U. Cameron, S. Gezelter, I. Kopeloff, W. J. Swiggard, M. Pope, and N. Bhardwaj. 1993. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med. 178:1067*.
64. Duramad, O., K. L. Fearon, J. H. Chan, H. Kanzler, J. D. Marshall, R. L. Coffman, and F. J. Barrat. 2003. IL-10 regulates plasmacytoid dendritic cell response to CpG-containing immunostimulatory sequences. *Blood 102:4487*.
65. Klagge, I. M., V. ter Meulen, and S. Schneider-Schaulies. 2000. Measles virus-induced promotion of dendritic cell maturation by soluble mediators does not overcome the immunosuppressive activity of viral glycoproteins on the cell surface. *Eur. J. Immunol. 30:2741*.
66. Ghanekar, S., L. Zheng, A. Logar, J. Navratil, L. Borowski, P. Gupta, and C. Rinaldo. 1996. Cytokine expression by human peripheral blood dendritic cells stimulated in vitro with HIV-1 and herpes simplex virus. *J. Immunol. 157:4028*.
67. Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med. 189:821*.
68. Thomas, R., L. S. Davis, and P. E. Lipsky. 1993. Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol. 150:821*.
69. McLellan, A. D., G. C. Starling, L. A. Williams, B. D. Hock, and D. N. J. Hart. 1995. Activation of human peripheral blood dendritic cells induces the CD86 co-stimulatory molecule. *Eur. J. Immunol. 25:2064*.
70. Pierre, P., S. J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R. M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature 388:787*.
71. Clatza, A., L. C. Bonifaz, D. A. Vignali, and J. Moreno. 2003. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J. Immunol. 171:6478*.
72. Kelsall, B. L., E. Stuber, M. Neurath, and W. Strober. 1996. Interleukin-12 production by dendritic cells: the role of CD40-CD40L interactions in Th1 T-cell responses. *Ann. NY Acad. Sci. 795:116*.
73. Peng, X., A. Kasran, P. A. Warmerdam, M. de Boer, and J. L. Ceuppens. 1996. Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-γ production. *Eur. J. Immunol. 26:1621*.
74. Gibson, S. J., J. M. Lindh, T. R. Riter, R. M. Gleason, L. M. Rogers, A. E. Fuller, J. L. Oesterich, K. B. Gorden, X. Qiu, S. W. McKane, et al. 2002. Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. *Cell. Immunol. 218:74*.
75. Gary-Gouy, H., P. Lebon, and A. H. Dalloul. 2002. Type I interferon production by plasmacytoid dendritic cells and monocytes is triggered by viruses, but the level of production is controlled by distinct cytokines. *J. Interferon Cytokine Res. 22:653*.
76. Dumais, N., A. Patrick, R. B. Moss, H. L. Davis, and K. L. Rosenthal. 2002. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. *J. Infect. Dis. 186:1098*.
77. Anders, H. J., B. Banas, Y. Linde, L. Weller, C. D. Cohen, M. Kretzler, S. Martin, V. Vielhauer, D. Schlondorff, and H. J. Grone. 2003. Bacterial CpG-DNA aggravates immune complex glomerulonephritis: role of TLR9-mediated expression of chemokines and chemokine receptors. *J. Am. Soc. Nephrol. 14:317*.
78. Raghavan, S., J. Nystrom, M. Fredriksson, J. Holmgren, and A. M. Harandi. 2003. Orally administered CpG oligodeoxynucleotide induces production of CXC and CC chemokines in the gastric mucosa and suppresses bacterial colonization in a mouse model of *Helicobacter pylori* infection. *Infect. Immun. 71:7014*.
79. Soumelis, V., I. Scott, Y.-J. Liu, and J. Levy. 2002. Natural interferon-α producing cells in HIV infection. *Hum. Immunol. 63:1206*.
80. Willey, R. L., R. Byrum, M. Piatak, Y. B. Kim, M. W. Cho, J. L. Rossio, Jr., J. Bess, Jr., T. Igarishi, Y. Endo, L. O. Arthur, et al. 2003. Control of viremia and prevention of SHIV induced disease in rhesus macaques immunized with inactivated SIV and HIV-1 particles. *J. Virol. 77:1163*.
81. Lifson, J. D., M. Piatak, Jr., J. L. Rossio, J. Bess, Jr., E. Chertova, D. Schneider, R. Kiser, V. Coalter, B. Poore, R. Imming, et al. 2002. Whole inactivated SIV virion vaccines with functional envelope glycoproteins: safety, immunogenicity, and activity against intrarectal challenge. *J. Med. Primatol. 31:205*.
82. Teleshova, N., I. Frank, and M. Pope. 2003. Immunodeficiency virus exploitation of dendritic cells in the early steps of infection. *J. Leukocyte Biol. 74:683*.
83. Marrack, P., J. Kappler, and T. Mitchell. 1999. Type I interferons keep activated T cells alive. *J. Exp. Med. 189:521*.
84. Matikainen, S., T. Sareneva, T. Ronni, A. Lehtonen, P. J. Koskinen, and I. Julkunen. 1999. Interferon-α activates multiple STAT proteins and upregulates proliferation-associated IL-2Rα, c-*myc*, and *pim*-1 genes in human T cells. *Blood 93:1980*.
85. Su, L., and M. David. 1999. Inhibition of B cell receptor-mediated apoptosis by IFN. *J. Immunol. 162:6317*.