LPS induces rapid IL-10 release by M-CSF-conditioned tolerogenic dendritic cell precursors

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Abstract: Dendritic cells (DC) obtained by culturing myeloid precursors in GM-CSF undergo maturation and induce an efficient T cell response when stimulated with microbial products. DC precursors themselves also recognize microbial products, and it remains unclear how these stimulated DC precursors modulate the immune response. We show here that M-CSF-conditioned human DC precursors responded to LPS, *Mycobacteria bovis*, and inflammatory cytokines by a rapid and robust production of IL-10, largely superior to that observed with immature DC or monocytes. The endogenous IL-10 restrained the DC precursors from converting into professional APC, as blocking the IL-10 receptor in the presence of LPS resulted in the formation of efficient T cell stimulators. LPS stimulation concomitant with DC differentiation gave rise to immature DC, which were tolerant to a secondary LPS exposure. Furthermore, the LPS-activated DC precursors reduced bystander DC maturation and anti-CD3/CD28-triggered T cell activation. These data suggest that when exposed to inflammatory or microbial signals, M-CSF-conditioned DC precursors can participate in the modulation of inflammation and immune response by rapid release of IL-10. *J. Leukoc. Biol.* 82: 133–141; 2007.

Key Words: *growth factor · cytokine · differentiation · activation · tolerance*

INTRODUCTION

Dendritic cells (DC) play a key role in initiating an adaptive, primary, immune response by their ability to capture antigen, migrate, and prime antigen-specific T cells [1]. In addition, there is good evidence that DC are implicated in inducing peripheral T cell tolerance to sampled self-antigens [2]. GM-CSF and M-CSF are related growth factors participating in macrophage development. M-CSF is considered more specific to the macrophage lineage, whereas GM-CSF, in addition, promotes DC differentiation in particular when coupled to Th2 cytokines IL-4 or IL-13. M-CSF is a steady-state growth factor produced constitutively by many cell types, including cells of the monomacrophage lineage and in particular, stromal cells [3]. Constitutive production of GM-CSF in tissue is low but is up-regulated in response to inflammation or danger signals, and activated macrophages and T cells can produce high levels of GM-CSF. It is therefore reasonable to assume that DC precursors, which reside in tissue before differentiation into DC, are exposed to M-CSF.

Mice lacking functional M-CSF (*op/op*) have reduced numbers of Langerhans cells (LC), the epidermal DC subset [4], and mice deficient in M-CSF receptor (M-CSFR) are devoid completely of LC [5]. Moreover, M-CSFR$^{-/-}$ mice appear to lack dermal DC. Thus, M-CSF seems to be involved in the development and/or maintenance of LC and dermal DC, possibly at the stage of DC precursor. In human models of DC development, it has been shown that cord blood monocytes cultured in M-CSF and IL-4 produce more IL-10 in response to LPS than when cultured in GM-CSF and IL-4 [6]. This suggests that M-CSF instructs myeloid cells to an anti-inflammatory program. However, the M-CSF/IL-4-cultured monocytes are fully differentiated DC and cannot address the role of DC precursors in the immune system. Alternately, monocytes cultured in M-CSF retain their capacity to differentiate to DC in the presence of GM-CSF and IL-4 [7], and we have shown recently that cord blood progenitor-derived CD14$^+$ cells kept in M-CSF retain the ability to differentiate to dermal DC and LC [8, 9]. These M-CSF-conditioned CD14$^+$ cells show phenotypic homologies with dermal CD14$^+$ cells, including expression of DC-SIGN, an important pathogen attachment factor, and are permissive to HIV and Dengue virus infection [8]. As these cells can differentiate to dermal DC or LC-type DC, we called them preDC. Although DC release TNF-α and undergo maturation, when infected by Dengue virus, the preDC release IL-10 but no TNF-α and do not undergo maturation [8]. This suggested that preDC, recognizing infectious organisms, could down-modulate the immune response through IL-10 production.

Therefore, we investigated IL-10 production by monocytes in response to LPS when precultured in M-CSF or GM-CSF and

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studied how LPS affected preDC, which are M-CSF-conditioned myeloid cells, in their ability to differentiate to DC, to mature, and to activate T cells. Our results suggest that M-CSF instructs myeloid cells to an anti-inflammatory function in response to LPS, and GM-CSF conditions an inflammatory program. The preDC released early IL-10 and could modulate the immune response by down-regulating T cell proliferation and DC maturation. However, GM-CSF could revert the anti-inflammatory program installed by M-CSF. Thus, we confirm that M-CSF enhances the production of IL-10 by differentiating monocytes [6] and extend the previous work by detailed IL-10 secretion kinetics and by characterizing the immune properties of M-CSF-conditioned DC precursors.

MATERIALS AND METHODS

Cell culture

Umbilical cord blood was collected after consent and processed according to institutional guidelines. $CD34^+$ cells were purified from cord blood using anti-CD34-coated magnetic beads (Miltenyi, Paris, France) and cultured for 5 days in RPMI-1640 medium containing 10% endotoxin-free FCS (Australian origin, Invitrogen, France), 25 ng/ml stem cell factor (R&D Systems, Lille, France), 3 ng/ml TNF-α (R&D Systems), and 200 U/ml GM-CSF (Schering-Plough, Kenilworth, NJ, USA). The cells were then washed and cultured in 25 ng/ml M-CSF (R&D Systems) for 6 days with refreshing M-CSF at Day 3, giving rise to a cell population containing mainly $CD14^+$ cells, which were purified using anti-CD14-coated magnetic beads (Miltenyi) and are referred to as preDC. To obtain DC, the preDC were differentiated in the presence of 500 U/ml (45 ng/ml) GM-CSF (Schering-Plough), 5 ng/ml IL-4 (Schering-Plough), and 2 ng/ml TGF-β (R&D Systems) for 2–3 days.

Peripheral blood buffy coats from healthy donors were obtained from the Établissement Français du Sang, Hôpital Hôtel-Dieu (France). Monocytes were enriched from mononuclear cells by centrifugation through a 52% Percoll gradient (Sigma-Aldrich, Saint Quentin Fallavier, France). Floating cells were collected and negatively selected using a monocyte isolation kit (Dynal, Compiègne, France). Monocytes were used directly or after a 5-day preculture in 50 ng/ml M-CSF or 100 ng/ml GM-CSF in complete medium with cytokine refreshment at Day 3. For data presented in **Table 1**, monocytes were cultured for 18 h in 25, 50, or 100 ng/ml M-CSF or in 100 ng/ml GM-CSF.

Cell stimulation

Monocytes, preDC, or DC were stimulated with different LPS concentrations *(Escherichia coli*; Sigma-Aldrich), 10 ng/ml IFN-γ (R&D Systems), 10 ng/ml TNF-α (R&D Systems), 5 ng/ml IL-1β (R&D Systems), 10 ng/ml IL-6 (R&D Systems), or 1×10^{-6} M PGE_2 (Sigma-Aldrich). For CD40 ligand (CD40L) simulation, cells were cocultured with mitomycin C-treated $CD40L^+$ mouse fibroblasts at a ratio of five preDC:one fibroblast. IL-10R-blocking experiments were performed using the anti-IL-10R1 antibody (MAB274; R&D Systems).

TABLE 1. Presence of IL-10 (pg/ml) 4 h after Stimulation by Indicated Doses of LPS

	Donor A		Donor B	
	LPS		LPS	
	2.5 ng/ml	50 ng/ml	2.5 ng/ml	50 ng/ml
Monocytes	0	0	0	0
M-CSF 25 ng/ml	1156	1354	233	278
M-CSF 50 ng/ml	1729	1638	236	230
M-CSF 100 ng/ml	1326	1684	137	159
GM-CSF 100 ng/ml	0	0	0	0

For this, two million cells were incubated with 15 μg/ml (2×) of blocking antibody for 30 min at 37°C, and then medium alone or supplemented with LPS was added to obtain a final concentration of 7.5 μg/ml neutralizing antibody. Recombinant human (rh)IL-10 was purchased from R&D Systems and used at 10 ng/ml. The mycobacterium *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) was kindly provided by Nathalie Winter (Institut Pasteur, Paris, France). preDC or DC were incubated for 4 h in complete medium lacking antibiotics at a multiplicity of infection (MOI) of one cell to five bacteria. The cells were then washed extensively in PBS and cultured for 20 h in complete medium containing antibiotics.

Cytokine measurements

All cell culture (10^6 cells/ml) supernatants were collected and stored at –20°C. IL-10 measurements were done by the sandwich ELISA technique using antihuman IL-10-specific antibody (Diaclone, Besançon, France). The IL-6 ELISA set was from Immunotools (Freiburg, Germany). The data are reported for 1×10^6 cells/ml.

Phenotypic analysis

Expression of specific markers was determined by flow cytometry on a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA) and the following antibodies from BD PharMingen (San Diego, CA, USA): CD14-FITC [macrophage P9 (MϕP9)], CD1a-PE (HI149), CD83-FITC (HB15e), and CD86-FITC (FUN-1); from Beckman-Coulter (Roissy, France): HLA-DR-FITC (Immu-357) and CD80-FITC (MAB104).

MLR

Naïve T cells were purified from cord blood $CD34^+$ progenitor-depleted PBMC by positive cell sorting using anti-CD4-coated magnetic beads (Dynal). After selection, beads were detached using the provided detach-bead enzyme mix. The T cell population purity was routinely >95%. The MLR medium was complete medium supplemented with 5% human AB serum (AbCys, Paris, France) and 1 mM sodium pyruvate (Invitrogen). For the MLR, stimulator cells, washed free of cytokines or LPS, were distributed in triplicate in graded doses in 96-well, round-bottom plates (Falcon, Becton Dickinson). To facilitate comparison of preDC and DC with respect to T cell stimulation, same donor $CD34^+$ cord blood progenitors were used to generate preDC and DC at different time intervals. Cord blood $CD4^+$ T cells (2×10^4) were added to each well in a final volume of 200 μl. T cell proliferation was evaluated after 5 days of culture following an overnight incubation with 1 μCi [^{3}H]methylthymidine. Results are expressed as mean cpm ± SD of triplicate cultures.

T cell activation

Peripheral adult T cells from buffy coats were isolated with anti-CD4 (Dynal) magnetic beads. Anti-CD3 antibody (10 μg/ml) in PBS was added to a flat-bottom, 96-well plate (Costar, Corning, NY, USA), incubated for 3 h at room temperature, and then seeded with 100 μl containing 10^5 T cells in complete medium, supplemented with 2 μg/ml anti-CD28 (Serotech, Oxford, UK) and 10% human AB serum. Finally, 100 μl supernatants from resting or LPS-stimulated preDC or medium, with or without IL-10 (20 ng/ml) or IL-2 (400 IU/ml), were added. After 5 days, T cells proliferation was assessed as described above. To avoid contact of LPS with T cells, which may have an effect on regulatory T cells [10], the preDC were pulsed for 3 h with LPS, washed extensively, and then kept in culture for 24 h. Still, in control experiments, we found that the addition of LPS to CD3/CD28-activated T cells had no effect on T cell proliferation (data not shown).

RESULTS

M-CSF instructs monocytes to rapid IL-10 production

In response to stimuli such as LPS, monocytes respond by releasing anti-inflammatory IL-10 and proinflammatory TNF-α/IL-6 [11]. In view of this dichotomous cytokine response, with important implications with regard to monocyte differen-

tiation into suppressor-type macrophages [12] or DC, we asked if monocytes can be instructed to trigger an anti-inflammatory or an inflammatory program exclusively. Monocytes were cultured for 5 days in M-CSF or in GM-CSF and then stimulated by LPS. Production of IL-10 and IL-6 was measured with time (**Fig. 1A**). M-CSF-conditioned monocytes produced IL-10 rapidly but little IL-6. In contrast, when precultured in GM-CSF, the monocytes released IL-6 rapidly but little IL-10. TNF-α secretion was detected in both conditions after 3 h of stimulation without a clear difference between M-CSF or GM-CSF pretreatment (data not shown). Culturing monocytes in M-CSF or GM-CSF alone did not lead to IL-10 or IL-6 release. To further analyze the potential of M-CSF to instruct monocytes to secrete early IL-10, we cultured monocytes only for 18 h in the presence of different concentrations of M-CSF or GM-CSF and then measured IL-10 4 h after LPS stimulation. As shown in Table 1, exposing monocytes to M-CSF for 18 h was sufficient to induce IL-10 secretion, whereas no IL-10 was released by control monocytes. However, concomitant stimulation of LPS + M-CSF did not lead to IL-10 secretion (data not shown), suggesting that M-CSF triggers a cell differentiation program and does not simply provide a synergistic signal to LPS. The data show that M-CSF prepares monocytes to an IL-10 anti-inflammatory program, and GM-CSF instructs for an IL-6 inflammatory response.

Cord blood-derived $CD14^+$ cells kept for 6 days in M-CSF retain the capacity to convert into DC [8]. We tested whether these $CD14^+$ DC precursors (preDC) secrete IL-10 in response to LPS. As shown in Figure 1B, when stimulated with 50 ng/ml LPS, preDC produced virtually as much IL-10 as monocytes after 24 h. However, in the presence of 2.5 ng/ml LPS, preDC still released 3500 pg/ml IL-10, whereas monocytes only produced little IL-10. In a kinetic evaluation with 2.5 ng/ml LPS (Fig. 1C), IL-10 was clearly detected (220 pg/ml) after 2 h for preDC and peaked at 9 h. In contrast, we detected low IL-10 levels (230 pg/ml) 8 h after stimulation for monocytes, and levels remained low throughout the experiment. Thus, preDC can release high levels of IL-10 rapidly in response to a low dose of LPS.

Proinflammatory signals trigger IL-10 release

We next tested stimuli other than LPS for their ability to trigger IL-10 production. preDC were cultured for 24 h in GM-CSF, TNF-α, IL-6, IL-1β, IFN-γ, as well as PGE_2, LPS, or CD40L-transfected fibroblasts or a combination thereof. The combination of TNF-α, IL-6, IL-1β, and PGE_2 was reported to trigger DC maturation efficiently [13]. As shown in **Figure 2A**, GM-CSF did not induce IL-10, but all of the other cytokines triggered IL-10 production. We then assessed these stimuli for their ability to convey to preDC the capacity to stimulate naive

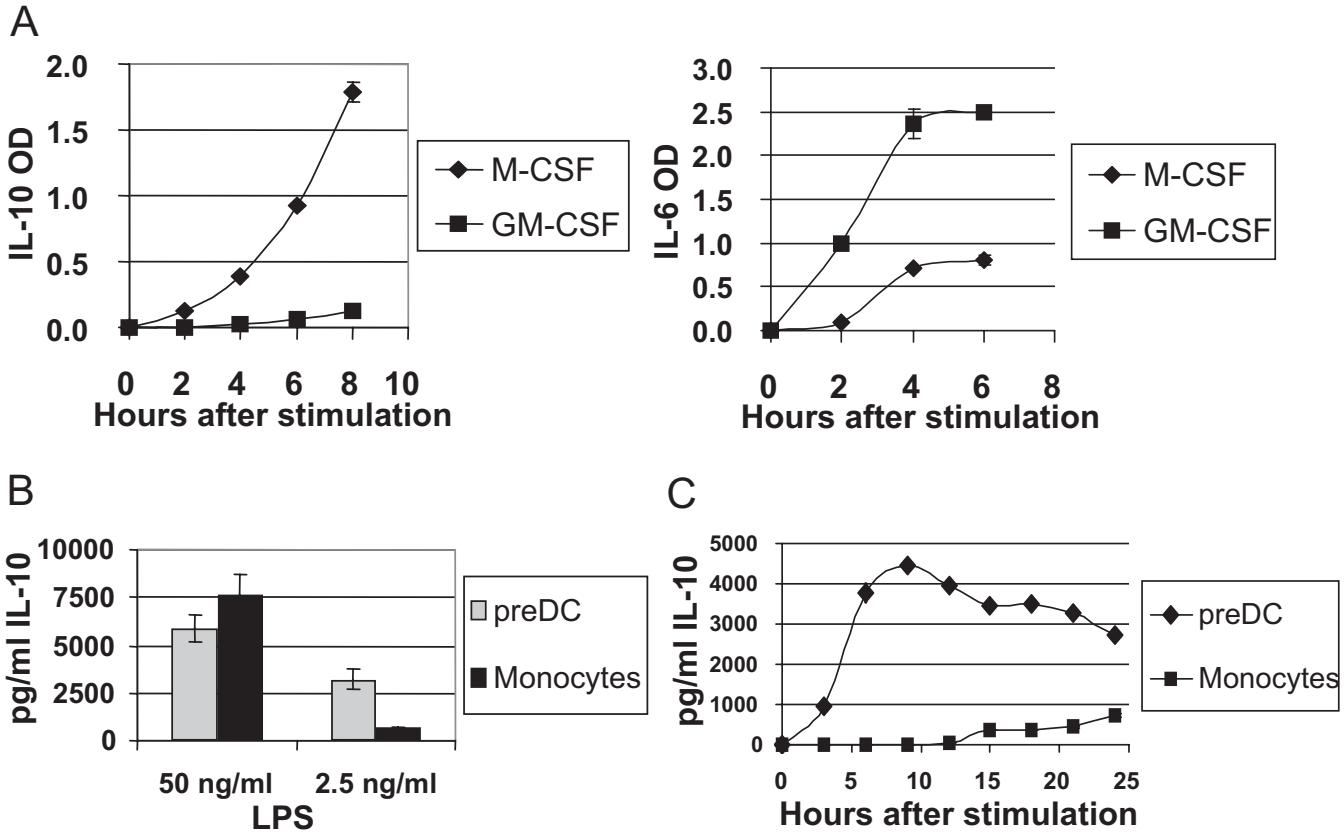

Fig. 1. M-CSF instructs monocytes to rapid IL-10 production. (A) Adult monocytes were cultured for 5 days with M-CSF or GM-CSF and then stimulated with 50 ng/ml LPS, and release of IL-10 and IL-6 was measured. The data are expressed as OD of the ELISA assay and are representative of three donors. (B) preDC or monocytes were stimulated with 2.5 ng/ml or 50 ng/ml LPS, and IL-10 production was measured after 24 h (mean±SD of IL-10 for 10^6 cells from five different donors). (C) preDC and monocytes are stimulated with 2.5 ng/ml LPS, and production of IL-10 was measured at different time-points for 24 h. The data are the mean ± SD of duplicates and representative of three donors.

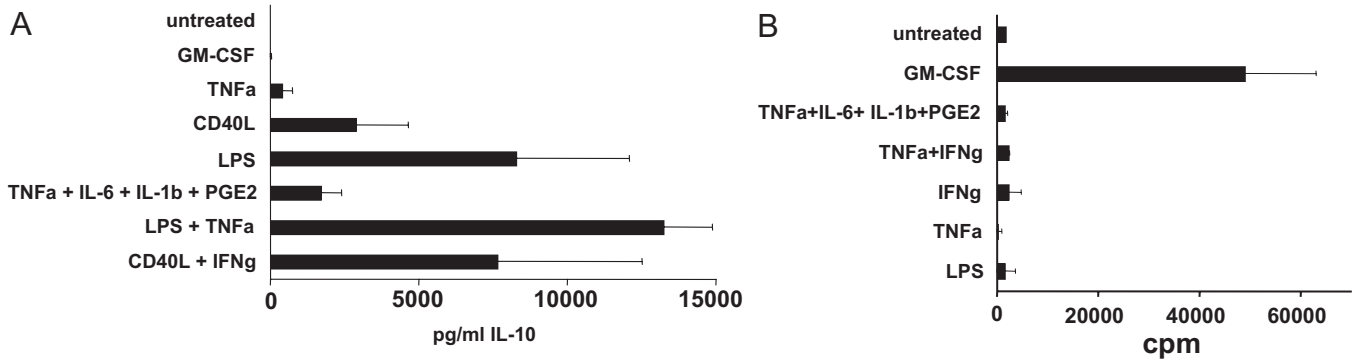

Fig. 2. Proinflammatory and microbial signals but not GM-CSF trigger IL-10 release. (A) preDC were cultured for 24 h in the presence of the indicated cytokines, LPS, or in the presence of CD40L$^+$ fibroblasts. IL-10, in the cell supernatant, was measured by ELISA and expressed as the mean ± SD for 10^6 cells of at least three different donors. (B) preDC were stimulated by the indicated cytokines or LPS for 24 h and then washed. They were added to allogenic cord blood CD4$^+$ T cells at a ratio of 1:1. After 5 days, T cell proliferation was measured by incorporation of [3H]methylthymidine for 24 h. The data, representative of two experiments, are expressed as mean cpm ± SD of triplicate wells.

T cells in a MLR (Fig. 2B). In almost a reciprocal manner to IL-10 release, none of the stimuli except GM-CSF instructed preDC to prime naive T cell proliferation. Therefore, preDC responded to a variety of proinflammatory cytokines by IL-10 production and remained unable to prime T cells. Reflecting the lower IL-10 release, it is likely that TNF-α is a weak stimulus for preDC. However, GM-CSF could instruct the preDC to activate T cells.

Differentiation to DC abolishes IL-10 production and induces T cell priming

The finding that GM-CSF promotes T cell priming is best explained by GM-CSF inducing DC formation. As we described previously [8, 9] and also summarized in **Table 2**, GM-CSF alone or more efficiently, GM-CSF with IL-4 and TGF-β induced the differentiation of preDC into CD1a$^+$ DC, and DC obtained in GM-CSF/IL-4/TGF-β are not LC, as they lack CCR6 and Langerin expression but are of dermal type [9]. We compared preDC and DC derived from preDC in GM-CSF/IL-4/TGF-β for IL-10 production in response to LPS and live *M. bovis* BCG. As shown in **Figure 3A**, although preDC produced high levels of IL-10 in response to LPS and BCG, a 48-h culture in GM-CSF/IL-4/TGF-β was sufficient to abolish IL-10 production almost completely in response to these microbial stimuli. We then tested the capacity to prime naive T cells in a MLR (Fig. 3B). Stimulating preDC with LPS or infecting the cells with BCG did not promote the capacity to prime T cells, even at high APC:T cell ratios. However, when cultured for 48 h in GM-CSF/IL-4/TGF-β, the resulting DC displayed a low level of T cell priming, which was enhanced strongly by LPS or BCG mycobacteria. Therefore, converting preDC into immature DC modifies the cells so that they respond to LPS or BCG by reducing IL-10 production and becoming potent T cell stimulators.

TABLE 2. Conversion of preDC into CD1a$^+$CD14$^-$ DC

	GM-CSF	GM-CSF + IL-4	GM-CSF + IL-4 + TGFβ
% of CD1a$^+$CD14$^-$	21.8 ± 3.6	65.8 ± 5.5	83.3 ± 3.8

Blocking IL-10 enables LPS-stimulated preDC to convert directly into professional APC

Given that preDC differentiation into DC is accompanied with a reduction of IL-10 release and that IL-10 is a known negative regulator of DC maturation [14], we asked whether elimination of IL-10 signaling concomitant with LPS stimulation would convert preDC directly into professional APC. Therefore, preDC were incubated with antagonistic anti-IL-10R antibody and stimulated with LPS. Twenty-four hours later, expression of maturation marker CD83, HLA-DR, and costimulatory molecules CD80 and CD86 was assessed (**Fig. 4A**, left side). Under these conditions, preDC acquired high expression of all these markers in a manner similar to DC stimulated by LPS. However, the majority of the cells remained CD14$^+$CD1a$^-$, although the level of CD14 decreased (Fig. 4A, right side). We then tested the ability of the CD14$^+$ cells stimulated with LPS in the presence of anti-IL-10R antibody to prime naive T cells. As shown in Figure 4B, the cells displayed a T cell priming activity as potent as LPS-matured DC. Therefore, endogenous IL-10 triggered by LPS inhibits direct conversion of preDC into professional APC.

DC derived from LPS-stimulated preDC are unable to prime T cells

To explore the outcome of simultaneous exposure to DC-differentiating cytokines and LPS, we cultured preDC in GM-CSF/IL-4/TGF-β with immediate addition of LPS. **Figure 5A** shows that DC differentiation was as efficient as in the absence of LPS. As expected, addition of LPS 48 h after exposure to DC-promoting cytokine addition induced the formation of mature DC with up-regulation of CD83, CD80, CD86, and HLA-DR. However, the presence of LPS during DC differentiation resulted in predominantly immature DC. We observed some cell death in these cultures, which, however, rarely exceeded 10% (data not shown). Although delayed LPS increased the level of naïve T cell proliferation (Fig. 5B, DC>LPS), DC

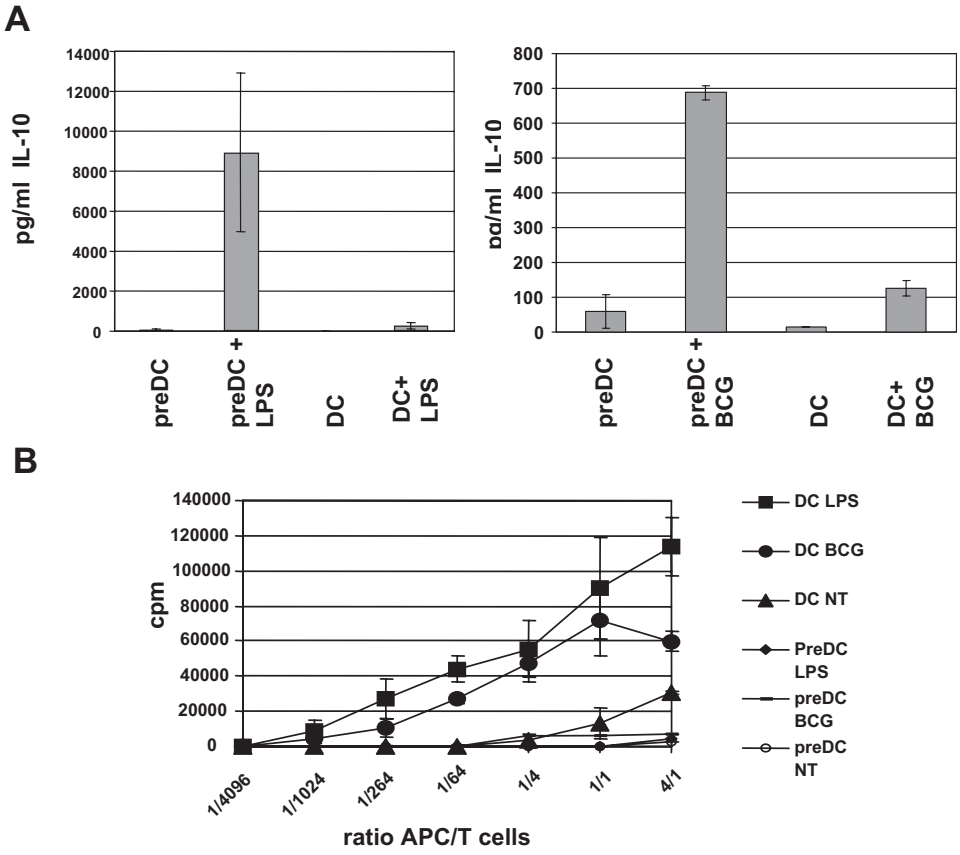

Fig. 3. Differentiation to DC abolishes IL-10 production and allows maturation in response to LPS and BCG. (A, left panel) preDC and DC derived from the same preDC in GM-CSF/IL-4/TGF-β were stimulated for 24 h with 1 μg/ml LPS or left untreated, and secretion of IL-10 was measured. The data represent the mean ± SD for 10^6 cells from five different donors. Right panel, preDC or the derived DC were incubated with live BCG for 3 h at MOI = 5 and then were washed and cultured in complete medium containing antibiotics for 24 h. The data are representative of two donors. (B) preDC or the derived DC were stimulated with 1 μg/ml LPS or infected by live BCG at MOI = 5. After washing, the cells were then added in graded doses to allogenic cord blood $CD4^+$ T cells. After 5 days, T cell proliferation was measured by incorporation of [3H]methylthymidine. The data are representative of at least two donors. NT, not treated.

differentiated in the presence of LPS (LPS-DC) showed a decreased stimulation, not only compared with LPS-matured DC but also with unstimulated DC. Re-exposed to LPS did not convey the cells with a T cell priming capacity (LPS-DC>LPS). This demonstrates that LPS does not disturb the development of DC from preDC; however, under these conditions, the DC are unable to prime T cells and become endotoxin-tolerant.

LPS-stimulated preDC modulate bystander DC maturation and T cell activation

In human dermis, $CD14^+$ monocytic cells are predominant compared with $CD1a^+$ DC. It is therefore relevant to consider the influence of these $CD14^+$ cells on $CD1a^+$ DC, and we addressed the question of whether LPS-stimulated preDC could control bystander DC maturation. As shown in **Figure 6A**, addition of LPS to a 1:1 coculture of DC and preDC resulted in a significant reduction in the proportion of $CD83^+$ mature DC. Next, we tested whether LPS-stimulated preDC could reduce bystander T cell activation. T cells were activated by anti-CD3/anti-CD28 antibodies in the presence of medium or cell-free supernatants of resting or LPS-stimulated preDC (Fig. 6B). The supernatant of LPS-stimulated preDC but not unstimulated preDC reduced T cell proliferation significantly. As control, addition of rhIL-2 or rhIL-10 enhanced or decreased T cell proliferation, respectively. Therefore, with LPS encounter, preDC can down-regulate bystander DC maturation and T cell activation.

DISCUSSION

M-CSF and GM-CSF are two growth factors, which affect proliferation and differentiation of the myeloid lineage. M-CSF is produced constitutively, particularly by fibroblasts, whereas important amounts of GM-CSF are released by activated macrophages and T cells during inflammation. In this report, we have investigated the cell plasticity and the immune response of myeloid DC precursors following conditioning by M-CSF and GM-CSF. M-CSF-conditioned cord blood-derived $CD14^+$ cells (preDC) or M-CSF-conditioned monocytes retain the capacity to differentiate into DC [7–9]. In response to microbial products or inflammatory cytokines, preDC or M-CSF-conditioned monocytes produced high levels of IL-10 rapidly but little IL-6, whereas pre-exposure to GM-CSF resulted in a rapid IL-6 release but little IL-10. Kinetic analysis showed that preDC produced faster and higher levels of IL-10 than monocytes in response to LPS. Moreover, preDC are more sensitive to LPS than monocytes. We further provided evidence that an 18-h incubation with M-CSF is sufficient to render monocytes capable of secreting early IL-10. There is no clear difference in CD14 expression between monocytes and preDC, and TLR2/4 expression is higher on monocytes than on preDC or M-CSF-conditioned monocytes (data not shown). Therefore, the different LPS/BCG response with respect to IL-10 among monocytes, preDC and DC is unlikely a result of different TLR2/4 levels. Rather, we favor the hypothesis that M-CSF triggers the assembly of an anti-inflammatory signaling cascade, possibly implicating glycogen synthase kinase 3 [15].

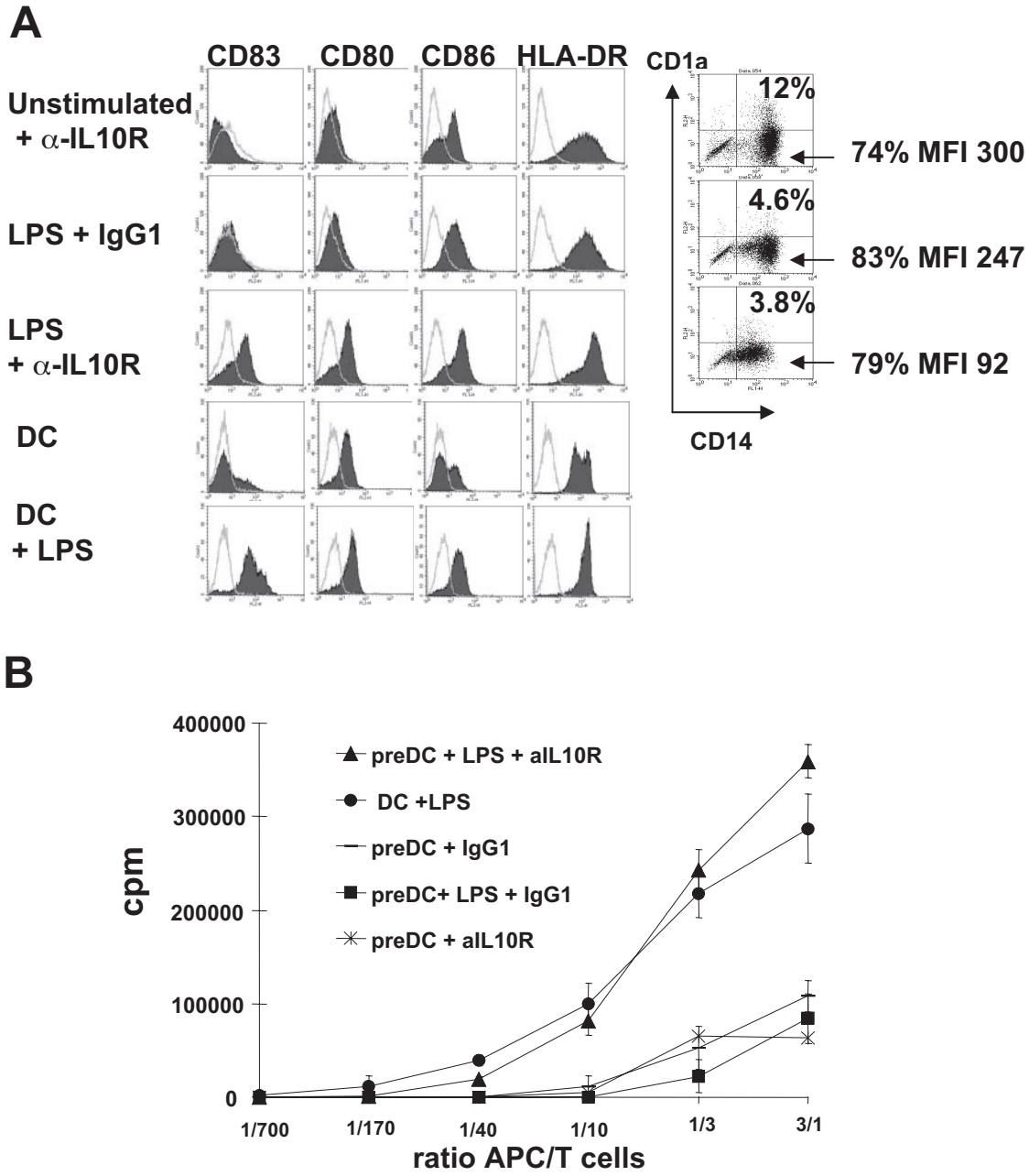

Fig. 4. Blocking IL-10 enables LPS-stimulated preDC to convert into professional APC. (A, left panel) preDC were incubated with an anti-IL-10R antibody (α-IL-10R) or its isotype control before exposing the cells to 1 μg/ml LPS. After 24 h, expression of cell surface markers was assessed by FACS together with control, unstimulated (no LPS) preDC and as positive control, DC and LPS-matured DC derived from preDC. Live cells were electronically gated; white histograms represent isotype controls, and specific labeling is shown in gray. (Right panel) Expression of CD1a versus CD14 was measured by FACS for unstimulated preDC—preDC treated with LPS in the presence of control antibody of anti-IL-10R antibody. The percentage of $CD1a^+CD14^+$ cells and $CD1a^-CD14^+$ cells is shown. Also, the mean fluorescence intensity (MFI) of CD14 expression of $CD1a^-CD14^+$ cells is indicated. (B) Cells treated as in A were washed and added in graded doses to allogeneic cord blood $CD4^+$ T cells. After 5 days, T cell proliferation was measured by incorporation of [3H]methylthymidine and expressed as mean cpm ± SD of triplicate wells. The data are representative of three experiments.

We observed that conversion into DC by GM-CSF/IL-4/TGF-β abolished IL-10 production almost completely, and the cells acquired potent T cell priming activity in response to LPS or BCG mycobacteria. These data show that according to the type of differentiation factor (M-CSF vs. GM-CSF), myeloid cells acquire an anti-inflammatory or an inflammatory activity, respectively. Yet, the M-CSF-cultured anti-inflammatory preDC remained fully capable of converting into DC, even during LPS-triggered IL-10 release. Thus, preDC are like monocytes in terms of capacity to differentiate into DC but are like anti-inflammatory macrophages in terms of cytokine expression. However, it is probable that a prolonged exposure to M-CSF will result in progressive incapacity to differentiate into DC [7]. Although simultaneous exposure of LPS and GM-CSF/IL-4/TGF-β still allowed DC formation, the DC displayed an immature phenotype and were unable to prime T cells. In addition, their re-exposure to LPS could not induce T cell priming. This phenomenon of endotoxin tolerance has also

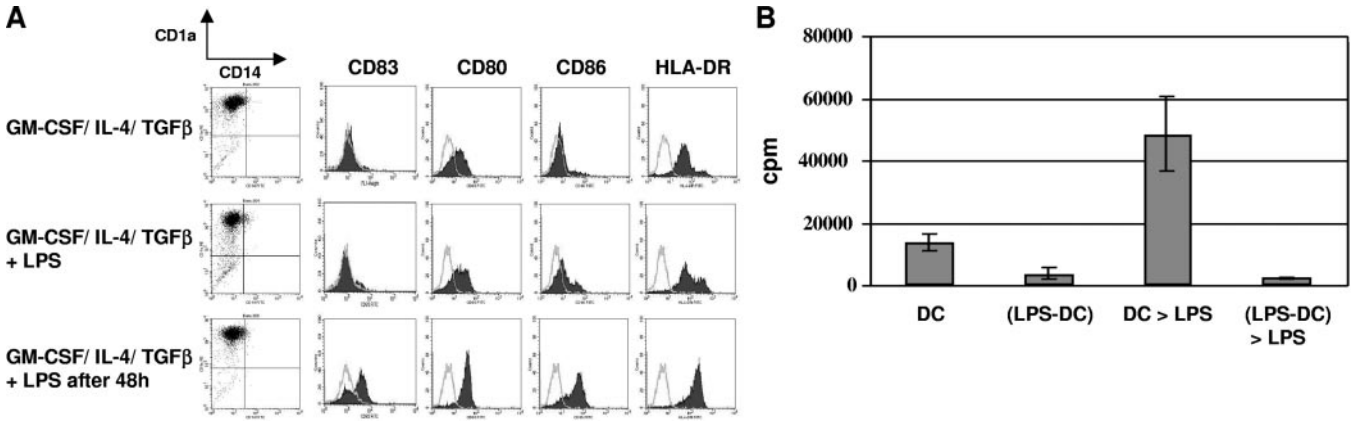

Fig. 5. DC derived from LPS-stimulated preDC are immature. (A) DC were derived from preDC under the following conditions: 3-day culture in GM-CSF/IL-4/TGF-β in the absence or in the immediate presence of 100 ng/ml LPS or when LPS addition was delayed by 48 h. Expression of CD1a versus CD14 as well as that of other cell surface markers was analyzed by FACS without electronically gating for live cells. (B) The level of naïve T cell priming was compared between DC derived from preDC in the presence of GM-CSF/IL-4/TGF-β (DC) and LPS added during DC differentiation (LPS-DC) or added after 48 h (DC>LPS). Also, LPS-DC were re-exposed to 100 ng/ml LPS for 24 h (LPS-DC>LPS). Shown is the T cell proliferation at an APC:T cell ratio of 1:1. Mean cpm ± SD from triplicate wells; each condition is representative of at least two experiments.

been observed with LPS-stimulated monocytes and may be related to reduced association of TLR4 with MyD88 and decreased IL-1R-associated kinase 1 activity [16]. Thus, autocrine IL-10 prevented the formation of mature DC and hence, T cell priming. In addition, we provided evidence that the supernatant of LPS-preDC antagonized maturation of bystander DC. This may be relevant in conditions of a mixed myeloid population such as in the skin, where $CD14^+$ cells colocalize with $CD1a^+$ dermal and Langerhans DC. Furthermore, the supernatant of LPS-stimulated preDC prevented polyclonal $CD4^+$ T cell proliferation, so that even under conditions of T cell activation, preDC can down-modulate the formation of a T cell-mediated immune response.

Collectively, the data suggest that myeloid DC precursors, when exposed to M-CSF, respond to LPS and probably to other danger signals by installing a tolerogenic environment, which inhibits formation of professional APC in an autocrine and a paracrine manner and prevents bystander T cell activation. We have not detected CCR7 expression on LPS-stimulated preDC, indicating that tolerance would be restricted to tissue and not carried to draining lymph nodes, unless mediated by tolerized, immature or semimature, migratory DC or via soluble IL-10. Our work is in line with a report showing that M-CSF/IL-4 but not GM-CSF/IL-4 induces cord blood monocytes to differentiate into cells that secrete IL-10 but not IL-12 upon LPS stimulation [6]. However, while the $CD14^+$ cells used here can convert readily into $CD1a^+$ DC in GM-CSF/IL-4, the cells used by Li et al. [6] lack CD14 and CD1a and can no longer differentiate into $CD1a^+$ DC when subsequently exposed to GM-CSF. Also, adult monocytes cultured in M-CSF for 3 days produced more IL-10 than when cultured in M-CSF/IL-4 (data not shown). Xu et al. [17] showed that M-CSF but not GM-CSF induces the differentiation of monocytes into IL-10-producing macrophages with intrinsic anti-inflammatory properties and are specialized in clearing apoptotic cells. This indicates that M-CSF may have a function in tissue homeostasis and peripheral tolerance to self-antigens.

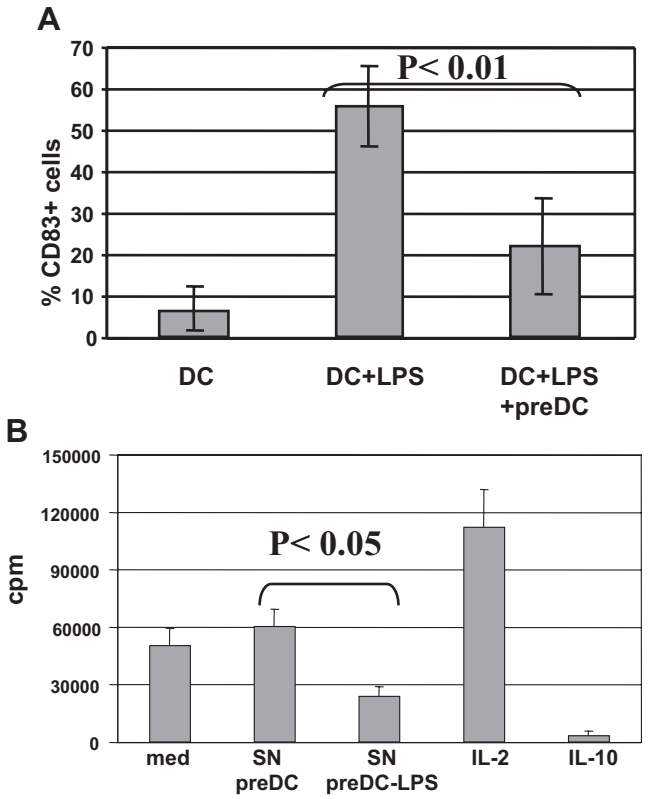

Fig. 6. LPS-stimulated preDC reduce DC maturation and T cell proliferation. (A) DC were cocultured with preDC (ratio of 1:1) and 50 ng/ml LPS. As control, DC were left untreated or treated with LPS in the absence of preDC. After 24 h, the cells were stained for CD1a and CD83. The percentage of $CD1a^+$ cells expressing CD83 is shown. The results are the mean ± SD from three experiments. Statistic significance was assessed with the Student's paired *t*-test. (B) Adult $CD4^+$ T cell was activated by anti-CD3/CD28 antibodies for 5 days in the presence of medium only, supernatant (SN) from preDC, supernatant from LPS-treated preDC, or medium containing IL-2 or IL-10. T cell proliferation was measured by incorporation of [3H]methylthymidine. The data are expressed as mean cpm ± SD from triplicate wells, representative of at least three experiments. Statistic significance was assessed with the Student's paired *t*-test.

preDC share cell surface markers with dermal $CD11b^+CD1a^-$ cells [8, 9], and human dermal $CD11b^+CD1a^-$ mono/macrophages produce high levels of IL-10 rapidly after UV irradiation [18]. It is therefore plausible that the dermal $CD11b^+$ mono/macrophages are DC precursors and that IL-10 influences their conversion into immunostimulatory, mature dermal DC. In support of this notion are the observations that intradermal injection of live bacteria in mice results in little DC mobilization to draining lymph nodes [19, 20], and that subcutaneous LPS triggers a higher immune response in mice when the IL-10R is blocked [21]. Recently, Bogunovic et al. [22] presented evidence that dermal DC, like Langerhans DC, arise from a tissue-residing, cycling precursor under steady-state conditions. This supports our use of the cycling $CD34^+$ progenitor to generate dermal-like $CD14^+$ cells and suggests that dermal mono/macrophages may be exposed to M-CSF throughout their development.

Yet, the fact that an adaptive immune response can be raised in response to immunization with adjuvants containing microbial products shows that a local immune tolerance can be broken. We have found that a means to convert LPS-stimulated preDC directly into professional APC is by blocking IL-10 signaling. Also, CD40L can override the IL-10 block and likewise induce formation of $CD14^+$ APC with potent T cell priming activity [23]. The Th2 cytokine IL-4 does not revert tolerance, although M-CSF/IL-4 monocytes have DC features [6], but the Th1 cytokine IFN-γ can convert LPS-stimulated monocytes into $CD14^-CD83^+$ mature DC through down-regulation of IL-10 production [24]. Alternatively, it is possible that the inflammation increases GM-CSF levels so that attracted monocytes will be conditioned to release proinflammatory cytokines and would differentiate into DC. In this context, it is relevant to point out that $CD16^+$ monocytes ($Gr\text{-}1^+$ in mouse) are preferentially recruited to inflamed sites [25]. These cells not only appear to release more proinflammatory cytokines than their $CD16^-$ counterpart [26] but also differentiate more rapidly to DC [27].

In conclusion, we propose that under steady-state conditions, M-CSF may condition tissue-residing monocytes or DC precursors developing in tissue to an anti-inflammatory program. This would curtail an efficient, T cell-mediated immune response but avert pathogen dissemination and immunopathology.

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