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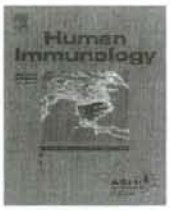

Rapid induction of OX40 ligand on primary T cells activated under DNA-damaging conditions

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Summary We have previously demonstrated that normal human T cells either long-term repeatedly stimulated or freshly activated *in vitro* in the presence of TGF-β express the cell surface T-cell costimulating molecule OX40 ligand (OX40L). To further elucidate the kinetics of OX40L expression by human T cells, we have examined whether cell proliferation was required for the expression of OX40L. Thus, normal fresh peripheral blood mononuclear cells were stimulated with immobilized anti-CD3 antibody in the presence of the DNA synthesis-blocking agents such as mitomycin C, 5-fluorouracil, or X-ray irradiation. Flow cytometric analyses demonstrated that a significant frequency of these DNA-damaged activated primary $CD4^+$ and $CD8^+$ T cells became $OX40L^+$ as early as 1 hour after treatment. The OX40L induction on the DNA-damaged activated T cells was inhibited by treatment with either RNA or protein synthesis inhibitors, actinomycin D, or cycloheximide, respectively. Induced OX40L on T cells was functional because it bound recombinant OX40. These data indicate that human primary T cells are programmed to rapidly express functional OX40L molecules after stimulation under DNA-damaging conditions, demonstrating that the induction of OX40L by T cells is independent of cell proliferation. The clinical implications of these new findings are discussed.

Introduction

Human OX40 ligand (OX40L) was first described by our laboratory as a gp34 molecule that is preferentially expressed on the cell surface of human T-cell lines transformed by human T-cell leukemia virus type I using a monoclonal antibody (mAb) [1]. Subsequently, gp34 was cloned [2] and finally identified as a member of the tumor necrosis factor (TNF) superfamily (TNFSF) [3]. OX40, its cognate receptor, was originally reported by Paterson *et al.* [4] as a cell surface antigen expressed by rat $CD4^+$ T-cell blasts that was recognized by MRC OX-40 antibody. OX40 is a member of the TNF receptor superfamily, which includes molecules such as Fas, CD27, CD30, CD40, 4-1BB, and others [4]. OX40 is transiently expressed on the cell surface of activated T cells [5]. The

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ABBREVIATIONS

5-FU	5-fluorouracil
Act-D	actinomycin D
CHX	cycloheximide
CsA	cyclosporine A
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FCM	flow cytometry
IgG	immunoglobulin G
IL	interleukin
i.p.	intraperitoneally
mAb	monoclonal antibody
MMC	mitomycin C
NK	natural killer
OX40L	OX40 ligand
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
sOX40	soluble human OX40
TCR	T-cell receptor
TGF-β1	transforming growth factor-β1
Th1	T helper 1
TNF	tumor necrosis factor
TNFSF	tumor necrosis factor superfamily
UV	ultraviolet

coexpression of OX40L and OX40 has been reported to be critical for T-cell response, which includes proliferation, survival, cytokine production, and the generation of memory cells [6,7] in a tumor necrosis factor associated factor (TRAF)2- and survival-dependent manner [8]. The delineation of the crystal structure of the OX40-OX40L complex demonstrated that whereas OX40L is a very compact, brick-shape structure that packs together to form flower-like trimers, the OX40 molecule binds at the monomer to monomer interface [9].

Initially, under normal conditions, the expression of OX40L was thought to be restricted to antigen-presenting cells such as dendritic cells (DCs) and B cells that were activated by ligation of the CD40L molecule or surface Ig [10,11], as well as select non-antigen-presenting cells such as endothelial cells [12]. OX40L expression by DCs was also induced by thymic stromal lymphopoietin [13]. It is, however, now becoming clear that functional OX40L can be induced on a wide variety of cells including activated T cells [14], natural killer (NK) cells [15], and mast cells [16], highlighting the multicell lineage expression of the OX40L molecule. It is reasoned that OX40-induced T-cell costimulation in fact occurs quite frequently via OX40-OX40L interaction at sites of inflammation, resulting in modulation of immune responses. Indeed, T-T-cell interactions during late stages of T-cell responses via OX40 and OX40L have been implicated in the long-term survival of antigen-specific $CD4^+$ memory effector T cells in the murine system [14]. It is of interest to note that whereas ligation of human OX40 by human OX40L stimulates human $OX40^+$ T cells, under Th1 conditions such OX40 stimulation by OX40L appears to result in the apoptosis of $CD4^+$ T cells [17]. Thus, it has been suggested that interactions between OX40L-OX40 control the fate of T-cell responses as a double-edge effector.

Recently, we reported that a high frequency of normal human T cells express functional OX40L when they were either long-term repeatedly stimulated *in vitro* or primarily stimulated once with anti-CD3 antibody in the presence of transforming growth factor β1 (TGF-β1) [18]. These observations prompted us to determine whether cell proliferation was necessary for the induction of OX40L on activated T cells. Thus, in the present study, we examined the effects of DNA synthesis-inhibiting agents on OX40L expression by fresh T cells activated via T-cell receptor (TCR) with immobilized anti-CD3 antibody. We report herein that lymphocytes including $CD4^+$ T and $CD8^+$ T cells express OX40L following a short-term period independent of cell proliferation, but dependent on both RNA and protein syntheses and activation.

Subjects and methods

Reagents

The medium used throughout these studies included RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum (Sigma; referred to as RPMI medium). Human recombinant cytokines used were interleukin (IL)-2 and TGF-β1 purchased from Peprotech (London, UK). The fluorescent mAbs used and their vendors are listed in parentheses. These included antihuman CD4, CD8, CD19, CD40, CD154, HLA-DR, and CD25 (Beckman-Coulter, Fullerton, CA), antihuman CD70 (CD27L) (Ancell, Bayport, MN), antihuman 4-1BB, 4-1BBL, TNF-α, and CD30 (BD Biosciences, Teterboro, NJ), antihuman Fas ligand (MBL Nagoya, Japan), antihuman TRAIL, CD30L, GITRL, CTLA-4, and GITR (R&D Systems, Inc., Minneapolis, MN), and OKT-3 and anti-HLA class I [W6/32, American Type Culture Collection (ATCC); ATCC, VA].

The mAbs produced in our laboratory included a mouse IgG1 anti-OX40L (clone 5A8) [19]; mouse IgG1 anti-human OX40 (clone B-7B5) [20]; and rat IgG2b anti-OX40 (clone W4-54, which was generated from a WKA rat and capable of blocking OX40/OX40L interaction) [18]. These in-house mAbs were purified from SCID mouse ascites fluids by gel filtration and labeled using FITC, POD, and Cy5 using commercial labeling kits according to the manufacturer's instructions. Biotinylated recombinant soluble human OX40 (sOX40, a form of murine IgG2a-Fc fusion protein) and OX40L (sOX40L, a form of murine CD8-fusion protein) were purchased from Ancell. Mitomycin C (MMC) was purchased from Kyowahakko (Tokyo, Japan). Actinomycin D (Act-D), 5-fluorouracil (5-FU), cycloheximide (CHX), cyclosporine A (CsA), and H_2O_2 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Metalloproteinase inhibitor (KB8301) for Fas-L detection was purchased from BD Pharmingen.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA kits utilized for the quantitation of human OX40L and OX40 were generated by our laboratory using combinations of in-house capture and detector mAbs. The detector mAbs were labeled utilizing a POD kit (Dojin, Kumamoto, Japan) according to the manufacturer's instructions. For standards, soluble human OX40L-murine CD8 fusion protein and human OX40-murine Ig (Ancell) were used. The weight ratio of OX40L and OX40 in the fusion proteins was calculated to be 46 and 42%, respectively, based on the published amino acid sequences of the molecules.

Stimulation of T cells *in vitro*

Human peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3 antibody (OKT-3) as described previously [18]. Briefly, PBMCs obtained by density gradient centrifugation on HistoPAQUE-1077 (Sigma) were suspended at 1×10^6 cells/ml in RPMI medium supplemented with 20 U/ml IL-2. The PBMCs were dispensed into individual wells of 24-well plates (BD Pharmingen) (1 ml/well) that had been precoated with anti-CD3 mAb. The PBMCs were cultured for 1 to 72 hours at 37°C in a 5% CO_2 humidified atmosphere in the presence or absence of MMC, Act-D, CHX, CsA, or TGF-β1, alone or in combination. In some experiments, PBMCs were pretreated with 50 μg/ml MMC for 45 minutes at 37°C, X-ray irradiated by exposure to a Hitachi MBR-1505R2, or UV-A or UV-B irradiated using SLUV-4 254/360 nm (AS ONE, Osaka, Japan). The UV dose was measured using a UV meter (UVP, Inc., Upland, CA). Cell viability was determined after an aliquot of the cells was stained with 0.1% eosin-Y. An anti-OX40 blocking mAb (W4-54) [18] was used to prevent the intercellular interaction between OX40 and OX40L [21]. For the quantitation of levels of OX40L and OX40 in the cells, cell lysates were prepared using a low-salt extraction buffer [1].

Flow cytometry (FCM)

FCM was carried out as described previously [18]. Briefly, cells to be analyzed were Fc-blocked by incubation of the cells with 2 mg/ml normal human pooled IgG on ice for 15 minutes. Aliquots of these cells were then subjected to staining using predetermined optimum concentrations of dye-conjugated mAbs for 30 minutes on ice. The cells were then washed using FACS buffer [phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide], fixed in 1% paraformaldehyde (PFA)-containing FACS buffer, and analyzed using a FACSCalibur, and the data obtained were analyzed using Cell Quest software (BD Pharmingen). To determine whether cell surface OX40L is functional, Fc-blocked cells were incubated with biotinylated recombinant sOX40 at a concentration of 2.5 μg/ml for 30 minutes on ice, followed by staining with PE-labeled streptavidin (Beckman Coulter) for 30 minutes on ice, and then analyzed by FCM.

In all the FCM analyses, isotype controls were used, and dead cells were gated out according to the forward and side scatter profile. In the appropriate experiments, samples were stained in conjunction with propidium iodide to exclude dead cells.

Animal studies

The immunodeficient BALB/c-Rag2$^{-/-}\gamma_c^{-/-}$ (referred to as BRG) mice [22] were obtained from Dr. M. Ito at the Central Institute for Experimental Animals (Kanagawa, Japan). Fresh human PBMCs (1×10^7 cells/head) were inoculated intraperitoneally (i.p.) into BRG mice, immediately followed by either MMC (620 μg/ head) or PBS inoculation. After 24 hours, the mice were sacrificed, and the cells harvested from the peritoneal cavity were pooled with spleen cells and aliquots subjected to phenotypic analysis by FCM. Multicolor analysis of the human cells was performed based on gating on cells expressing HLA class I.

Results

Induction of OX40L expression on T cells activated under DNA-damaging conditions

To test whether cell proliferation was required for the expression of OX40L by T cells, aliquots of fresh PBMCs were treated with either medium or a representative DNA synthesis inhibitor drug, MMC, and then stimulated *in vitro* with immobilized anti-CD3 mAb for 3 days. Aliquots of PBMCs cultured in the presence of TGF-β were used for the purposes of positive control for the expression of OX40L. As illustrated in Figure 1a, a significant number of MMC-treated cells within the lymphocyte gate were positive for OX40L. The percentage of OX40L-expressing cells treated with MMC was four times greater than the PBMCs stimulated in the presence of TGF-β1. PBMCs treated with MMC and stimulated with anti-CD3 in the presence of TGF-β did not demonstrate any further increase in the frequency of the OX40L$^+$ lymphocytes. Approximately two thirds of the OX40L-positive MMC-treated lymphocytes were also OX40 positive (Figure 1a), indicating that both OX40L and OX40 are coexpressed by cells activated in the presence of a DNA-damaging agent, as we have previously demonstrated for long-term cultured T cells [18]. Thus, it is clear that cell proliferation is not necessary for the expression of OX40L on activated human lymphocytes. As far as we know, the present data are the first to demonstrate that an inhibitor of DNA synthesis induces the cell surface expression of OX40L following TCR-mediated activation of human lymphocytes.

To determine which lymphocyte subsets expressed OX40L in these MMC-treated *in vitro* anti-CD3 activated PBMCs, dual-color staining was performed on lymphocytes gated according to forward and side scatter profiles. Figure 1b illustrates that OX40L was expressed predominantly by both CD4$^+$ and CD8$^+$ T cells and a minor (10%) population of lymphocytes expressing CD19 (B cells). No detectable induction of OX40L was observed on CD56$^+$ NK or CD14$^+$ monocytes (data not shown) under the present conditions.

To exclude the possibility that T cells become OX40L$^+$ through activation via their Fc receptors that may be ligated by the immunoglobulin G (IgG) Fc region of plate-bound anti-CD3 mAb, as has been demonstrated for human NK cells that were activated via the Fc-γ receptor CD16 [15], three additional agents that induce cell activation were utilized. These included the use of phytohemagglutinin A, phorbol 12-myristate 13-acetate (PMA), and immunobeads conjugated with anti-CD3/anti-CD28. As illustrated in Figure 1c, all three agents induced the expression of OX40L on the MMC-treated T cells but to varying degrees. Figure 1c also illustrates that among these agents, the CD3/CD28 mAb-conjugated beads were the most potent and PMA was the least potent in their ability to induce not only OX40L but also OX40 following MMC treatment of T cells, suggesting an indirect CD28 involvement for the induction of OX40L and OX40.

In efforts to determine whether the inhibition of DNA synthesis was effective in OX40L induction by not only primary T cells but also previously stimulated T cells, normal human T cells stimulated once with anti-CD3 mAb were restimulated either in the presence or in the absence of MMC for 1 day and then OX40L expression was analyzed. As illustrated in Figure 1d, MMC treatment increased the percentage of OX40L$^+$ cells from 20 to 51% of the restimulated T cells, demonstrating that both fresh and previously activated T cells were susceptible to OX40L induction by activation under DNA synthesis-inhibiting conditions.

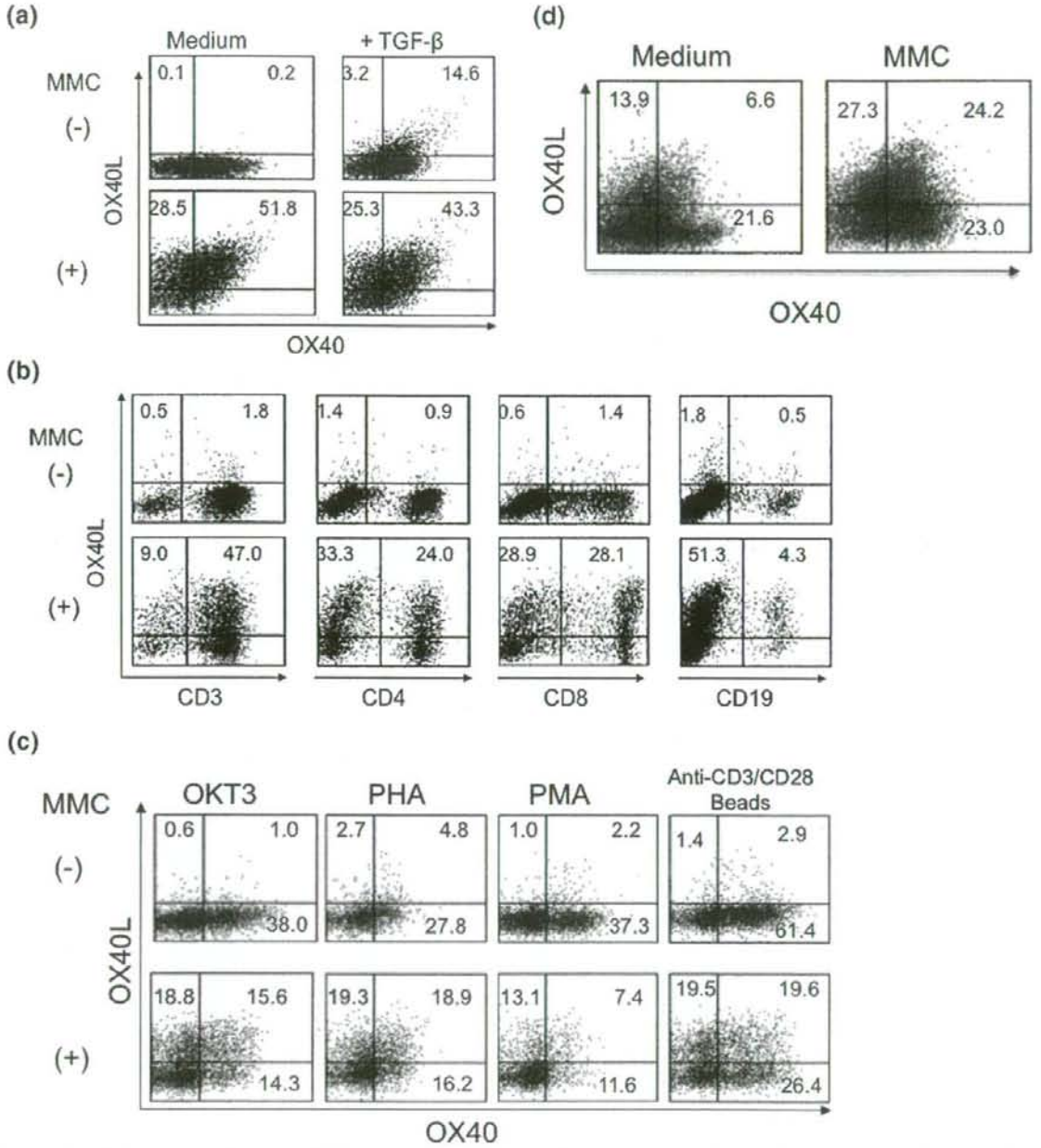

Figure 1. Induction of cell surface OX40L by primary T cells by MMC treatment and cell activation. (a) Fresh PBMCs were either untreated or pretreated with MMC (50 μg/ml for 45 minutes at 37°C), stimulated with immobilized OKT-3 in the presence or absence of 20 ng/ml TGF-β1 for 3 days, and then examined for OX40L and OX40 expression by FCM. (b) Fresh PBMCs were either pretreated with MMC (50 μg/ml MMC for 45 minutes at 37°C) or untreated, stimulated with immobilized OKT-3 for 2 days, and then examined for OX40L expression on select lymphocyte subpopulations by FCM. (c) Fresh PBMCs were stimulated with immobilized OKT-3, 10 μg/ml phytohemaglutinin A, 25 ng/ml PMA, or anti-CD3/anti-CD28-conjugated magnetic beads for 24 hours either in the presence or in the absence of 10 μg/ml MMC and then examined for OX40L and OX40 expression by FCM. (d) T cells that were previously activated with immobilized OKT-3 for 3 days were restimulated with immobilized OKT-3 in the presence or absence of MMC for 24 hours. OX40L and OX40 expression was examined by FCM. Representative data of three independent experiments using two different donors are illustrated.

Effect of other DNA synthesis inhibitors, ultraviolet (UV), and stresses on OX40L induction

To determine whether OX40L induction was unique to MMC treatment of *in vitro*-activated T cells or a general attribute of inhibitors of DNA synthesis, a variety of other DNA-damaging methods such as X-ray irradiation and 5-FU treatment were tested. As illustrated in Figure 2a, both treatments induced OX40L on activated T cells in a dose-dependent manner. The optimum doses of MMC, 5-FU, and x-ray for the maximum induction of OX40L on activated T cells were 6.3 to 12.5 μg/ml, 100 μg/ml, and 8-16 Gy, respectively. Treatment of PBMCs with optimal levels of 5-FU and MMC in combination followed by *in vitro* activation with anti-CD3 slightly enhanced the level of OX40L expression by T cells but the cell viability was reduced, possibly due to increased cytotoxicity (data not shown).

In an attempt to explore other conditions that might induce OX40L expression by T cells, we tested the exposure of aliquots of PBMCs to the DNA-damaging UV irradiation and exposure to heat and oxidative shock. Preliminary results of these studies indicated that an exposure of PBMCs to either UV-A or UV-B for a long period was very cytotoxic because most cells died by 24 hours following exposure (data not shown). However, exposure to sublethal doses of UV-B, but not UV-A, followed by short-term culture in the presence of immobilized anti-CD3 for 18 hours demonstrated a moderate increase of $OX40L^+$ cells above that seen with medium treatment (Figure 2b). In addition, neither exposure to heat shock nor oxidative stress by exposure of PBMCs to H_2O_2 had any detectable effect on the induction of OX40L expression following anti-CD3 activation (Figure 2c). These data highlight the effect of DNA stress on the induction of OX40L following *in vitro* activation of T cells.

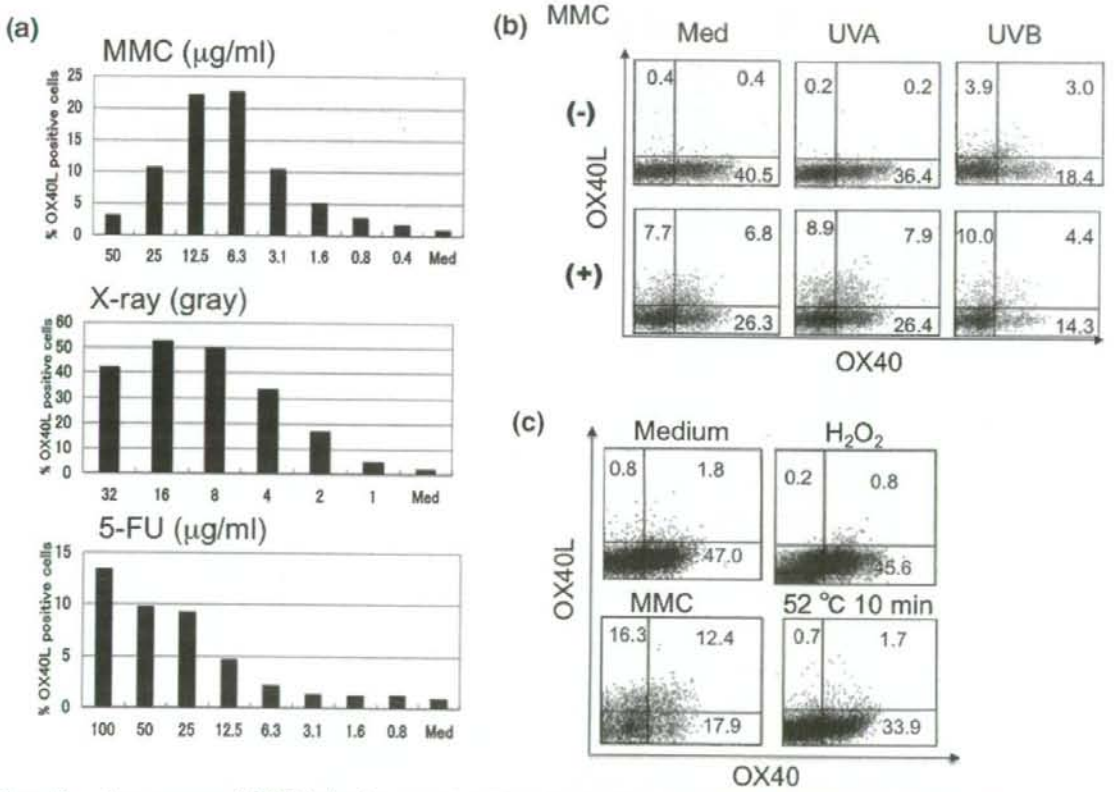

Figure 2. Dose response of OX40L induction on activated lymphocytes by exposure to DNA-damaging drugs and X-ray irradiation and the effect of UV irradiation, oxidation, or heat stress. (a) Fresh PBMCs were either untreated or pretreated by exposure to various doses of x-ray and stimulated with immobilized OKT-3 either in the presence or in the absence of grated concentrations of MMC or 5-FU for 24 hours. The frequency (%) of OX40L-positive cells was determined by FCM. (b) PBMCs were irradiated with sublethal doses of UV (UV-A at 365 nm for 81 seconds or UV-B at 254 nm or 60 seconds at 10 $\mu W/cm_2$) and then stimulated with immobilized OKT-3 for 18 hours in the presence or absence of MMC (10 μg/ml). Then, live lymphocytes were examined for the expression of OX40L and OX40 using standard FCM. (c) PBMCs were either untreated or preheated for 10 minutes at 52°C and then stimulated with immobilized OKT-3 for 24 hours in the presence or absence of 12.5 μg/ml H_2O_2 or 10 μg/ml MMC. The viable lymphocytes were then examined for OX40L and OX40 expression by FCM. Representative data of five independent experiments using three different donors are illustrated.

Effect of inhibitors of RNA and protein synthesis and T-cell activation on OX40L induction

We next tested the effects of inhibitors of RNA and protein synthesis on OX40L induction following anti-CD3 activation. As illustrated in Figure 3, neither Act-D nor CHX induced OX40L expression by activated T cells. Of interest was the finding that these two drugs not only inhibited the MMC-induced OX40L expression by T cells, but also inhibited OX40 expression. Thus, it is clear that new RNA and protein syntheses were required for the induction of not only OX40L but also OX40 by activated T cells. In addition, CsA, which is known to block T-cell activation at an early activation step, also interfered with both OX40L and OX40 induction, supporting the view that cell activation was an important requirement for OX40L expression by activated T cells.

Time course of OX40L expression and OX40L localization

Time-course experiments were performed to study the kinetics of OX40L induction. Fresh PBMCs were cultured in anti-CD3 mAb-coated plates in the presence of MMC for 1 to 72 hours and then examined for OX40L expression. As summarized in Figure 4a, the expression of OX40L became apparent as early as 1 hour after activation, and the percentage of positive cells gradually increased over time up to 72 hours. The decrease in cell viability following 72 hours in culture limited our ability to examine the induction of OX40L beyond this time period.

It was reasoned that one explanation for our failure to detect OX40L by non-MMC-treated *in vitro* anti-CD3 activated T cells could be due to the potential shedding of this molecule into the culture medium. We thus quantitated the levels of soluble OX40L in the culture supernatants and cell lysates of non-MMC-treated anti-CD3 activated cells using our in-house-developed OX40L and OX40 sandwich ELISA kits. As illustrated in Figure 4b, whereas OX40L was readily detected in the control MMC-treated activated cell lysates, there was no detectable level of OX40L in the culture supernatants of the MMC-untreated activated PBMCs. In addition, the level of cell-associated OX40L in the MMC-untreated activated PBMC was significantly lower than that of MMC-treated activated PBMCs. Of interest, the levels of OX40 in the cell lysates demonstrated an opposite trend with higher relative levels in MMC untreated compared with MMC-treated anti-CD3 activated cells. These data demonstrate that our failure to detect OX40L on non-MMC-treated *in vitro* anti-CD3-activated cells was not secondary to the shedding of this molecule. An alternative explanation could be that our failure to detect OX40L is due to homotypic binding of the OX40 to its cognate ligand OX40L in a manner that prevents the binding of the mAb being utilized for flow analysis. To address this issue, we treated PBMCs with a blocking anti-OX40 mAb (clone W4-54) during cultivation. The addition of such a blocking antibody still failed to demonstrate any detectable increase in the levels of OX40L on the cells following anti-CD3 activation and, furthermore, did not lead to any detectable levels of soluble OX40L in the culture supernatants (data not shown). These data further support the idea that both inhibition of DNA synthesis and activation are required for the induction of OX40L expression by activated T cells.

OX40L induced on T cells is functional

To examine whether OX40L molecules expressed by the MMC-treated activated human T cells were functional, we tested the ability of these cells to bind soluble biotinylated OX40 along with OX40L. Figure 5 (top) illustrates that MMC-treated activated T cells clearly bind soluble OX40 and at much higher levels than that of control MMC-untreated activated T cells. To rule out a role for the FcR because the soluble OX40 utilized was a fusion protein with Fc portion of the mouse IgG2a, sample cells were preincubated with purified mouse IgG2a at 100 μg/ml and then analyzed for sOX40 binding. The blocking of FcR failed to influence the binding profile of sOX40, providing suggestive evidence that the binding of sOX40 was not likely due to FcR involvement (data not shown). In addition, the blocking of potential homotypic OX40-OX40L interaction by the addition of a blocking anti-OX40 mAb resulted in enhanced binding of sOX40 by both

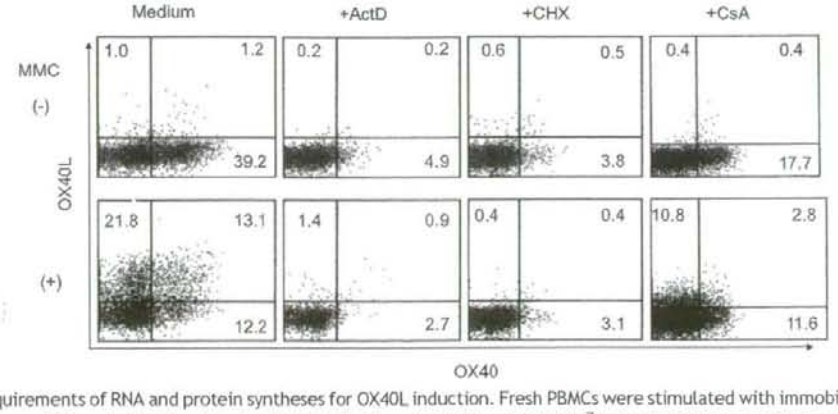

Figure 3. Requirements of RNA and protein syntheses for OX40L induction. Fresh PBMCs were stimulated with immobilized OKT-3 in the presence or absence of 10 μg/ml MMC and/or 1 μg/ml Act-D, 10 μg/ml CHX, or 5.5×10^{-7} M CsA for 24 hours and then examined for OX40L expression by FCM. Representative data of five independent experiments using three different donors are illustrated.

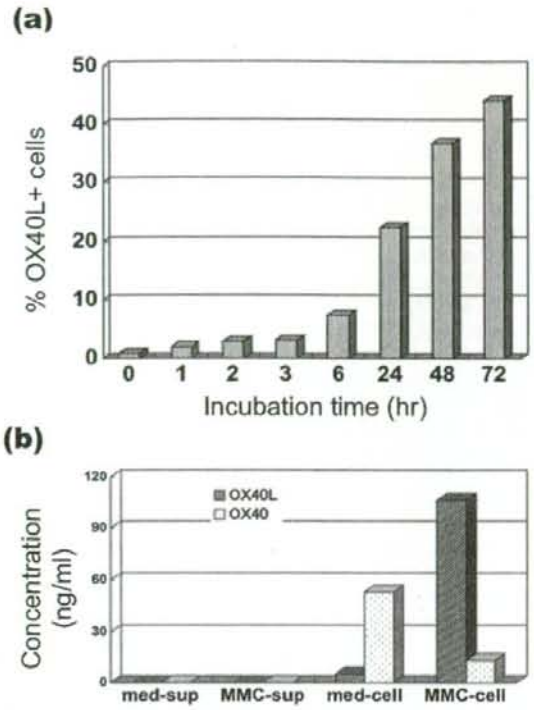

Figure 4. Time course of OX40L induction and its localization. (a) Fresh PBMCs were activated with immobilized OKT-3 in the presence of 10 μg/ml MMC for various periods of time. At each time point, the frequency of viable OX40L$^+$ lymphocytes within the lymphocyte gate was analyzed by FCM. (b) Fresh PBMCs were activated with immobilized OKT-3 either in the presence of 10 μg/ml MMC or in medium for 24 hours, and then culture supernatants (-sup) and cell lysates (1×10^6 cells/ml) (-cell) were examined for concentrations of OX40L and OX40 using our in-house ELISA kits. Representative data of five independent experiments using three different donors are illustrated.

MMC-treated and untreated T cells (data not shown), indicating that a certain level of homotypic OX40-OX40L interaction does occur at the cell surface. Thus, these data suggest that OX40L molecules on the T cells were indeed functional, at least as determined by their ability to bind to their cognate receptor. On the contrary, similar studies conducted on analyzing the binding of sOX40L demonstrated that MMC-treated activated T cells displayed relatively lower levels of binding of sOX40L compared with MMC-untreated controls, indicating that a balance must exist in the expression of OX40L and OX40 being synthesized by T cells depending on the conditions of T-cell activation.

Induction or enhancement of OX40L, CD27L, and CD30L expression in the MMC-treated activated T cells *in vitro* and *in vivo*

In efforts to determine whether exposure to a DNA stress-inducing agent uniquely influenced OX40L expression or whether such exposure generally influences the expression of other TNFSF of cell surface molecules following *in vitro* activation, we also examined the expression of CD27L, CD30L, FasL, TRAIL, and TNF-α in addition to a variety of other cell surface molecules. Results of these studies demonstrated that the MMC treatment following anti-CD3 activation led to significant increases in the expression of CD27L and CD30L on T cells, but the expression levels of the other three TNFSF molecules known as death-receptor ligands, FasL and TRAIL and TNF-α, were not significantly different under these stimulation conditions.

Finally, in efforts to determine whether such induction of OX40L on T cells by MMC treatment and activation also occurred *in vivo*, we utilized our humanized mouse model. Human T cells are immediately activated following transfer into a xenogeneic mouse [23]. Thus, we did not use any exogenous stimulating agents in these experiments. Fresh human PBMCs were transplanted i.p. into immunodeficient mice followed by MMC injection. After 24 hours, these mice were sacrificed and cells from the spleen and peritoneal cavity were collected and then examined for the expression of OX40L together with CD27L and CD30L. As illustrated in Figure 6b, HLA class I-gated live human lymphocytes recovered from the MMC-treated mice expressed high levels of OX40L, as well as CD27L and CD30L, in contrast to MMC-untreated controls. These results indicate that induction or enhanced expression of OX40L, CD27L, and CD30L by human T cells indeed occurred *in vivo* by exposure to DNA-damaging agents and cell activation.

Discussion

One of the objectives of the present study was to address the original question as to whether cell proliferation was

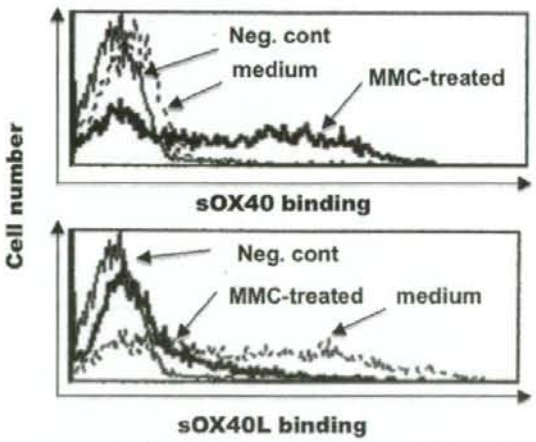

Figure 5. The OX40-binding capacity of the OX40L induced on MMC-treated activated T cells. Fresh PBMCs were activated with immobilized OKT-3 either in the presence or in the absence of 10 μg/ml MMC for 24 hours. Cell samples were washed, preincubated with Fc-blocking buffer, and then stained with biotinylated soluble OX40 or OX40L followed by streptavidin-PE staining and FCM analysis. Representative data of five independent experiments using three different donors are illustrated.

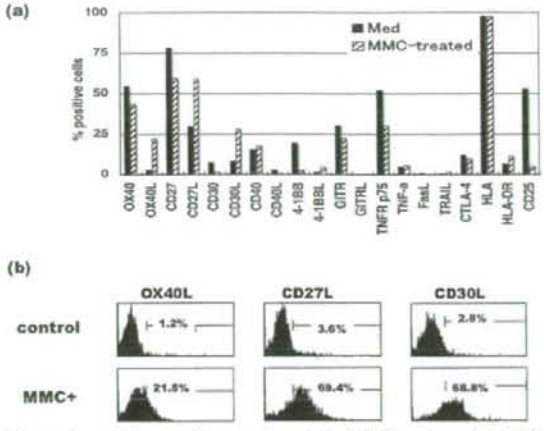

Figure 6. Enhanced expression of the TNFSF molecules CD30L and CD27L, together with OX40L on MMC-treated activated T cells both *in vitro* and *in vivo*. (a) Fresh PBMCs were activated with immobilized OKT-3 either in the presence of 10 μg/ml MMC or in medium for 24 hours, and then the viable lymphoid cells were analyzed for cell surface phenotype using a variety of mAbs. The percentage of positive cells is reported. Representative data of five independent experiments using at least three different donors are illustrated. (b) Immunodeficient mice (two animals per group) were engrafted with 1×10^7 fresh human PBMCs i.p. followed by immediate injection with 620 μg MMC or PBS (control). After 24 hours, these mice were sacrificed and their spleen cells and cells within the intraperitoneal cavity were harvested and pooled. The levels of cell surface expression of OX40L, CD27L, and CD30L on HLA class I$^+$ human lymphocytes were examined by FCM. Representative data of three independent experiments using two different donors are illustrated.

necessary for OX40L induction on activated T cells. Of interest is our finding that T-cell activation under DNA synthesis-inhibiting conditions rapidly induces the cell surface expression of OX40L, which is dependent on both RNA and protein syntheses *in vitro* and *in vivo*. The DNA damaging agents that induce OX40L expression include irradiation with x-ray and UV and anticancer drugs. Therefore, the phenomenon of OX40L induction on T cells may be not as rare an event in the normal life of humans.

The precise mechanism for the present observation is unknown at present. However, there are several potential explanations for the observed results. First, OX40L is synthesized as a result of a DNA repair process, in which OX40L$^+$ T cells may rescue OX40$^+$ T cells by stimulating an antiapoptotic pathway via OX40 signaling [8,24]. On the other hand, OX40L may function as a rescue molecule for such DNA-damaged activated T cells when stimulated with OX40. Second, high levels of OX40L on T cells may stimulate coexisting OX40-positive T cells to ameliorate emergency conditions induced by exposure to radiation and anti-DNA drugs via suppressing potent T helper 1 (Th1) conditions [13]. Third, OX40L expression may favor the clearance of DNA-damaged apoptotic cells by scavenger cells such as macrophage or dendritic cells. Although it is not known whether OX40L functions as an "eat-me" signal of apoptotic T cells [25], OX40L may facilitate the adhesion between apoptotic T cells and phagocytes such as DCs that are induced to express OX40 under certain conditions [26]. Because DCs are stimulated by exposure to apoptotic PBMCs activated via TCR [27], it is of interest to test whether OX40L on dying activated T cells is involved in the enhancement of antigen-presenting functions of DCs.

The potential benefits of OX40 stimulation by recombinant OX40L or agonistic antibody [28-30] on the therapy of some diseases, especially cancer, have been extensively investigated utilizing murine models [6,31,32]. One of the important roles of OX40 expression during immunotherapy is to maintain antigen-specific effector memory T cells [14,33,34]. In addition, results of some recent studies have revealed that OX40 triggering blocks regulatory T-cell function and thus facilitates T-cell-mediated tumor rejection [35,36]. These findings suggest that triggering of OX40 could have important therapeutic implications. In this regard, OX40L-expressing autologous T cells freshly prepared *in vitro* or *in vivo* may be good candidates that would be safe and have limited unnecessary side effects. Furthermore, because soluble recombinant OX40L molecules are less effective in activation than oligomers [29], cell-associated OX40L expressed on the cell surface may facilitate OX40 ligation more strongly than the soluble recombinant OX40L. Because MMC and irradiation have been generally used for the treatment of cancer in humans, simultaneous administration with humanized anti-CD3 antibody may induce a substantial number of OX40L-expressing T cells *in vivo*. Alternatively, such OX40L-positive T cells can be easily generated *in vitro* in large quantities for adoptive transfer studies by long-term cultivation of PBMCs in the presence of IL-2 and anti-CD3/anti-CD28 antibodies. Such therapeutic strategies must be clearly verified using our humanized mouse model.

Several agents that trigger OX40L expression in a variety of cell lineages have been previously reported, including thymic stromal lymphopoeitin, TNF-α, lipopolysaccharide (LPS), or CD40 ligation for DCs [11,13,37], CD40 ligation and mitogens for B cells [10,38], IL-12 for murine T cells [14], CD16 or Ig-like receptor ligation for human NK cells [15], Fc-εRI ligation for human mast cells, TL1A and death receptor 3 ligand for murine CD4$^+$CD3$^-$ accessory cells, and IL-4 and TGF-β for human T cells [18]. On the other hand, down-regulating factors for OX40L expression have also been reported, including IL-12 for human T cells [18], type-I interferon for human mast cells [39], and IL-4 for murine T cells [14]. It remains to be established as to the mechanisms by which IL-12 and IL-4 function differently on OX40L induction between human and murine T cells. In addition, our preliminary study demonstrated no apparent induction of OX40L on murine splenocytes following x-ray irradiation and stimulation (G. Matsuzaki, K. Okuma and Y. Tanaka, unpublished data). Thus, there might be species-specific differences associated with OX40L induction or suppression by T cells and potentially also the biological outcomes of such OX40L-OX40 interactions. One example for the latter is that whereas mouse CD4$^+$ T cells acquire longevity following ligation of OX40 [14], human Th1 CD4$^+$ T cells have been shown to undergo apoptosis as a result of OX40 ligation [17]. A possible explanation for these discrepancies may be related to exposure to different environmental conditions and differences in the process of aging in the two species. Whereas mouse T cells are obtained from young mice (younger than a

few months) that have been housed in an specific pathogen free (SPF) environment, human T cells are obtained from adults having been exposed to irradiation, various microorganisms, and anti-DNA substances. Thus, it can be speculated that, as a result of aging in a natural environment (non-SPF environment), human immune systems are more frequently exposed to OX40L than the immune systems of naive mice. Because OX40L transgeneic mice suffer from immunological disorders such as autoimmune diseases [40], it is reasonable to assume that human T cells are less responsible for OX40L stimulation than murine T cells as a result of homeostasis maintenance.

Taking into consideration that DNA damage is a daily event in human life and one of the major contributors to the senescence of cells, it also seems possible that there could be a relationship between OX40L expression and senescence of T cells. Indeed, OX40L can be induced in T cells after long-term and repeated activation *in vitro* [18,41] without exposure to additional exogenous DNA-damaging agents. Furthermore, levels of soluble OX40L in serum samples are higher in aged humans [42] than in young adults. Thus, it will be interesting to explore both the relationship between telomere length and OX40L expression in T cells and the biologic meaning of OX40L expression for T-cell senescence and homeostasis.

Previously, Kim *et al.* [43] reported that 4-1BB (CD137), which is a member of the TNF receptor superfamily and normally induced on activated T cells as a costimulatory molecule, is rapidly induced on resting T cells by exposure to DNA-damaging agents to protect T cells from genotoxic stress. The major difference between 4-1BB and OX40L is that 4-1BB can be induced only by DNA damage without activation, whereas OX40L induction requires both signals. In addition, as illustrated in Figure 6, 4-1BB and its ligand expressing fresh lymphocytes were decreased by activation in the presence of MMC. Thus, the mechanism of and physiological role for OX40L induction by T cells are independent of 4-1BB activation although human OX40 and 4-1BB form a heterodimer in activated T cells and the heterodimer functions to suppress T-cell immune responses [44].

Further studies are in progress to determine the mechanisms by which DNA damage impacts activated T cells by expressing or downmodulating immunological molecules, including CD27L and CD30L, and their physiological roles in immunity. These studies may be relevant not only for the development of new immunotherapeutic strategies but also for our understanding of how DNA-damaged T cells influence the health of patients suffering from radiation exposure or receiving anticancer drugs.

Acknowledgments

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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 I (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

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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-1β, TNF-α and prostaglandin E_2 (PGE_2) 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 μg/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 m*M* Na_2-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% CO_2 humidified incubator for 3 days. The cultures were pulsed at different time points with either KLH (10 μg/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% NaN_3 (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 μg/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% CO_2 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 μg/ml) and soluble anti-CD28 mAb (1 μg/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 dispended 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

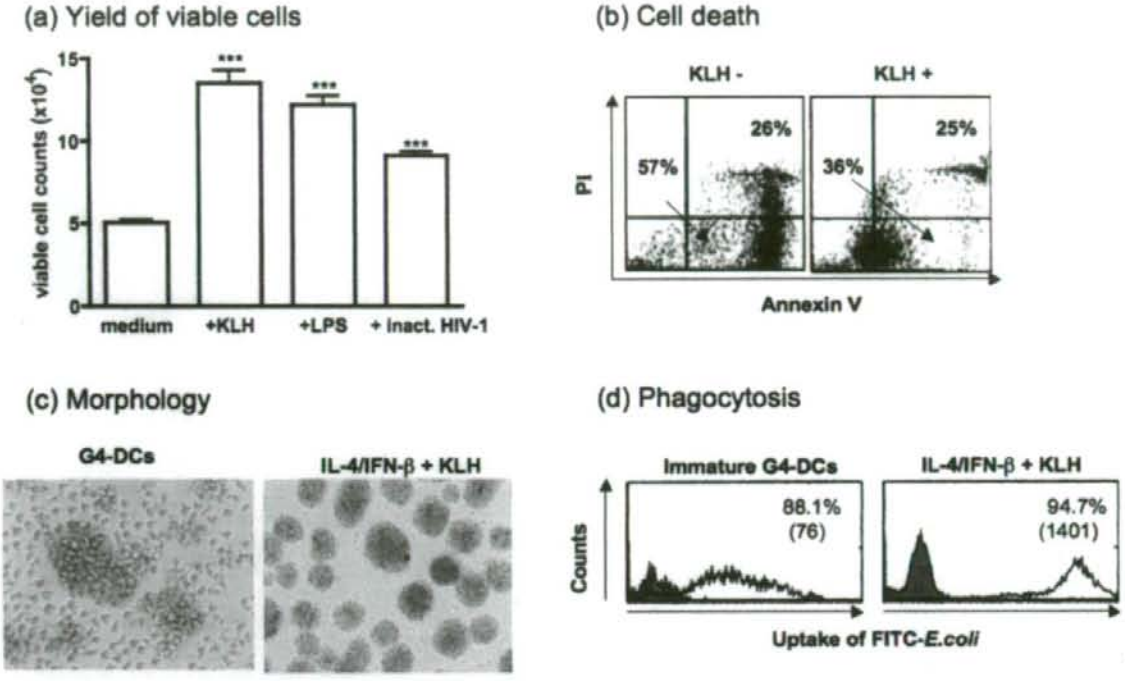

Figure 1. The addition of KLH, LPS, or inactivated HIV-1 promotes the survival of monocytes cultured in media containing IL-4 and IFN-β. $CD14^+$ monocytes purified from PBMCs by a negative selection method (3×10^5 cells/well) were cultured in the presence of IL-4 and IFN-β, followed by the addition 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 INF-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 ×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 ± 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

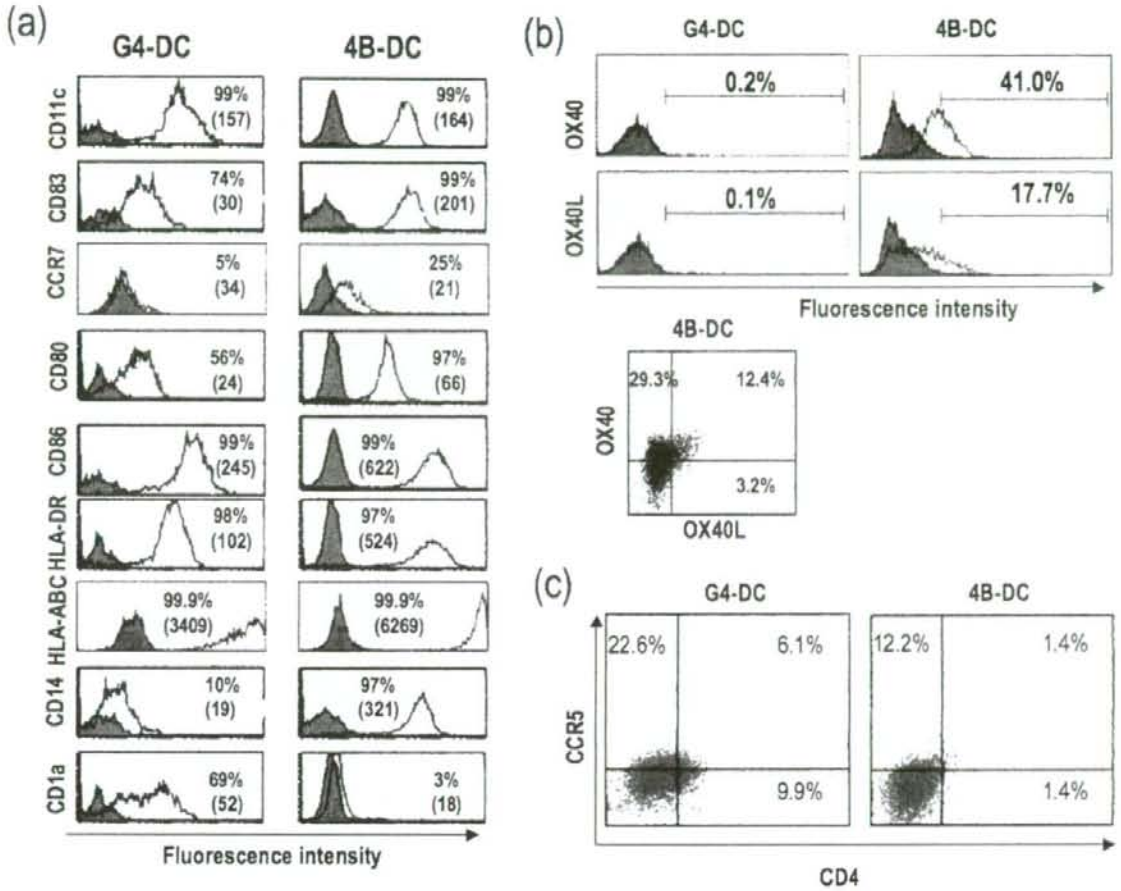

Figure 2. Flow cytometry facilitated phenotypic characterization of IL-4/IFN-β and KLH-cultured monocytes (4B-DCs) and for comparison G4-DCs. (a) 4B-DCs and G4-DCs were examined for cell surface expression of CD11c, CD83, CCR7, CD80, CD86, HLA-DR, HLA-ABC, CD14, and CD1a as described in the Materials 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 ± 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

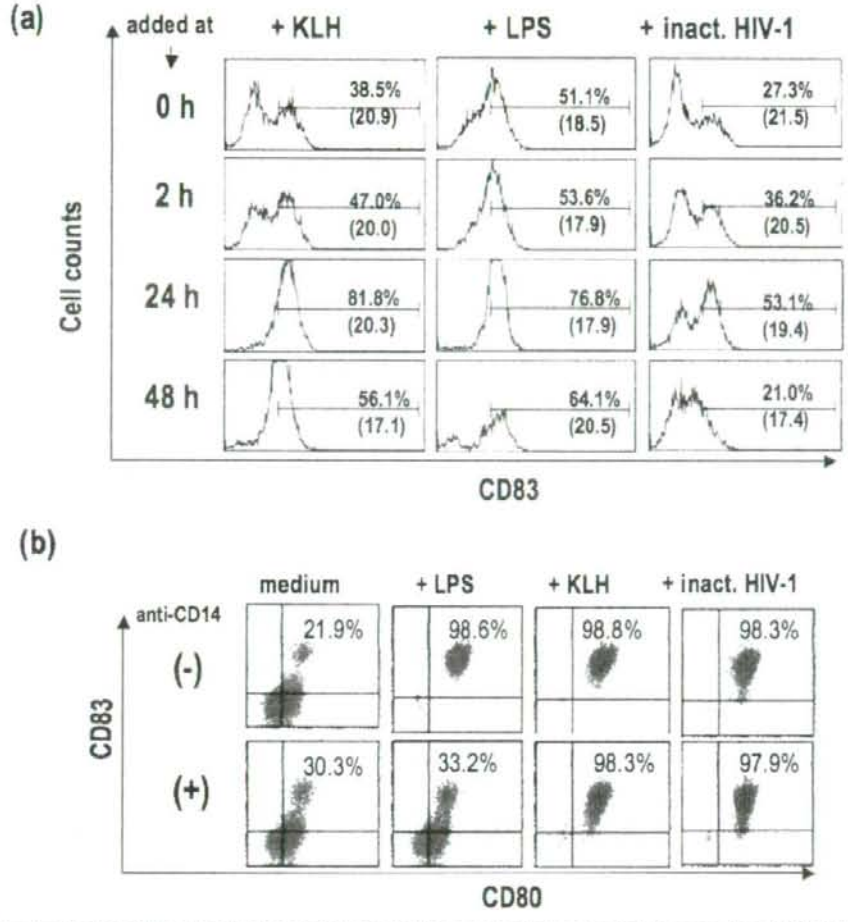

Figure 3. Optimum time for the addition of antigens needed for stimulation and pathways for 4B-DC generation. (a) $CD14^+$ monocytes were cultured in the presence of IL-4 and IFN-β and at indicated times KLH, LPS, or inactivated HIV-1 was added to each culture, and all cultures were harvested on Day 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 μg/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 ± 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

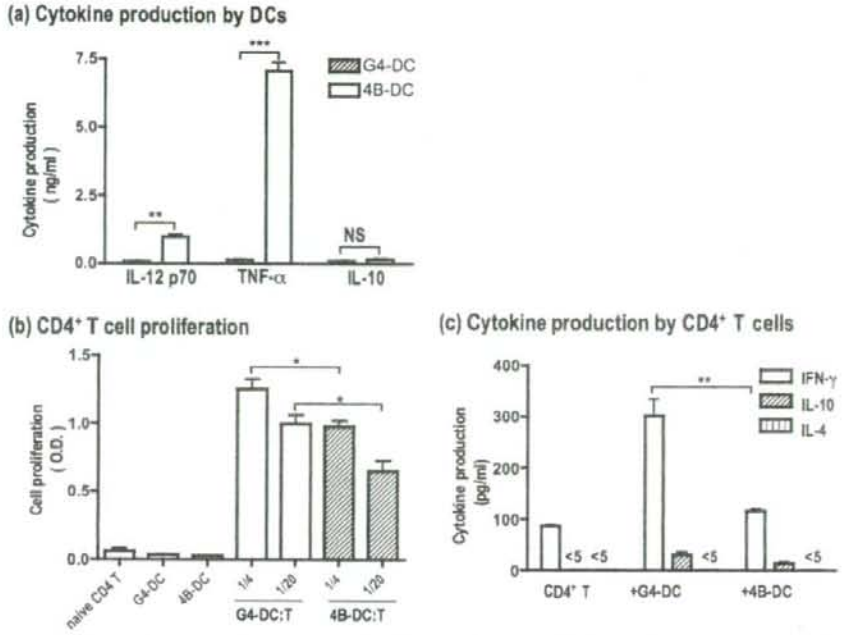

Figure 4. Difference in cytokine production and comparison of the allostimulation potential of naïve $CD4^+$ T cells by 4B-DCs and G4-DCs. 4B-DCs (Day 3) were generated using KLH and conventional G4-DCs (Day 7) were generated by maturation with IFN-β for last 2 days. (a) Supernatants of the two DC cultures 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 ± 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

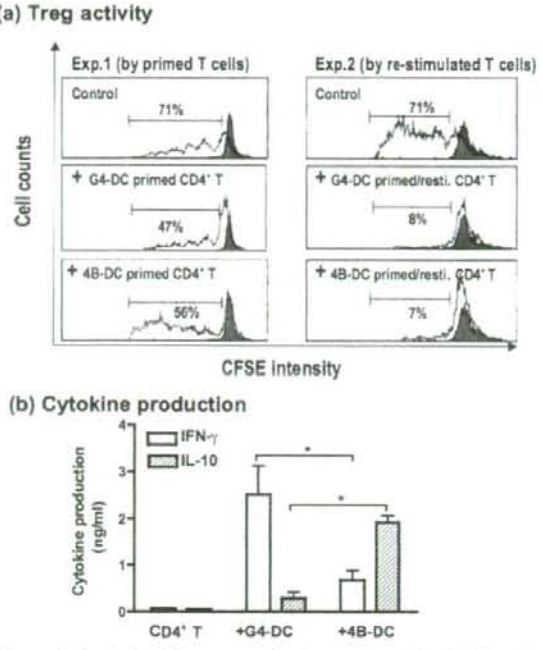

Figure 5. Analysis of Treg generation by coculture with 4B-DCs and the synthesis of IL-10 by restimulated $CD4^+$ T cells. Allogeneic KLH-matured 4B-DC- or conventional G4-DC-primed $CD4^+$ T cells as shown in Fig. 4b, or those cells after restimulation were examined for cytokine production and Treg 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 ± SD of the data derived from the three samples.

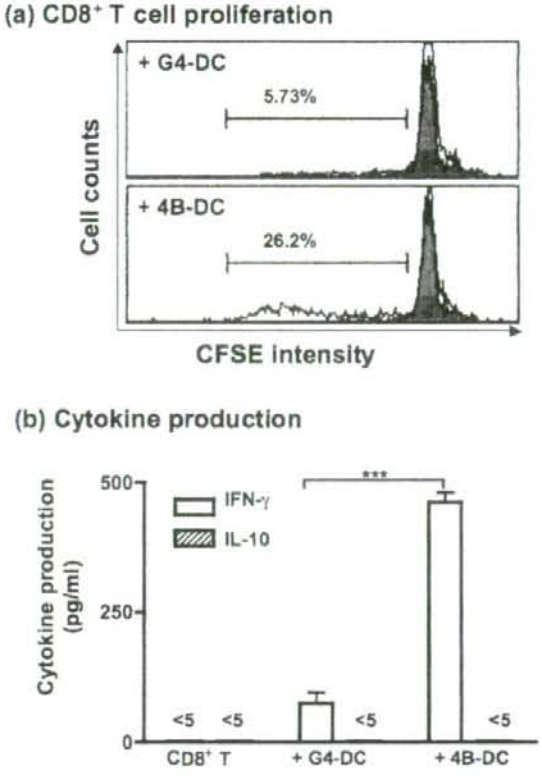

Figure 6. Stimulation of $CD8^+$ T cells with 4B-DCs. CFSE-labeled purified $CD8^+$ T cells were cocultured with allogeneic KLH-matured 4B-DCs or G4-DCs at T to DC ratio of 4:1 in RPMI medium containing IL-2 (20 U /ml) in 96 well U-bottom plate for 6 days. (a) Proliferation of the CFSE-labeled $CD8^+$ 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 ± 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 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-1β, TNF-α, and PGE_2 (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-CDs 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