

Generation of Mature Dendritic Cells with Unique Phenotype and Function by *In Vitro* Short-Term Culture of Human Monocytes in the Presence of Interleukin-4 and Interferon- β

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Dendritic cell (DC)-based immunotherapy has been utilized for the treatment of not only a number of human malignancies but also a select group of infectious diseases. Conventional techniques for the generation and maturation of DCs require 7 days of *in vitro* culture, which prompted us to seek alternative methods that would hasten the generation of functional human myeloid DCs *in vitro*. Following the use of a number of cytokines/growth factors, we found that *in vitro* culture of purified human monocytes, in media containing interleukin (IL)-4, together with interferon (IFN)- β for 24 hrs, followed by the addition of non-specific antigenic stimuli, such as keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS), or inactivated human immunodeficiency virus (HIV)-1 induced the monocytes to differentiated by 3 days into mature DCs (4B-DCs). These 4B-DCs expressed high levels of CD83 and CD11c, as well as markers of immune activation, including CD80 and CD86, human leukocyte antigen (HLA) class I and II, and CD14, but not CD1a. Anti-CD14 blocking antibody interfered with generation of 4B-DCs by LPS, but not by KLH or HIV-1. Interestingly, 4B-DCs, but not conventional DCs generated using macrophage-colony stimulating factor and IL-4 (G4-DCs), expressed OX40 and OX40L. 4B-DCs showed phagocytic activity, and spontaneously produced IL-12 and tumor necrosis factor (TNF)- α , but not IL-10.

4B-DCs promoted proliferation of allogeneic naïve CD4⁺ T cells, producing IFN- γ at lower levels than those stimulated with G4-DCs. 4B-DCs were more potent stimulators of allogeneic bulk CD8⁺ T cells producing IFN- γ than G4-DCs. These data indicate that 4B-DCs are unique and may provide a relatively more rapid alternative tool for potential clinical use, as compared with conventional G4-DCs. *Exp Biol Med* 233:721–731, 2008

Key words: dendritic cell, IL-4, type-I IFN, immune stimulation, OX40, human monocytes

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells that play crucial roles in the initiation and regulation of immune responses (1). DCs reside in an immature state at the sites of potential pathogen entry, and generally recognize invading pathogens via a family of Toll-like receptors (TLRs), complement receptors, and lectin receptors (2). This interaction with pathogens induces DCs to undergo maturation and migrate into secondary lymphoid organs, where they present processed forms of the antigen to stimulate antigen-specific T cells, and thus initiate immune responses (3). Results from a number of recent studies have highlighted the potential of DCs to serve as natural cellular “adjuvants” (4). This functional attribute has led to clinical trials of DC-based immunotherapy for not only a number of animal tumor models and human malignancies (5) but also against a number of infectious disease agents. This view is highlighted by the recent finding that immunization of human immunodeficiency virus type 1 (HIV-1) infected patients with autologous DCs sensitized with chemically inactivated autologous HIV-1 led to a marked sustained decrease in viral load (6, 7), which was reasoned to be

This work was supported in part by grants from a Grant-in-Aid for Research on HIV/AIDS and Health Sciences focusing on Drug Innovation from the Ministry of Health, Labor and Welfare of Japan; and Japan Human Science Foundation.

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Received December 12, 2007.
Accepted January 4, 2008.

DOI: 10.3181/0712-RM-333
1535-3702/08/2336-0721\$15.00
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secondary to DC-mediated enhancement of both virus-specific cellular and neutralizing antibody-responses. This finding was further supported utilizing a human peripheral blood leukocyte-populated severe combined immune deficiency (hu-PBL-SCID) mouse model, in which the human peripheral blood mononuclear cell (PBMC)-engrafted mice, following immunization with inactivated HIV-1-pulsed conventional DCs, were shown to generate higher relative levels of HIV-1-specific T- and B-cell immune responses sufficient to protect these animals against challenge with virulent HIV-1 isolates (8, 9).

A variety of methodologies to generate functional DCs *in vitro* have been published. The findings from these studies show that the phenotype and function of *in vitro* monocyte-derived DCs are influenced by a wide variety of factors, including methods utilized to isolate and prepare purified populations of monocytes and the *in vitro* culture conditions utilized (10). Immunostimulating myeloid DCs have been conventionally generated *in vitro* by culturing human peripheral blood monocytes in the presence of granulocyte and granulocyte macrophage colony-stimulating factor (GM-CSF) together with interleukin (IL)-4 for 4–6 days followed by maturation stimuli for 1–2 days. In order to acquire functional immunostimulating capacity, DCs need to mature because immature DCs have been shown to be immunosuppressive due to their ability to selectively induce the generation and expansion of regulatory T cells (Treg) (11). Thus, conventional immature DCs have been used following maturation by incubation in media containing tumor necrosis factor (TNF)- α , CD40 ligand, or lipopolysaccharide (LPS). Type-I interferon (IFN- α and IFN- β) has also been used to mature immature DCs (12, 13). Recently, attempts have been made to reduce the time required for preparing mature DCs *in vitro* aimed at reducing the cost and the labor involved. One representative method is to incubate the enriched population of monocytes in a cocktail of IL-6, IL-1 β , TNF- α and prostaglandin E₂ (PGE₂) on day 1 following the culture of monocytes in GM-CSF and IL-4 (14, 15). Another method described proposes the use type-I IFN and GM-CSF (12, 16). Although several lines of evidence exist that suggest the inhibitory nature of type-I IFN for the generation of myeloid DCs *in vitro* (17, 18), results from other studies show that type-I IFN-derived myeloid DCs are potent stimulators for antigen-specific not only CD4⁺ T, but also CD8⁺ T cell responses including those against HIV-1 (13, 16, 19, 20).

The use of type-I IFN by some and IL-4 by others for the *in vitro* maturation of DCs (21, 22) prompted our laboratory to test whether the use of these two cytokines in combination in the absence of GM-CSF could facilitate differentiation of functional DCs during a short-term culture period. We herein report the results of our findings that document our success in the generation of functional DCs from monocytes within 3 days that are distinct from conventional DCs in phenotype, cytokine production, and function.

Materials and Methods

Reagents. Medium used throughout these studies consisted of RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum (FCS; Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μ g/ml streptomycin (hereafter referred to as RPMI medium). The recombinant human cytokines used included human IL-4, GM-CSF (PeproTech, London, UK), IFN- β (Torey, Tokyo, Japan), and IL-2 (provided by the National Institutes of Health Acquired Immune Deficiency Syndrome [AIDS] Research and Reference Reagent Program). Anti-human CD3 (OKT-3) and anti-human CD28 monoclonal antibodies (mAb) were obtained from ATCC (Manassas, VA) and R&D Systems, Inc. (Minneapolis, MN), respectively. Aldrithiol-2 (AT-2), keyhole limpet hemocyanin (KLH), LPS from *Escherichia coli*, and low-endotoxin bovine serum albumin (BSA) were purchased from Sigma Chemical. A human monocyte negative isolation and a human naïve CD4⁺ T cell isolation kit were purchased from Dynal Biotec Inc. (Oslo, Norway) and Miltenyi Biotec (Gladbach, Germany), respectively. Enzyme-linked immunosorbent assay (ELISA) kits for human IL-12 p70 and TNF- α were purchased from Biosource International, Inc. (Camarillo, CA). ELISA kits for human IL-10 and IFN- γ were purchased from R&D Systems, Inc. A bromodeoxyuridine (BrdU)-incorporated ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). The Vybrant carboxyfluorescein succinimidyl ester (CFSE) cell tracer kit and fluorescein isothiocyanate (FITC)-labeled *E. coli* (FITC-*E. coli*) were purchased from Invitrogen (Carlsbad, CA). Fluorescent-dye labeled mouse mAbs used included FITC-IgG1 anti-CD80, FITC-IgG2a anti-CD1a, FITC-IgG2b anti-human leukocyte antigen (HLA)-DR, phycoerythrin (PE)-IgG1 anti-CD11c, PE-IgG2b anti-CD86, Cy5-IgG2a anti-CD14, Cy5-IgG1 anti-CD3, FITC- or PC5-IgG2b anti-CD83, PE-IgG2a anti-CCR7 (BD Pharmingen, San Diego, CA), PE-IgG2a anti-HLA-ABC (DAKO, Glostrup, Denmark), and isotype-matched control mAbs (Beckman Coulter, Fullerton CA). Anti-human CCR5 used was Cy5-IgG1 (rat) anti-human CCR5 (clone T312) (23). For examination of OX40 and its ligand (OX40L), fluorescently labeled or unlabeled mouse IgG1 anti-human OX40 (clone B7B5) (24), anti-human OX40L (clone TAG34) (25), or control IgG1 (clone TAXY7) (26) were used in combination with FITC-goat anti-mouse IgG (American Qualex, San Clemente, CA). A rat IgG1 anti-OX40 mAb, W4-54, was used to block intercellular transfer of OX40 (27).

Generation of DCs. PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by a density gradient centrifugation method on lymphocyte separation medium (Sigma Chemical). Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na₂-EDTA. Monocytes were purified from these PBMCs by using the CD14⁺

monocyte negative isolation kit. An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% CD14⁺ cells. These monocytes were resuspended at a concentration of 5×10^5 cells/ml in RPMI medium and 0.5–1.0 ml of cell suspension was dispensed into individual wells of 24-well plates (BD Pharmingen), and then cultured in the presence of IL-4 (10 ng/ml) and IFN- β (1000 U/ml) at 37°C in a 5% CO₂ humidified incubator for 3 days. The cultures were pulsed at different time points with either KLH (10 μ g/ml), LPS (10 ng/ml), or HIV-1_{IIIB} (p24 level of 10 ng/ml). The HIV was inactivated with Aldrithiol-2 as described previously (9) prior to its addition to the cultures. Cultures containing conventional DCs were prepared in parallel for purposes of comparison designed to mature concurrently with the DCs generated according to our modified protocol. The conventional DCs were derived by culturing the enriched population of monocytes in the presence of GM-CSF (500 ng/ml) and IL-4 (20 ng/ml) for 5 days followed by maturation by the addition of 1000 U/ml IFN- β treatment for an additional 2 days (9).

Flow Cytometry. Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% NaN₃ (fluorescence activated cell sorting [FACS] buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a predetermined optimal concentration of the appropriate fluorescently labeled mAbs against human cell surface molecules on ice for 30 min. In some experiments, cells were indirectly stained with mouse mAbs at 2.5 μ g/ml for 30 min, followed by goat anti-mouse IgG-FITC. After washing with FACS buffer, cells were fixed in 1% paraformaldehyde (PFA)-containing FACS buffer. The cells were analyzed by standard flow cytometry using a FACS-Calibur assisted by Cell Quest software (BD Pharmingen).

Phagocytic function was examined using FITC-labeled *E. coli* as described previously (28). Briefly, sample cells (2×10^5) in 0.2 ml of RPMI medium were incubated with FITC-*E. coli* particles at a cell to bacterium ratio of 1:15 for 30 min at 37°C in a 5% CO₂ humidified incubator. The mixture was washed twice with FACS buffer, and fixed with FACS buffer containing 1% PFA. The relative levels of uptake of FITC-*E. coli* by the cells were determined by standard flow cytometry.

Stimulation of Naïve CD4⁺ T and Bulk CD8⁺ T Cells. Enriched population of naïve CD4⁺ T cells (2×10^4 /ml) were isolated from normal human PBMCs by using the naïve CD4⁺ T cell isolation kit (>90% purity as assessed by FACS analysis), and then cocultured with allogeneic DCs (0.5×10^4 cells/ml) in RPMI medium without the exogenous addition of IL-2 in 96-well U-bottom plates (BD Pharmingen) in a volume of 0.2 ml/well. Cultures were performed in triplicate. After 7 days, cell proliferation was assessed by using the BrdU-incorporation ELISA kit. CFSE-labeling of T cells was carried out according to the manufacturer's protocol. CFSE-labeled or unlabeled CD8⁺

T cells were stimulated with allogeneic DCs at a DC to T cell ratio of 1:4 in RPMI medium supplemented with IL-2 (20 U/ml) in 96-well U-bottom plate for 5 days. Proliferation of CFSE-labeled CD8⁺ T cells was assessed by standard flow cytometry. For expansion of CD4⁺ T cells stimulated by DCs, the CD4⁺ T cells were stimulated with immobilized OKT-3 mAb (5 μ g/ml) and soluble anti-CD28 mAb (1 μ g/ml) in IL-2-free RPMI medium for 3 days.

Treg Assay. CFSE-labeled naïve CD4⁺ T cells (2×10^4 /well) were cocultured with autologous highly enriched population of CD14⁺ cells (2×10^4 /well). The CD14⁺ cells were precoated with anti-CD3 mAb (OKT-3) that bound the CD14⁺ cells via the Fc receptors. These cocultures were incubated in the presence or absence of varying numbers of cells being tested for Treg activity. Thus, CD4⁺ T cells that were cocultured with allogeneic 4B-DC or G4-DC were tested for their ability to serve as Tregs. In addition, CD4⁺ T cells restimulated with anti-CD3/CD28 mAb (2×10^4 cells/well) were subsequently assessed for Treg activity. Cultures were performed in triplicate using 96-well U-bottom plates. Following coculture with the Tregs for 3 days, the cells were harvested and the CFSE profile of the responder cells was examined by standard flow cytometry.

In Vitro HIV-1 Infection Assay. Sensitivity of DCs to infection *in vitro* with HIV-1 R5 strain JR-CSF was determined as follows. Target DCs were washed and dispensed into wells of a 96-well U-bottomed plate at 1×10^5 cells/well in a volume of 0.1 ml of RPMI medium and then infected with R5 HIV-1 JR-CSF at a multiplicity of infection (m.o.i.) of 0.1 and incubated overnight at 37°C. After washing the cells three times, the cells were cultured in 0.2 ml RPMI medium at 37°C. HIV-1 replication was monitored by the quantitation of HIV-1 p24 produced in the culture supernatants for 7 days using the commercially available HIV-1 p24 ELISA kit (Zepto Metrix, Buffalo, NY).

Statistical Analysis. Data were tested for significance using the Student's *t* test by using Prism software (GraphPad Software). The following symbols were used to denote levels of statistical significance: * denotes $P \leq 0.05$, ** denotes $P \leq 0.01$ and *** denotes $P \leq 0.001$.

Results

Monocytes Cultured *In Vitro* in the Presence of IL-4 and IFN- β Require an Additional Source of Stimulation in Order to Survive and Differentiate into Mature DCs. A variety of cytokines/growth factors in varying combinations and concentrations were first evaluated for their potential to induce human monocytes to differentiate into a DC type of morphology *in vitro*. Of these combinations, the most optimal DC type phenotype was observed with the use of IL-4 and IFN- β . However, by 3 days most human monocytes cultured in the presence of IL-4 and IFN- β did not survive due to apoptosis and/or necrosis. Therefore, we attempted to determine whether the addition of exogenous antigens such as KLH, LPS, or inactivated HIV-1 to the cultures would lead to rescue from

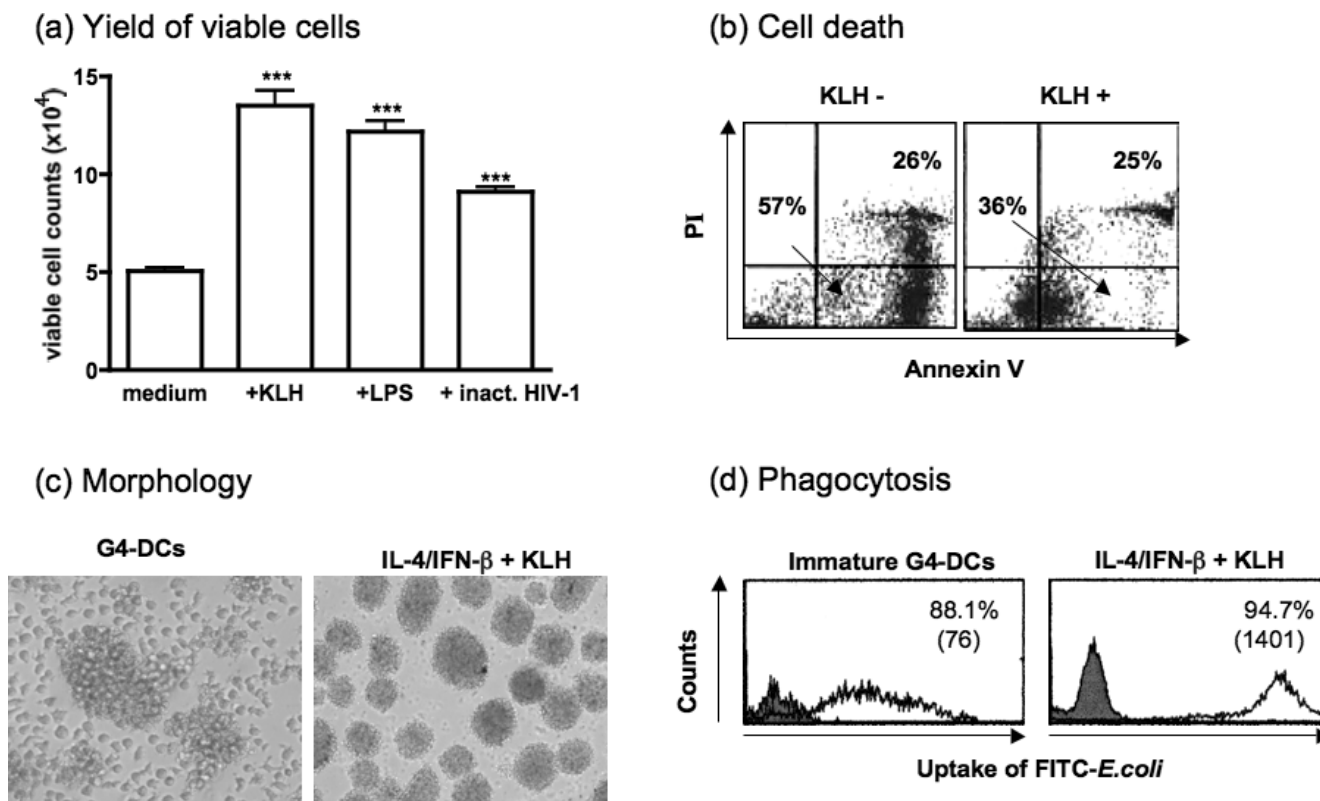


Figure 1. The addition of KLH, LPS, or inactivated HIV-1 promotes the survival of monocytes cultured in media containing IL-4 and IFN- β . CD14⁺ monocytes purified from PBMCs by a negative selection method (3×10^5 cells/well) were cultured in the presence of IL-4 and IFN- β , followed by the addition of KLH, LPS, or inactivated (inact.) HIV-1 on Day 1 and harvested on Day 3. (a) Yield of total number of viable cells/well. (b) Flow cytometric profile of an aliquot of the monocytes incubated with IL-4 and IFN- β , with and without KLH for 3 days and stained with Annexin V-FITC and PI. (c and d) Comparison of the morphology of and phagocytosis of *E. coli* by the KLH-stimulated monocytes cultured in IL-4 and IFN- β (Day 3) and conventional DCs (termed G4-DCs) that had been generated for 7 days from CD14⁺ monocytes using GM-CSF and IL-4, followed by maturation by IFN- β during last 2 days. The cells were observed under a phase-contrast vertical microscope at an original magnification of $\times 100$. Percent-FITC-positive cells and the mean fluorescence intensity (MFI) are shown in parentheses. Data shown are representative of one of three independent experiments with the standard deviation of $<10\%$. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples.

cell death. As shown in Fig. 1a, the addition of KLH, LPS, or inactivated HIV-1 to the monocyte cultures at a predetermined optimum time interval of Day 1 (see below) led to a 2–3-fold higher increase in the yield of viable cells on Day 3 of culture. A representative profile of annexin V and propidium iodide (PI) staining of monocytes cultured in the presence/absence of KLH is shown in Fig. 1b. As seen, the profile obtained demonstrates that the inclusion of KLH led to marked diminution in the levels of annexin V and PI staining of the *in vitro* cultured cells. Monocytes cultured in parallel in media containing only either IL-4 or IFN- β alone showed poor survival (data not shown). In contrast with conventional DCs derived by culture in media containing IL-4 and GM-CSF followed by further maturation with the addition of IFN- β (hereafter referred to as G4-DCs) which form large aggregates, these IL-4/IFN- β and KLH cultured monocytes not only survived, but also appeared to demonstrate distinct morphology (Fig. 1c). These IL-4/IFN- β and KLH cultured monocytes were shown to not only retain their phagocytic function but appeared to become

more efficient as compared with immature G4-DCs, as determined by the uptake of FITC-*E. coli* (Fig. 1d).

As shown in Fig. 2a, IL-4/IFN- β and KLH cultured monocytes expressed high levels of CD83 and CD11c, demonstrating that these cells could be phenotypically defined as mature myeloid DCs (hereafter referred to as 4B-DCs). These KLH-matured 4B-DCs expressed either higher frequencies and/or mean densities of most of the cell surface markers studied except for CD1a. The most marked differences noted were the expression of CCR7 by a significant frequency of 4B-DCs, the universal expression of CD14 and the absence of CD1a by the 4B-DCs. This phenotypic profile was consistently noted and was irrespective of the use of KLH, LPS, or inactivated HIV-1 as a source of antigen stimulation on day 1 (data not shown). Interestingly, a significant frequency of the 4B-DCs was positive for OX40, a marker of activated T cells, and a smaller frequency expressed its ligand OX40L (Fig. 2b). Two-color analysis of OX40/OX40L expression showed that most if not all of the OX40L-expressing 4B-DC population of cells also expressed OX40. It should be

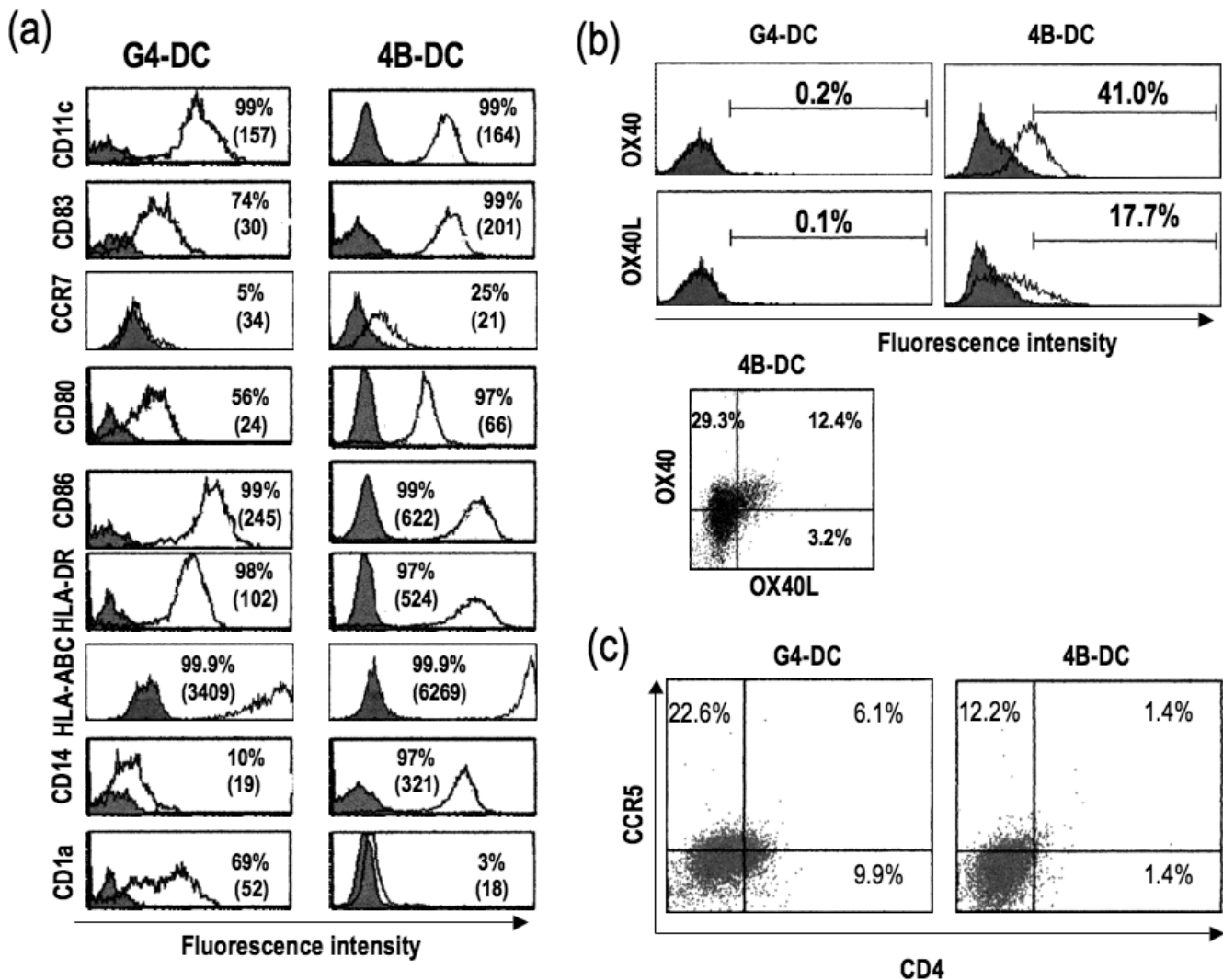


Figure 2. Flow cytometry facilitated phenotypic characterization of IL-4/IFN- β and KLH-cultured monocytes (4B-DCs) and for comparison G4-DCs. (a) 4B-DCs and G4-DCs were examined for cell surface expression of CD11c, CD83, CCR7, CD80, CD86, HLA-DR, HLA-ABC, CD14, and CD1a as described in the Materials and Methods section. Percent-positive cells and MFI in parenthesis are presented. (b) Expression of OX40 and OX40L on these DCs as determined by an indirect and a direct immunofluorescence, upper and lower graph, respectively, are shown. (c) Expression of CD4 and CCR5 as determined using rat anti-human CCR5 T312-FITC and mouse anti-human CD4 OKT-4-Cy5. Each of the data sets shown is representative of three independent experiments with the standard deviation of <10%. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples.

emphasized that the conventional G4-DCs (Fig. 2b) and fresh monocytes (data not shown) were negative for OX40/OX40L, indicating that 4B-DCs acquired OX40 as a result of differentiation from monocytes under the present culture conditions.

The levels of cell surface expression of the HIV-1 receptor CD4 and co-receptor CCR5 are shown in Fig. 2c. Relatively lower levels of CD4 and CCR5 were expressed by 4B-DCs as compared with G4-DCs, which was consistent with the observation that the 4B-DCs were relatively resistant to *in vitro* infection with R5 HIV-1 JR-CSF strain (data not shown).

Optimum Time for the Addition of Antigens and Pathways for 4B-DC Generation. Kinetic studies were carried out in efforts to determine the optimum time that was

required for the addition of the antigens such as KLH, LPS, and inactivated HIV-1 for the generation of 4B-DCs. As seen in Fig. 3a, the optimum time for the addition of KLH, LPS, or inactivated HIV-1 was 24 hours. Interestingly, addition of these stimulating agents at the time of initiation of the culture did not result in maximum differentiation, which supports the view that at least a few hours of exposure of the enriched population of monocytes to IL-4 and IFN- β was required for the full differentiation of 4B-DCs.

It was reasoned that because a small amount of LPS (as low as 10 pg/ml) could support 4B-DC generation (data not shown), it was possible that the effects of KLH and inactivated HIV-1 on 4B-DCs maturation was potentially due to contamination of each of these agents with LPS. To exclude this possibility, anti-CD14 neutralizing mAb was

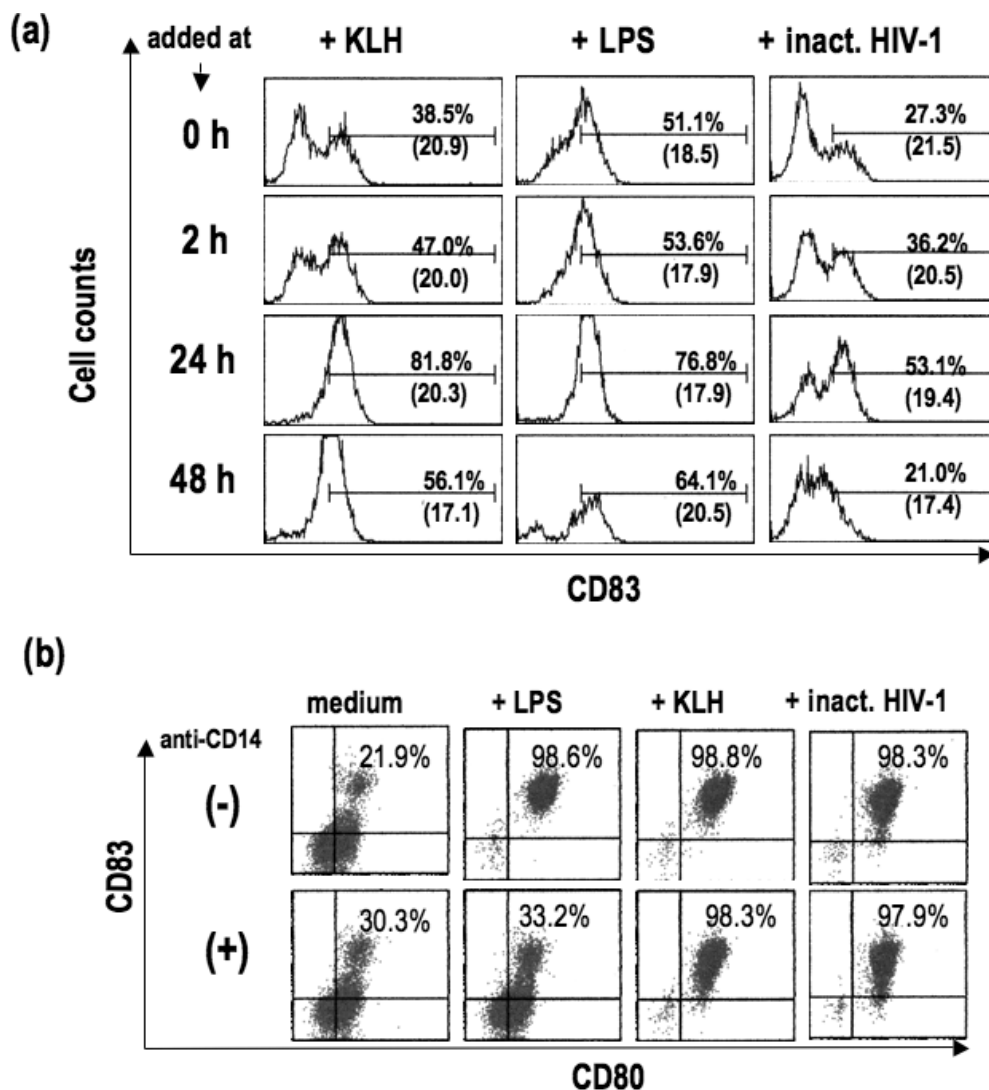


Figure 3. Optimum time for the addition of antigens needed for stimulation and pathways for 4B-DC generation. (a) CD14⁺ monocytes were cultured in the presence of IL-4 and IFN- β and at indicated times KLH, LPS, or inactivated HIV-1 was added to each culture, and all cultures were harvested on Day 3. An aliquot of the cells were analyzed for the expression of CD83. Percent-positive cells and MFI in parentheses are shown. (b) CD14⁺ monocytes were cultured in the presence of IFN- β and IL-4 overnight, and then treated with anti-CD14 or for purposes of control an isotype identical mAb (at final 10 μ g/ml) at 37°C for 1 hour. Then, LPS, KLH, or inactivated HIV-1 was added to the cultures and cultured for an additional 2 days. The expression of CD83 and CD80 were determined by standard flow cytometry. Data shown are representative of one of three independent experiments with the standard deviation of <10%. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples.

added to the culture to block LPS binding (Fig. 3b). As judged by the expression of CD83 and CD80, the differentiation of 4B-DCs from monocytes with the addition of LPS was completely blocked by the addition of anti-CD14 mAb. However, there appeared to be negligible effects of the addition of the same dose of anti-CD14 mAb on the ability of KLH and inactivated HIV-1 on 4B-DC generation. Therefore, it appears that there are at least two differentiation pathways for 4B-DCs: a CD14-dependent and a CD14-independent pathway under these culture conditions.

Cytokine Production and Naïve CD4⁺ T Cell Allostimulation by 4B-DCs. In attempts to define functional characteristics of 4B-DCs, the cytokine synthesizing profile and allostimulating properties of these cells

were compared with those of G4-DCs. Thus, culture supernatants from the KLH-matured 4B-DCs and the conventional G4-DCs were quantitated by ELISA for levels of IL-12 p70, TNF- α , and IL-10. As seen in Fig. 4a, supernatant fluids from the 4B-DCs produced higher relative levels of IL-12 p70 and TNF- α than the conventional mature G4-DCs. The level of IL-10 production by both 4B-DCs and G4-DCs was low. The results of the cytokine analysis induced by the maturation of 4B-DCs using LPS and inactivated HIV-1 was essentially similar to that of KLH except that there were differences in the relative levels of IL-12 p70 and TNF- α induced by LPS and inactivated HIV-1 (data not shown). Thus, there appeared to be a hierarchy, with LPS inducing the highest levels of

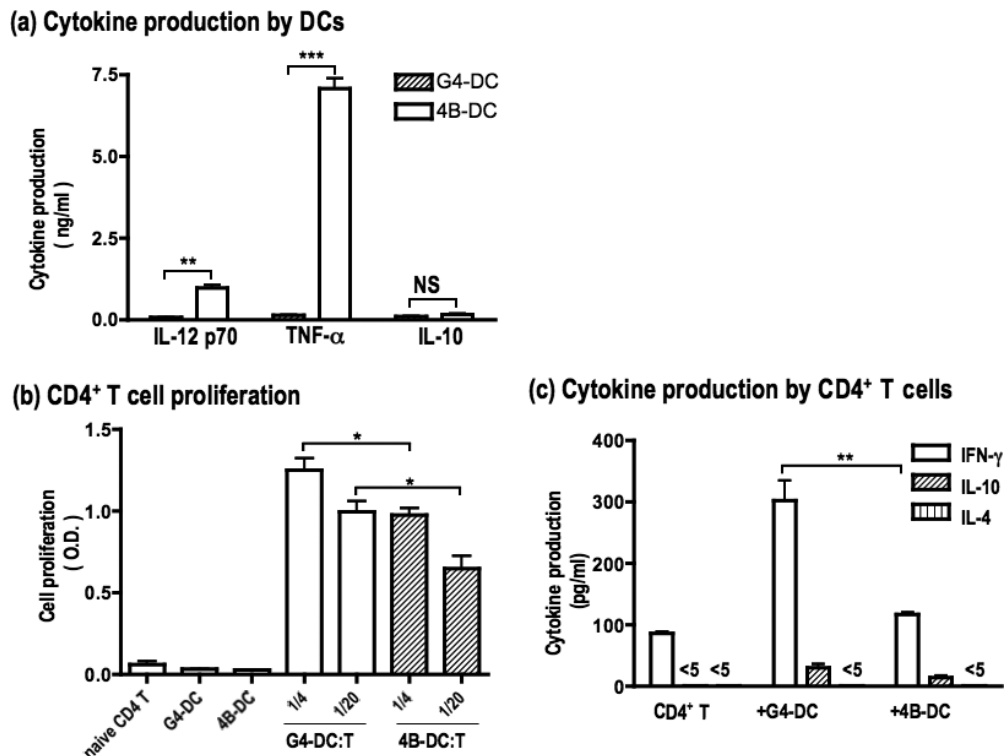


Figure 4. Difference in cytokine production and comparison of the allostimulation potential of naïve CD4⁺ T cells by 4B-DCs and G4-DCs. 4B-DCs (Day 3) were generated using KLH and conventional G4-DCs (Day 7) were generated by maturation with IFN- β for last 2 days. (a) Supernatants of the two DC cultures were examined for the levels of IL-12 p70, TNF- α , and IL-10 by ELISA. (b) Allogeneic naïve CD4⁺ T cells were cocultured with either 4B-DCs or G4-DCs for 7 days, and cell proliferation (optical density [O.D.]) of the naïve CD4⁺ T cells were quantitated by using BrdU-incorporation ELISA kits. (c) Cytokine levels produced in the supernatant fluids by these cocultured cells (Day 7) were determined by ELISA. Data shown are representative of one of three independent experiments with the standard deviation of <10%. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples. NS, not significant.

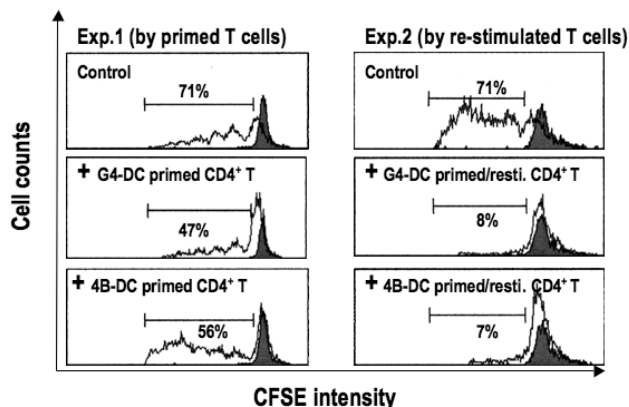
IL-12 p70 and TNF- α , followed by KLH, and the lowest level induced by inactivated HIV-1.

The allostimulating function of KLH-matured 4B-DCs was next compared with that of G4-DCs using the standard mixed leukocyte reaction (MLR). Purified allogeneic naïve CD4⁺ T cells were cocultured with either 4B-DCs or G4-DCs at a T to DC ratio of 4:1 or 20:1 for 7 days and the levels of cell proliferation were assessed by BrdU incorporation. As shown in Fig. 4b, the ability of 4B-DCs to induce alloproliferation of the naïve CD4⁺ T cells was slightly weaker than that of G4-DCs. To determine the cytokine profile of these allostimulated CD4⁺ T cells induced by 4B-DCs and G4-DCs, the levels of IFN- γ , IL-4, and IL-10 in the culture supernatants of these cultures on day 7 were quantitated by ELISA. As shown in Fig. 4c, the 4B-DC-stimulated T cells secreted lower levels of IFN- γ in comparison to those stimulated with conventional G4-DCs. Production of IL-4 was not detectable (<5 pg/ml) and that of IL-10 was low in these two culture fluids. These data indicate that 4B-DCs were capable of stimulating bulk naïve CD4⁺ T cell differentiation into Th1-like cells but their potential was lower than conventional G4-DCs.

In Vitro Restimulation of 4B-DC-Primed CD4⁺ T Cells. Because 4B-DCs were less effective than G4-DCs in induction of CD4⁺ T cell proliferation as described above,

we reasoned that this difference could be due to the differential activation of Tregs by the former. However, these CD4⁺ T cells allo-activated by either 4B-DCs or G4-DCs failed to show detectable levels of Treg activity. Thus as seen in Fig. 5a, Experiment 1, the responder CD4⁺ T cells cultured in the absence (control) or presence of allo-4B-DC-primed CD4⁺ T cells, as well as those primed with allo-G4-DCs (at cell to cell ratio of 1:1) gave the same CFSE profile, indicating that the Treg frequency in the CD4⁺ T cells primed either with 4B-DCs or G4-DCs was either absent and/or undetectable under these culture conditions. To provide for a more sensitive assay for the detection of potential Tregs, these alloprimed CD4⁺ T cells were restimulated by incubation with immobilized OKT-3 mAb together with soluble anti-CD28 mAb for 2 days and then assayed for Treg activity and IL-10 production as described in the Methods section. Interestingly, CD4⁺ T cells from both the 4B-DCs and G4-DCs primed and *in vitro* expanded cells showed Treg activity at a responder to Treg cell ratio of 1:1 as shown by marked inhibition of autologous naïve CD4⁺ T cell proliferation (Fig. 5a, Experiment 2), indicating that there is no significant difference in Treg-inducing activity between 4B-DCs and G4-DCs. However, as seen in Fig. 5b the 4B-DC-primed and restimulated CD4⁺ T cells produced higher levels of IL-10 and lower levels of IFN- γ

(a) Treg activity



(b) Cytokine production

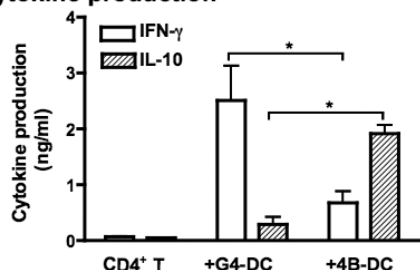
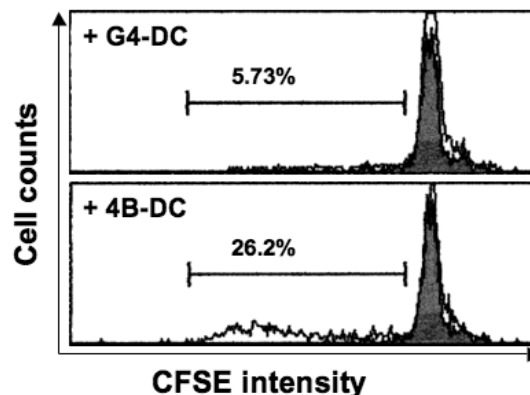


Figure 5. Analysis of Treg generation by coculture with 4B-DCs and the synthesis of IL-10 by restimulated CD4⁺ T cells. Allogeneic KLH-matured 4B-DC- or conventional G4-DC-primed CD4⁺ T cells as shown in Fig. 4b, or those cells after restimulation were examined for cytokine production and Treg activity. (a) 4B-DC- or G4-DC-primed CD4⁺ T cells, and those restimulated with anti-CD3/CD28 mAbs were added to CFSE-labeled autologous naïve CD4⁺ T cells cultures at a 1:1 ratio. Then soluble OKT-3 and autologous monocytes were added to the cultures. After 3 days, proliferation of the CD4⁺ T cells was analyzed by flow cytometry. (b) Cytokine production by the restimulated culture supernatants was quantitated by ELISA. Data shown are representative of one of three independent experiments with the standard deviation of <10%. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples.

than those that were G4-DC-primed/restimulated, showing a somewhat difference between the two DC populations in determination of T cell differentiation.

CD8⁺ T Cell Stimulation by 4B-DCs. Finally, the biological effects of 4B-DCs on CD8⁺ T cells were examined by standard MLR. Highly purified (>95%) CD8⁺ T cells that were labeled with CFSE were cocultured with allogeneic 4B-DCs or G4-DCs at a ratio of 4:1 (responder to stimulator) for 6 days. As shown in Fig. 6a, 4B-DC-induced alloproliferation of CD8⁺ T cells was more effectively than those induced by G4-DCs although the relative proliferative potential of these DC preparations for allogeneic CD8⁺ T cells was weaker than that noted above for CD4⁺ T cells. Supernatant fluids from such cultures were also examined for levels of IFN- γ and IL-10. As seen in Fig. 6b, the allo-4B-DC-stimulated CD8⁺ T cells synthesized significantly more IFN- γ than those stimulated with G4-DCs. IL-10 was not detected in these culture supernatant fluids. These data suggest that 4B-DCs were committed to

(a) CD8⁺ T cell proliferation

(b) Cytokine production

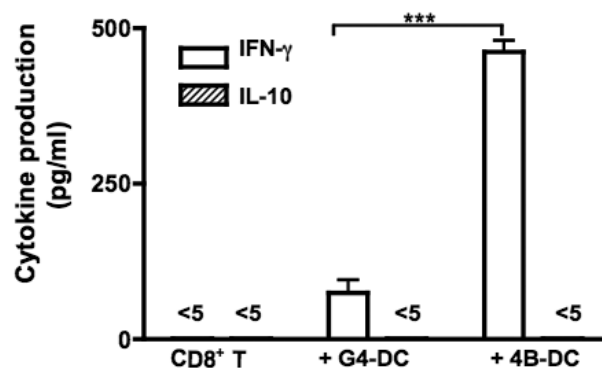


Figure 6. Stimulation of CD8⁺ T cells with 4B-DCs. CFSE-labeled purified CD8⁺ T cells were cocultured with allogeneic KLH-matured 4B-DCs or G4-DCs at T to DC ratio of 4:1 in RPMI medium containing IL-2 (20 U/ml) in 96 well U-bottom plate for 6 days. (a) Proliferation of the CFSE-labeled CD8⁺ T cells was assayed by flow cytometry. The frequency of proliferating cells of is shown. (b) IFN- γ production by non-labeled CD8⁺ cells in the culture supernatants were quantitated by ELISA. Data shown are representative of one of three independent experiments with the standard deviation of <10%. Each experiment utilized three different blood donors and the data depict mean $\pm$ SD of the data derived from the three samples.

stimulate CD8⁺ T cells rather than CD4⁺ T cells, which was in contrast to the case of the G4-DCs.

Discussion

The present study was conducted in efforts to define *in vitro* methodologies that could potentially hasten the *in vitro* maturation of myeloid DCs from human monocytes. Results of our studies show that indeed *in vitro* culture of highly enriched population of CD14⁺ human monocytes in the presence of IL-4 and IFN- γ for 1 day, followed by the addition of KLH, LPS, or AT-2-inactivated HIV-1 IIIB strain for 2 days, led to the generation of mature and functional myeloid DCs. These 4B-DCs displayed several distinct phenotypes from conventional DCs (Figs. 1 and 2) and from those reported as rapidly-induced DCs that were derived by using a cocktail of IL-6, IL-1 β , TNF- α , and PGE₂ (14, 15). The 4B-DCs expressed 3–6-fold higher

levels of CD83, CD80, CD86, HLA class-I, and class-II antigens than mature conventional G4-DCs from the same donors. In contrast to conventional G4-DCs, 4B-DCs expressed higher relative levels of CD14 but not CD1a which is a typical phenotype for precursor Langerhans cells (29), suggesting that 4B-DCs are sensitive to LPS stimulation but deficient in presentation of lipid antigens to T cells (30).

Another apparent phenotypic difference between 4B-DCs and G4-DCs was that 4B-DCs expressed the T cell activation marker OX40 under the present culture conditions. Although OX40L has been reported to be induced on DCs by treatment with CD40L or thymic stromal lymphopoietin (31, 32), to the best of our knowledge, this is the first study to report the expression of OX40 on human DCs. Our data suggest that the OX40 was endogenously induced on DCs and not transferred from contaminating activated OX40⁺ T cells (33) because the cultures were prescreened for such contaminating cells and OX40 was detected on 4B-DCs generated in the presence of anti-OX40 mAb (W4-54) that has been previously shown to block the passive transfer of OX40 (27). As it has been previously suggested that the interaction between OX40 and OX40L on T cells promotes survival of the T cells (34), because some of 4B-DCs were positive for OX40L, it is thus possible that the dual expressions of these antigens on 4B-DCs may have been involved in the control of survival and/or function of these cells through an autocrine and/or paracrine pathway. The mechanism of OX40 induction and its biological function remains to be determined, and experiments using anti-OX40 agonistic mAbs and soluble recombinant OX40L are currently in progress.

Another difference noted between 4B-DCs and conventional G4-DCs was that the 4B-DCs were nonadherent to the bottom of the plastic culture vessels, formed clusters, and formed large clumps in culture in 3 days. This morphology of 4B-DCs was very distinct from that of so-called typical "dendritic" cell, suggesting differences in the expression patterns of adhesion-related molecules which may include DC-SIGN, ICAM-3, extracellular matrix proteins, or their receptors.

The requirement for the addition of KLH, LPS, or inactivated HIV-1, as an additional stimulant after short-time culture of CD14⁺ monocytes for survival and differentiation of 4B-DCs remains to be determined, and is a subject of studies in progress. Some of these data indicate that LPS is likely activating the progenitor cells via both the CD14 and the TLR4-mediated signal pathway (35). On the other hand, KLH appears to mediate its effect via the mannose receptor-mediated pathway (36), and HIV-1-induced maturation likely involves the TLR7/TLR8 pathways. The latter data is supported by the finding that single strand viral RNAs have the potential to signal via the TLR7/TLR8 pathways (37). Because type-I IFN is known to skew the differentiation of monocytes into TLR7-expressing DCs (16), the monocytes cultured in the present media containing

IFN- β and IL-4 for 1 day might acquire TLR7 during this initial culture period. Our preliminary studies showed that CpG, the ligand of TLR9, could not generate mature 4B-DCs (data not shown), indicating that TLR9 pathway is not likely involved. It will be of interest to further examine which other TLRs can support 4B-DC generation.

Experiments on cytokine production patterns showed that 4B-DCs produced higher relative levels of IL-12 p70 and TNF- α than G4-DCs, and that IL-10 was low in supernatant fluids from both the cultures. This pattern is in contrast to those of the other type-I IFN-derived DCs in the presence of GM-CSF that produced less IL-12 than conventional DCs (16, 17). However, like the other type-I IFN-derived DCs (38), 4B-DCs showed reduced potential to induce alloproliferation of naïve CD4⁺ T cells and the synthesis of IFN- γ , as compared with G4-DCs. One possible reason for this may be related to the induction or stimulation of Tregs as reported by Carbonneil *et al.* (38). However, we could not detect any Tregs activity in these primary allo-4B-DC-stimulated CD4⁺ T cells. Nevertheless, restimulation of the 4B-DC-primed CD4⁺ T cells resulted in production of higher levels of IL-10 and less levels of IFN- γ than those primed with G4-DCs (Fig. 5). Thus, it is still possible that endogenously produced IL-10 might be involved in the low proliferation of the responder naïve CD4⁺ T cells stimulated allo-4B-DCs. Another possibility is that 4B-DCs had a shorter life span than G4-DCs (data not shown), which may be not enough for continuous stimulation of CD4⁺ T cells during the later stages.

There have been several studies performed aimed at exploring the potential beneficial effects of type-I IFN on monocyte-derived DCs in reference to immune protection against viral infections. Carbonneil *et al.* (20) reported that IFN- α together with GM-CSF induced monocytes to differentiate into functional and HIV-resistant DCs that are capable of inducing potent HIV-specific CD8⁺ T cell responses. Similarly, Santodonato *et al.* (39) demonstrated that 3-day DCs generated from monocytes in the presence of IFN- α and GM-CSF stimulated Epstein-Barr virus-specific CD8⁺ T cell responses. Thus it seems likely that DCs induced in the presence of type-I IFN, which express high levels of MHC class-I/II antigens, may favor CD8⁺ T rather than CD4⁺ T cell induction and have the potential to be highly effective against viral infections. Indeed, our present data showed that 4B-DCs were more potent to stimulate allogeneic bulk CD8⁺ T cells than G4-DCs. Another benefit of type-I IFN-induced or -treated DCs may be that they are resistant to productive infection with viruses including HIV-1 (20), influenza virus (40), and even Ebola virus (41), which may be applicable in clinical DC-based immunotherapy trials for such virally infected patients. As indicated herein, DC-based immunotherapy platforms continue to be utilized in a variety of clinical settings meant to either induce and augment pro-inflammatory immune responses (in the case of vaccines against infectious agents), modulate immune responses (such as in patients with autoimmune

diseases), and/or suppress immune responses (such as in organ/tissue allotransplantation). Thus, care needs to be taken in the use of proper sets of DCs and their biological characteristics thoroughly tested in animal models prior to their use in human studies. In particular, there is a need for making sure that utilization of DC-based immunotherapy does not inadvertently initiate an autoimmune response.

Studies are in progress to explore whether the present 4B-DCs are able to induce HIV-1-specific CD8⁺ and CD4⁺ T cell responses *in vitro* and *in vivo* using our hu-PBL-SCID mouse model (9).

We thank the National Institutes of Health AIDS Research and Reference Reagent Program for supplying IL-2.

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