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IL-10 Induces CCR6 Expression During Langerhans Cell Development While IL-4 and IFN-γ Suppress It

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Immune responses are initiated by dendritic cells (DC) that form a network comprising different populations. In particular, Langerhans cells (LC) appear as a unique population of cells colonizing epithelial surfaces. We have recently shown that macrophage-inflammatory protein-3α/CCL20, a chemokine secreted by epithelial cells, induces the selective migration of LC among DC populations. In this study, we investigated the effects of cytokines on the expression of the CCL20 receptor, CCR6, during differentiation of LC. We found that both IL-4 and IFN-γ blocked the expression of CCR6 and CCL20 responsiveness at different stages of LC development. The effect of IL-4 was reversible and most likely due to the transient blockade of LC differentiation. In contrast, IFN-γ-induced CCR6 loss was irreversible and was concomitant to the induction of DC maturation. When other cytokines involved in DC and T cell differentiation were tested, we found that IL-10, unlike IL-4 and IFN-γ, maintained CCR6 expression. The effect of IL-10 was reversible and upon IL-10 withdrawn, CCR6 was lost concomitantly to final LC differentiation. In addition, IL-10 induced the expression of CCR6 and responsiveness to CCL20 in differentiated monocytes that preserve their ability to differentiate into mature DC. Finally, TGF-β, which induces LC differentiation, did not alter early CCR6 expression, but triggered its irreversible down-regulation, in parallel to terminal LC differentiation. Taken together, these results suggest that the recruitment of LC at epithelial surface might be suppressed during Th1 and Th2 immune responses, and amplified during regulatory immune responses involving IL-10 and TGF-β. *The Journal of Immunology*, **2001, 167: 5594–5602.**

Dendritic cells (DC)[2] are bone marrow-derived professional APCs that are required for initiation of immune responses. They are found in virtually every tissue and fluid at different maturation states interconnected by defined pathways of circulation (1–5). In addition, DC populations differ in their origin and/or function. In particular, DC subsets have been reported to exert different functions with regard to the regulation of B cell proliferation (6, 7) and differentiation of T cell responses toward type I or type II (8–10). Also, $CD11c^-$ plasmacytoid DC, identified in humans, have been reported to correspond to the natural IFN-α-producing cells (11, 12), and lymphoid-derived DC, identified in mice, have been proposed to play a role in maintenance of peripheral tolerance (3, 13). Finally, Langerhans cells (LC) represent a population of DC only found in epithelia, and whose specific function is not yet fully elucidated.

The selective recruitment of a specific DC population at the site of infection will most likely be determinant for the type of immune response initiated. Recently, we and others have observed that macrophage-inflammatory protein-3α, also known as CCL20 (14), was the only chemokine produced by the epithelium that selectively attracts LC precursors (15, 16). CCL20 has also been shown to selectively induce migration of memory T cells with skin and gut epithelium-homing capacities as well as a subpopulation of gut intraepithelial T lymphocytes (17–19). Its unique activity suggests a key role of CCL20 in the control of LC and lymphocyte subset recruitment at epithelial surfaces and in the regulation of epithelial immunity. The understanding of the regulation of CCL20 secretion as well as of the expression of its receptor, CCR6, during LC development, should open avenues to understand and potentially control the specific function of epithelial LC. In the present study, we have studied the effects of IL-4, IFN-γ, IL-10, and TGF-β, cytokines involved in T cell (20–23) and DC differentiation, on the expression of CCR6 during LC development.

Several studies have shown that LC are regulated independently from other DC populations. In particular, TGF-β has been identified as a mandatory factor for the development of LC both in vitro and in vivo (24–26). In contrast, IL-4 that is required for the development of DC from monocytes (27, 28) blocks the development of LC from $CD34^+$ hemopoietic progenitor cells (HPC) (29). IL-10 has also been shown to block DC development and maturation (30–33). We found that IL-4 and IFN-γ block the expression of CCR6 as well as the responsiveness to CCL20. TGF-β irreversibly down-regulates CCR6 expression during final LC differentiation. In contrast, IL-10 positively regulates CCR6 expression on $CD34^+$-derived precursor cells and differentiated monocytes without affecting their capacity to differentiate into LC or mature DC. These results suggest that during Th1 and Th2 responses, IFN-γ and IL-4 might prevent epithelial colonization by LC. During mucosal-type immune responses, IL-10 might increase LC precursor recruitment and TGF-β expressed at mucosal surfaces, and might arrest LC when they reach epithelium.

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[2] Abbreviations used in this paper: DC, dendritic cell; HPC, hemopoietic progenitor cell; LAMP, lysosome-associated membrane glycoprotein; LC, Langerhans cell; rh, recombinant human; SCF, stem cell factor.

0022-1767/01/$02.00

Materials and Methods

Hemopoietic factors, reagents, and cell lines

Recombinant human GM-CSF (rhGM-CSF; sp. act., 2×10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml. rhTNF-α (sp. act., 2×10^7 U/mg; Genzyme, Boston, MA) was used at an optimal concentration of 2.5 ng/ml (34). Recombinant human stem cell factor (rhSCF; sp. act., 4×10^5 U/mg; R&D Systems, Abington, U.K.) was used at an optimal concentration of 25 ng/ml. rhIL-4 (sp. act., 2×10^7 U/mg; Schering-Plough) was used at a saturating concentration of 50 U/ml. rhIL-10 (sp. act., 10^7 U/mg; Schering-Plough) was used at 100 ng/ml. rhIFN-γ (sp. act., 10^7 U/mg; Sigma-Aldrich, St. Louis, MO) was used at 20 ng/ml. rhTGF-β1 (sp. act., 5×10^7 U/mg; R&D Systems) was used at 10 ng/ml. LPS (Sigma-Aldrich) was used at 10 ng/ml. Recombinant human chemokines macrophage-inflammatory protein-3α/CCL20 (sp. act., 4×10^5 U/mg) and monocyte chemoattractant protein-3/CCL7 (sp. act., 1×10^4 U/mg) were obtained through R&D Systems. mAbs against Langerin (clone DC-GM4) (35) and E-cadherin (clone HECD-1; Takara BioWhittaker Europe) were used to characterize LC. The maturation state of DC was determined by using the following mAbs: anti-CD83 (Immunotech, Marseille, France), anti-CD86 (BD PharMingen, San Diego, CA), and anti-DC LAMP (clone 104.G4) (36).

Generation of DC from cord blood CD34$^+$ HPC

Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34$^+$ Ag were isolated from mononuclear fractions through positive selection, as described (34, 37), using anti-CD34$^+$ mAb (Immu-133.3; Immunotech), goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and Minimacs separation columns (Miltenyi Biotec). In all experiments, the isolated cells were 80–99% CD34$^+$. After purification, CD34$^+$ cells were cryopreserved in 10% DMSO.

Cultures were established in the presence of GM-CSF, TNF-α, and SCF, as described (34), in endotoxin-free medium consisting of RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS (Flow Laboratories, Irvine, U.K.), 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-ME, and 100 μg/ml gentamicin (Schering-Plough) (referred to as complete medium). After thawing, CD34$^+$ cells were seeded for expansion in 25- to 75-cm^2 culture vessels (Linbro; Flow Laboratories) at 2×10^4 cells/ml. Optimal conditions were maintained by splitting these cultures at days 5 and 10 with medium containing fresh GM-CSF with or without other cytokines (cell concentration: 1–3 $\times 10^5$ cells/ml). At day 12, between 70 and 90% of the cells are CD1a$^+$ DC in GM-CSF condition.

Generation of DC from peripheral blood monocytes

Monocytes were purified by immunomagnetic depletion (Dynabeads; Dynal Biotech, Oslo, Norway) after preparation of PBMC, followed by a 52% Percoll gradient. The depletion was performed with anti-CD3 (OKT3), anti-CD19 (4G2), anti-CD8 (OKT8), anti-CD56 (NKH1; Beckman Coulter, Palo Alto, CA), and anti-CD16 (ION16; Immunotech) mAbs. Monocytes were cultured for 6–7 days in the presence of GM-CSF and IL-4 for the generation of DC (27) or in presence of GM-CSF plus IL-10 with or without IL-4 (cell concentration: 1–3 $\times 10^5$ cells/ml).

Induction of maturation of monocytes differentiated in presence of IL-10

Monocytes were cultured in presence of GM-CSF plus IL-10 until day 7. Differentiated monocytes were washed and then activated for 2 days in the presence of TNF-α (2.5 ng/ml) plus LPS (10 ng/ml).

Chemotaxis assay

Cell migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; Neuroprobe, Pleasanton, CA) (38). Briefly, human recombinant CCL20 and CCL7 were diluted to 1 μg/ml and 100 ng/ml in RPMI 1640 medium, respectively, and were added to the lower wells of the chemotaxis chamber. A total of 10^5 cells/well in 50 μl RPMI 1640 medium was applied to the upper wells of the chamber, with a standard 5-μm pore polyvinylpyrrolidone-free polycarbonate filter (Neuroprobe) separating the lower wells. The chamber was incubated at 37°C in humidified air with 5% CO_2 for 1 h. Then cells that have migrated to the underside of the filter were stained with Field's A and Field's B (BDH Laboratory Supplies, Dorset, England) and counted using an image analyzer (software, Vision Explorer and ETC 3000; Graphtek, Mirmande, France) in two randomly selected low-power fields (magnification ×20). Each assay was performed in duplicate, and the results were expressed as the mean SD of migrating cells per two fields.

Transwell (5-μm pore; Costar, Cambridge, MA) experiments were performed to characterize heterogeneous populations. Serial dilutions of chemokines were added to 24-well plates. A total of 5×10^5 cells was added to transwell inserts. Plates were incubated for 1.5 h at 37°C. After removal of the transwell inserts, cells were counted and stained for flow cytometry, to differentiate between CD1a$^+$ and CD14$^+$ cells from either CD34$^+$ HPC or monocytes.

Calcium fluorimetry

Intracellular Ca^{2+} concentration was measured using the fluorescent probe Indo-1, according to the technique reported by Grynkiewicz et al. (39). In brief, cells were washed in PBS and resuspended at 10^7 cells/ml in complete RPMI 1640 medium (see above). Then cells were incubated for 45 min at room temperature with 3 μg/ml Indo-1 AM (Molecular Probes, Eugene, OR) in the dark. After incubation, cells were washed and resuspended in HBSS/1% FCS at 10^7 cells/ml. Before measurement of intracellular Ca^{2+} concentration, cells were diluted 10-fold in HBSS/10 mM HEPES/1.6 mM $CaCl_2$ preheated at 39°C. Samples were excited at 330 nm with continuous stirring, and the Indo-1 fluorescence was measured as a function of time at 405 nm (dye is complexed with Ca^{2+}) and 485 nm (Ca^{2+}-free medium), in a 810 Photomultiplier Detection System (software, Felix; Photon Technology International, Monmouth Junction, NJ). Results are expressed as the ratio of values obtained at the two emission wavelengths.

Analysis of chemokine receptor expression by FACS

Expression of CCR6 and CCR5 was determined by using the following mAbs conjugated to PE: anti-CCR6 (clone 53103.111; R&D Systems) and anti-CCR5 (clone 2D7; BD PharMingen). For indirect staining, the cells were incubated with biotin-conjugated anti-CCR2 mAb (clone 48607.211; R&D Systems) or unconjugated anti-CCR7 mAb (clone 2H4; BD PharMingen), and subsequently stained with PE-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) or with PE-conjugated goat anti-mouse IgM (Jackson ImmunoResearch), respectively. On CD34$^+$-derived DC and monocyte-derived DC, double staining was performed with anti-CD1a FITC (BD PharMingen) and/or anti-CD14 FITC (BD PharMingen).

Results

IL-4 and IFN-γ block CCR6 expression on LC precursors through different mechanisms

We and others have previously shown that CCL20, produced by epithelial cells, induces the selective migration of LC precursors (15, 16). To analyze the regulation of LC recruitment in the periphery during immune responses, we investigated the modulation of CCR6 expression by different cytokines. First, we have focused our attention on the effects of IL-4 and IFN-γ on CCR6 expression. CD34$^+$ HPC were cultured in presence of GM-CSF plus TNF-α plus SCF until day 6 and then cultured only with GM-CSF to preserve their immaturity.

CCR6 was detected starting at day 6, optimally expressed from day 8 to 10, and progressively lost from day 13 (Fig. 1*A*). When IL-4 was added at day 3 (not shown) or at day 6 on CCR6$^+$ LC precursors (Fig. 1*A*), CCR6 expression was completely abolished from day 8 to 14. As a consequence, IL-4 added at day 3 induced 70–95% suppression of CCL20 responsiveness at day 8, and 95% from day 10 to 14 (Fig. 1*C*). In contrast, the responsiveness to monocyte chemoattractant protein-3/CCL7 was not affected by IL-4 (Fig. 1*D*). Similar effects were observed with IL-13 (not shown). This down-regulation of CCR6 was most likely the result of the blockade of LC differentiation, as we previously described (29), and as illustrated by the down-regulation of Langerin (Fig. 1*B*), without alteration of maturation markers (CD86, CCR7). In line with this hypothesis, as for LC differentiation (our unpublished observations), the effect of IL-4 was reversible (Fig. 2). Upon withdrawal of IL-4 for 5 days (day 13), the expression of CCR6 on LC was recovered at 70% compared with cells that have never been exposed to IL-4 (Fig. 2*A*), and the response to CCL20 was nearly completely restored (75–90%) (Fig. 2*B*). While CCR6

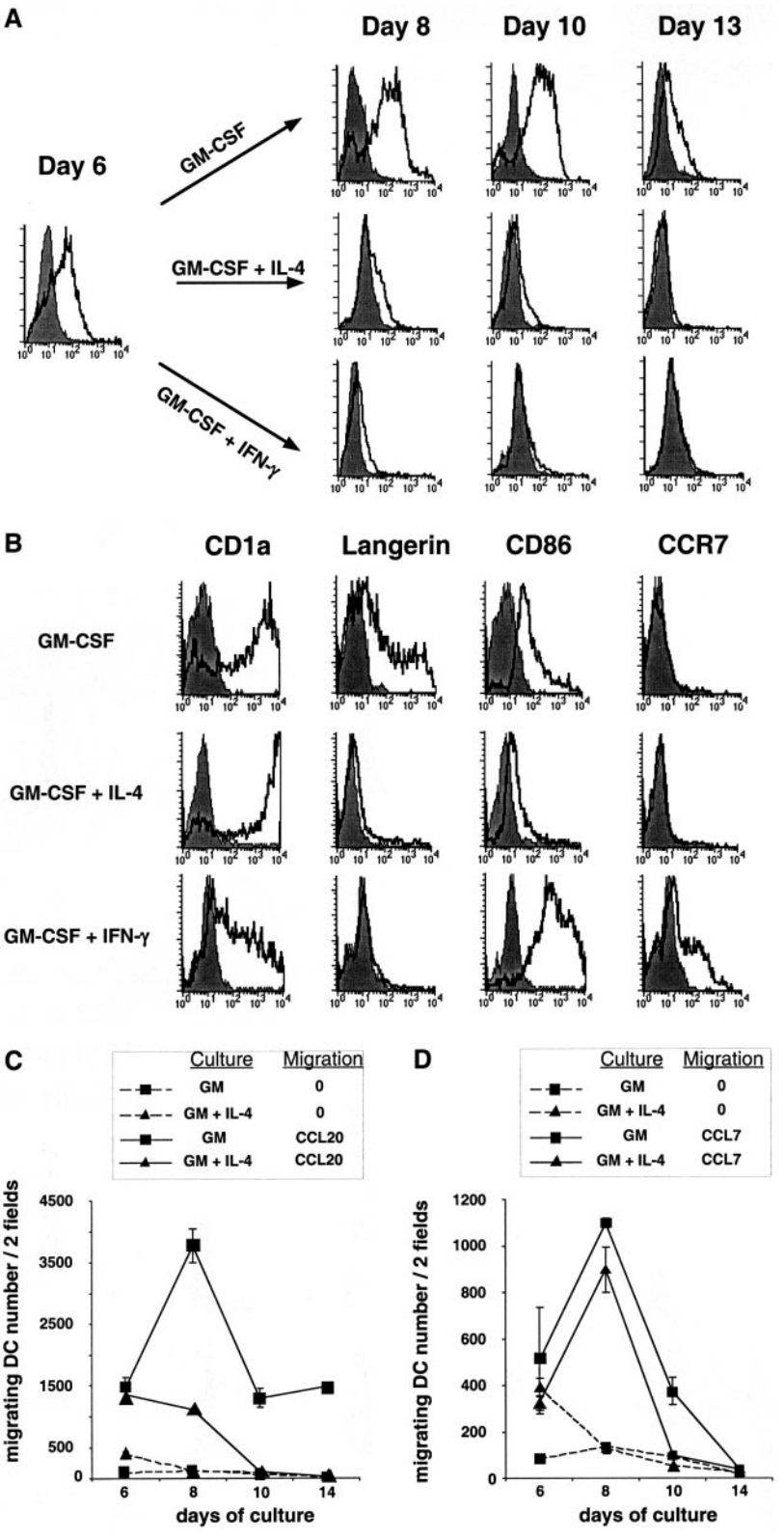

FIGURE 1. IL-4 and IFN-γ induce the down-regulation of both CCR6 expression and CCL20 responsiveness on $CD34^+$-derived $CD1a^+$ LC through different mechanisms. *A* and *B*, $CD34^+$ HPC were cultured in presence of SCF plus GM-CSF plus TNF-α for 6 days. The cells were then seeded in presence of GM-CSF alone or GM-CSF plus IL-4 (50 U/ml) or GM-CSF plus IFN-γ (20 ng/ml). At the indicated time points, individual culture samples were recovered, and the expression of CCR6 on $CD1a^+$ cells was determined by double staining analyzed by FACS (*A*). At day 10, individual culture samples were recovered, and the expression of the indicated markers was determined by FACS analysis (*B*). Filled histograms represent isotype match controls. *C* and *D*, $CD34^+$ HPC were cultured in presence of SCF plus GM-CSF plus TNF-α for 6 days, and at day 3, IL-4 (50 U/ml) was added in one-half of the culture. At day 6, the cells were then seeded in presence of GM-CSF alone or GM-CSF plus IL-4, respectively. At the indicated time points, individual culture samples were recovered and the cells were tested for their response to CCL20 (*C*) and to CCL7 (*D*). Migration assays were performed in Boyden microchambers. Results are expressed as number of migrating cells per two low power fields (magnification ×20). Results are representative of more than three independent experiments.

protein was rapidly down-regulated in presence of IL-4, its mRNA was still present (not shown), offering a possible explanation of the reversible action of IL-4.

In presence of IFN-γ from day 3 (not shown) or from day 6 on $CCR6^+$ LC precursors (Fig. 1*A*), CCR6 expression was completely abolished from day 6 to 14. The loss of CCR6 expression induced by IFN-γ also resulted in the suppression of the migration to CCL20 (see Fig. 2*B*), without affecting that to CCL7 (not shown). In contrast to IL-4, IFN-γ induced the strong up-regulation of CD86 and CCR7 (Fig. 1*B*), suggesting that the loss of CCR6 expression reflected the IFN-γ-induced DC maturation, as previously reported (40). In line with the IFN-γ-induced maturation, even 5 days after the removal of IFN-γ, the LC were still CCR6 negative (Fig. 2*A*) and CCL20 unresponsive (Fig. 2*B*).

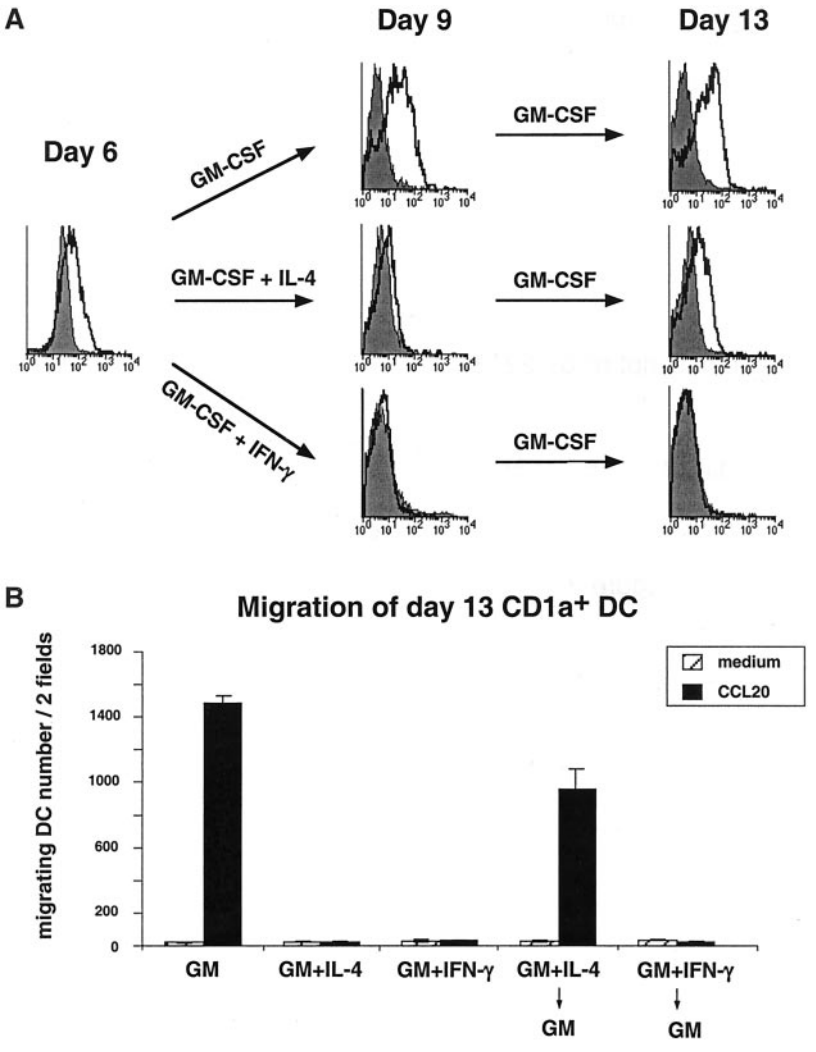

FIGURE 2. The effect of IL-4, but not that of IFN-γ, on CCR6 expression and CCL20 responsiveness is reversible. $CD34^+$ HPC were cultured in presence of SCF plus GM-CSF plus TNF-α for 6 days. The cells were then seeded in presence of GM-CSF alone or GM-CSF plus IL-4 (50 U/ml) or GM-CSF plus IFN-γ (20 ng/ml). At day 9, one-half of the cultures were harvested, and the cells were washed and reseeded in the presence of GM-CSF alone. At day 13, individual culture samples were recovered and the cells were tested. *A*, At the indicated time points, CCR6 expression on $CD1a^+$ DC was determined by double staining analyzed by FACS. Open histograms represent CCR6 staining, and filled histograms represent isotype match controls. Results are representative of five experiments. *B*, At day 13, individual culture samples were recovered, and the cells were tested for their capacity to migrate in response to CCL20. Migration assays were performed in Boyden microchambers. Results are expressed as number of migrating cells per two low power fields (magnification ×20). Results are representative of more than three independent experiments.

These observations show that IL-4, through reversible suppression of LC differentiation, and IFN-γ, through induction of irreversible LC maturation, both block LC migration in response to CCL20 concomitantly to the loss of CCR6 expression.

Dual role of TGF-β on CCR6 expression depending on the stage of LC differentiation

TGF-β has been shown to play a critical role in the development of LC from $CD34^+$ progenitors (25, 29). Thus, we investigated the effect of TGF-β on CCR6 expression during LC development. When TGF-β was added at the initiation of the culture, no alteration of CCR6 expression was observed on $CD1a^+$ LC precursors (Fig. 3*A*). Also, TGF-β did not impact on their responsiveness to CCL20 nor to CCL7 in migration assay (Fig. 3*C*). In contrast, the addition of TGF-β at day 6, which up-regulates Langerin on $CCR6^+$ LC precursors (Fig. 3*B*), induced the down-regulation of CCR6 expression from day 8 to 12 with the concomitant suppression of LC migration in response to CCL20, without affecting that to CCL7 (Fig. 3*D*). When TGF-β was removed at day 8, no recovery of CCR6 expression nor CCL20 responsiveness was observed (not shown).

These results show that although TGF-β is involved in the development of the LC phenotype, TGF-β induces the down-regulation of CCR6 during final LC differentiation.

IL-10 up-regulates CCR6 expression on $CD34^+$-derived DC without altering their capacity to differentiate into LC

IL-10 was added at day 6 of the culture, and CCR6 expression was analyzed every 2 days from day 8 to 12 (day 10 is shown in Fig. 4*A*). We found that IL-10 arrested the cells at the $CD14^+$ and $CD1a^+$ precursor stage (not shown). Surprisingly, all these cells expressed high levels of CCR6, with 2- to 4-fold increase in mean fluorescence intensity compared with $CD34^+$-derived DC in GM-CSF alone (Fig. 4*A*). The effects of IL-10 on other chemokine receptors involved in DC trafficking were then investigated. CCR2 expression that is restricted to the $CD14^+$ precursors was sustained in presence of IL-10 in accordance with the maintenance, in presence of IL-10, of cells in that stage of differentiation (Fig. 4*A*). CCR5 expression was not detected in presence of GM-CSF alone, and it was slightly induced by IL-10. Regarding CCR7, its expression was not detected in both conditions.

Upon withdrawal of IL-10 at day 10, cells cultured in presence of TGF-β differentiated and acquired characteristic LC markers such as Langerin and E-cadherin within 4 additional days (Fig. 4*B*).

These observations show that among the different cytokines tested, IL-10 is the only factor allowing the up-regulation of CCR6 expression during $CD34^+$-derived DC development without altering their capacity to differentiate into LC.

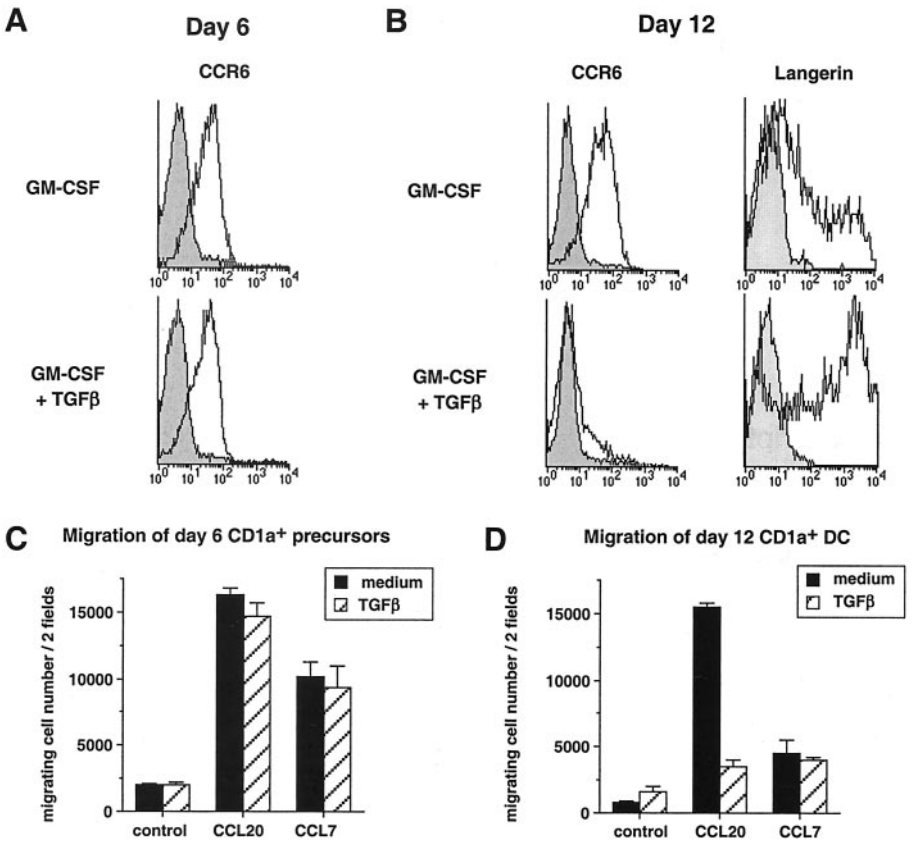

FIGURE 3. Dual role of TGF-β on CCR6 expression and CCL20 responsiveness depending on the stage of LC differentiation. $CD34^+$ HPC were cultured in presence of SCF plus GM-CSF plus TNF-α for 6 days, in presence or absence of TGF-β (10 ng/ml) (*A* and *C*). Alternatively, TGF-β was only added at day 6 together with GM-CSF (*B* and *D*). At days 6 and 12, individual culture samples were recovered and cells were tested. *A* and *B*, Expression of CCR6 and Langerin (*B* only) (open histograms) was determined on total cells by FACS analysis. Filled histograms represent isotype match controls. *C* and *D*, Cells were tested for their capacity to migrate in response to CCL20 and CCL7. Migration assays were performed by seeding 5×10^5 cells in transwells of 5 μm for 1.5 h. The migrated cells were analyzed for CD1a expression. Results are expressed as the number of $CD1a^+$-migrated cells measured by FACS. Results are representative of three independent experiments.

IL-10 selectively induces CCR6 expression and CCL20 responsiveness on differentiated monocytes while preserving their capacity to differentiate into mature DC

These observed effects on $CD34^+$-derived DC led us to investigate the effect of IL-10 on monocytes and monocyte-derived DC that do not express CCR6. Monocytes were cultured in presence of GM-CSF with or without IL-4, in presence or absence of IL-10 during 7 days. As shown in Fig. 5*A*, IL-10 induced CCR6 expression both in presence or absence of IL-4. Then the activity of CCL20 was assessed by calcium flux (Fig. 6*A*) and by chemotaxis assay (Fig. 6*B*), and in accordance with CCR6 expression, the IL-10-cultured cells responded to CCL20. However, in presence of IL-4, the IL-10-cultured cells displayed a lower calcium flux and no migratory capacity in response to CCL20. Of note, shorter time points have also been tested (1, 3, and 5 days), and no induction of cell surface expression of CCR6 nor chemotactic response to CCL20 has been detected under these conditions (not shown). Moreover, the induction of the expression and the functionality of CCR6 by IL-10 required the presence of GM-CSF (not shown). In parallel experiments, we could not detect any effects of TGF-β on monocyte-derived DC.

The regulation of other chemokine receptors was also investigated under these conditions. As shown in Fig. 5*B*, IL-10 had no effect on the regulation of CCR2, CCR5, and CCR7 during the differentiation of monocytes in presence of GM-CSF. In particular, CCR2 expression that is lost upon culture was not preserved by IL-10.

After removal of IL-10 and in presence of inflammatory mediators and bacterial products (TNF-α plus LPS, Fig. 5*C*) or T cell signals (not shown), cells differentiated into mature DC, as illustrated by the up-regulation of the costimulatory molecules CD83 and CD86, and the induction of the DC maturation marker DC-LAMP. The phenotype of the mature DC was comparable with cells cultured without IL-10 (not shown).

These observations show that in presence of IL-10, differentiated monocytes can acquire responsiveness to CCL20 while preserving their capacity to differentiate into mature DC. Moreover, compared with the other main chemokine receptors involved in DC trafficking, the IL-10 effect is selective for CCR6.

Discussion

LC represent a unique population of DC colonizing epithelial surfaces at the border between the body and the external milieu. As such, these cells most likely play a critical role in the regulation of epithelial immunity. In the present study, we show that immunoregulatory cytokines are likely to have an impact on the recruitment of LC at site of injury through the regulation of CCR6.

We found that IL-4 and IL-13 block CCR6 expression during LC development and down-regulate its expression on differentiated LC precursors, most likely as a consequence of a reversible suppression of LC differentiation. This observation is in agreement with a recent study showing that IL-4 down-regulates CCR6 expression on DC (41). This inhibitory effect of IL-4 may explain the absence of CCR6 on DC differentiated from monocytes in presence of GM-CSF plus IL-4 (42, 43). However, these results may appear in contrast with a recent report showing that monocytes cultured in presence of GM-CSF, IL-4, and TGF-β express CCR6 and respond to CCL20 (44).

Several lines of evidences suggest that IL-4 amplifies the Th2 responses by inducing both receptors (CCR3, CCR4, and CCR8 (45)) and ligands (CCL22 and CCL17 (46, 47)) involved in Th2 cell recruitment and by blocking the expression of ligands for Th1-selective receptors (CXCL9, CXCL10, and CXCL11 (48, 49)). The presently described effect of IL-4 on CCR6 expression may be

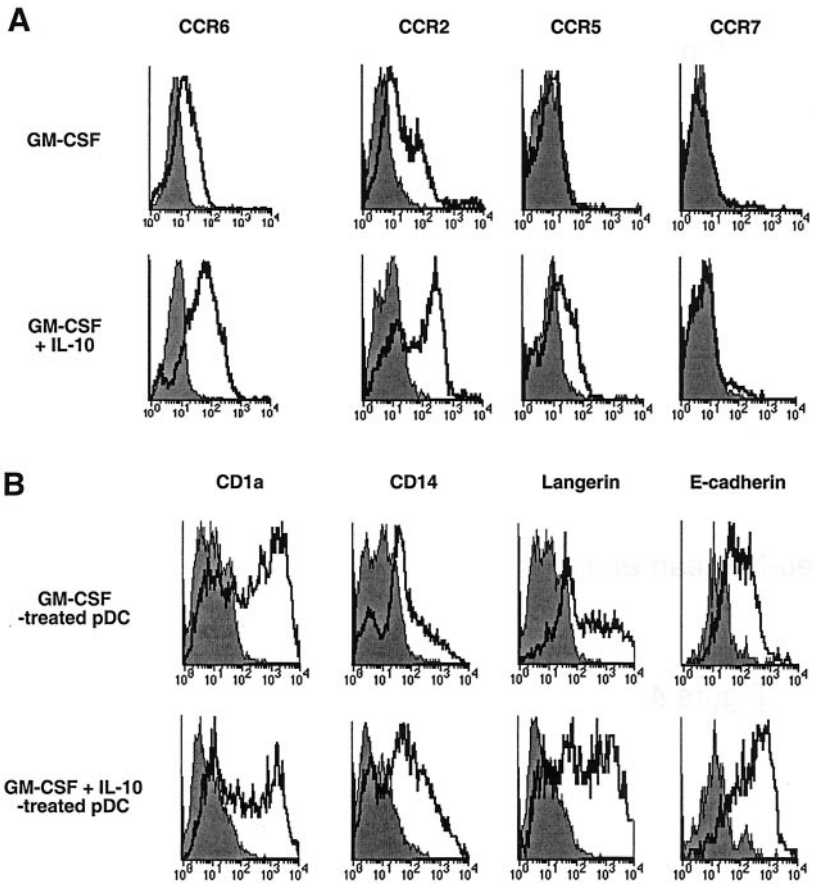

FIGURE 4. IL-10 positively regulates CCR6 expression on $CD34^+$ HPC-derived $CD1a^+$ LC precursors that preserve their capacity to differentiate into LC. $CD34^+$ HPC were cultured in presence of SCF plus GM-CSF plus TNF-α until day 6 and in presence of GM-CSF alone or GM-CSF plus IL-10 (100 ng/ml) from day 6 to 10. *A*, Cells were recovered, and the expression of CCR6 and other chemokine receptors was determined by FACS analysis. *B*, Individual culture samples were recovered and reseeded in presence of GM-CSF plus TGF-β during 4 days. At day 14, cells were recovered and the expression of indicated DC markers was determined by FACS analysis. Filled histograms represent isotype match controls. Results are representative of three independent experiments.

linked to an amplification of Th2 responses. It is tempting to speculate that during Th2 responses, the local production of IL-4 might prevent the recruitment of LC at site of injury, and the induction of immune response other than Th2. However, we also found that IFN-γ blocks the expression of CCR6 as well as CCL20 responsiveness. Unlike IL-4, IFN-γ amplify Th-1 responses by inducing CXCR3 in T cells (50) as well as its ligands CXCL9, CXCL10, and CXCL11 in many cell types (51), and by preventing the expression of CCR4 ligands (50). Thus, LC recruitment might also be suppressed during ongoing Th1-type immune responses. This may appear in contrast with our previous observation of CCL20 up-regulation in close association with infiltrating LC and memory T cells in lesional skin from psoriasis (15, 52), a pathology that is associated with a Th1 profile (production of IL-2, IL-12, and IFN-γ). In the present study, we have demonstrated that the ability of IFN-γ to induce the irreversible loss of CCR6 expression is linked to its effect on DC maturation, as shown by the up-regulation of costimulatory molecules (40) and the induction of CCR7 expression. Besides, this result is in line with the observation that maturing DC-$LAMP^+$ LC are present in lesional psoriatic skin (15). Thus, IFN-γ might switch chemokine responsiveness and allow DC to respond to other chemokines such as CCL19 and CCL21 involved in the constitutive DC trafficking into lymphoid organs. Additionally, the observed CCR6 down-regulation induced by IFN-γ and also by IL-4 may act as a stop signal to prevent the recruitment of additional LC precursors, and thus attenuate the local inflammation, when the cytokines leak into the circulation.

Because the effects of IL-4 and IFN-γ on CCR6 down-regulation may suggest that LC are involved in immune-type reactions different from polarized Th1/Th2 responses, we have investigated the effects of TGF-β and IL-10, cytokines recognized for their role in the development of regulatory T cells (53). Surprisingly, we found that IL-10 maintains the expression of CCR6 on precursor cells that preserve their capacity to differentiate into LC. So, this data clearly indicate that IL-10-treated DC are not committed in a defined pathway, nor completely differentiated. This observation might be related to the previously described arrest of LC maturation by IL-10 (30). Furthermore, we observed that IL-10 up-regulates CCR6 expression on monocyte-derived DC, and this receptor is functional, as demonstrated by the capacity of IL-10-treated cells to migrate in response to CCL20. The investigation of other important chemokine receptors involved in DC trafficking (CCR2, CCR5, and CCR7) demonstrated that the observed effect of IL-10 was selective for CCR6. Importantly, these IL-10-treated cells were not terminally differentiated macrophages, as they had the capacity to differentiate into mature DC upon inflammatory or T cell signals. Seven days of culture in presence of GM-CSF and IL-10 were required to optimally induce CCL20 responsiveness on monocytes, suggesting that the effect of IL-10 on CCR6 expression by monocyte-derived DC is not optimal as a possible consequence of the inhibitory effect of IL-4 on CCR6 expression. As monocytes have been shown to differentiate into DC in absence of IL-4 within 2 days following a transendothelial migration (54, 55), IL-10 may under some environmental conditions allow monocytes (or DC precursors) to acquire the capacity to reach the epithelium in response to CCL20. Furthermore, in contrast to the inhibitory effect of IL-10 on CCL20 secretion by monocytes (56), CCL20 expression by epithelial cells, which are the main in vivo source, was not decreased by IL-10 (not shown).

Unexpectedly, when $CCR6^+CD1a^+$ LC precursors were exposed to TGF-β, a cytokine involved in LC development, a profound reduction of CCR6 expression was observed. This may appear in contradiction with the described effects of TGF-β on CCR6

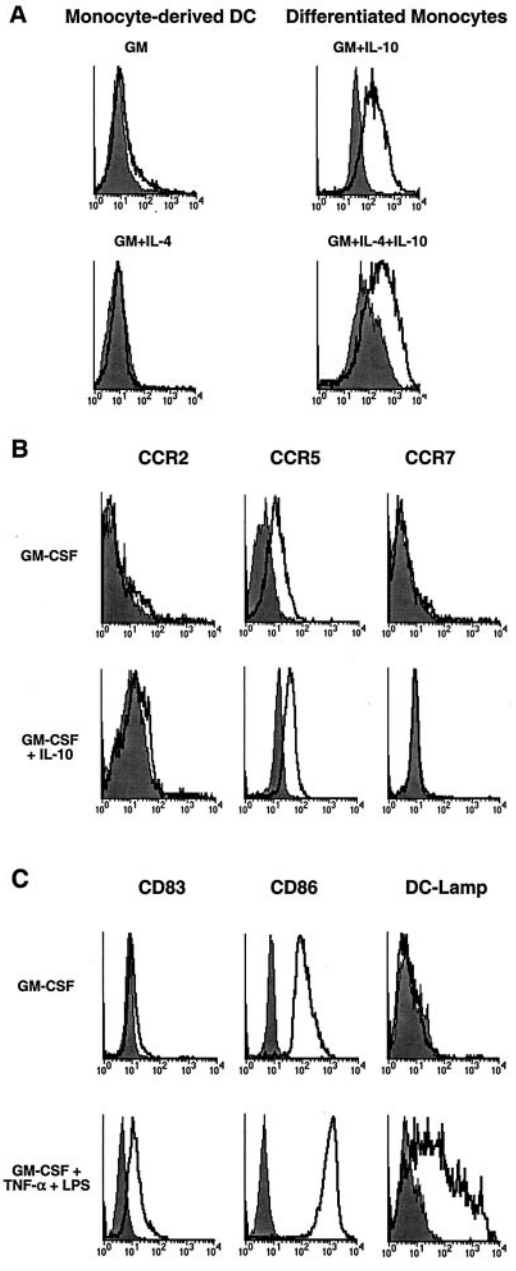

FIGURE 5. IL-10 selectively up-regulates CCR6 expression on differentiated monocytes while preserving their ability to differentiate into mature DC. Monocytes were cultured for 7 days in presence of GM-CSF, in presence or absence of IL-4, and with or without IL-10. *A*, The individual culture samples were recovered, and the expression of CCR6 on total cells was determined by FACS analysis. *B*, The expression of the other chemokine receptors, CCR2, CCR5, and CCR7, on monocytes cultured in presence of GM-CSF with or without IL-10 was determined by FACS analysis. *C*, Monocytes cultured in presence of GM-CSF with or without IL-10 for 7 days were harvested, washed, and reseeded in the presence of GM-CSF alone or GM-CSF plus TNF-α (2.5 ng/ml) plus LPS (10 ng/ml) during 2 days. At day 9, the expression of the costimulatory molecules CD83 and CD86 as well as the DC maturation marker DC-LAMP on total cells was determined by FACS analysis. Filled histograms represent isotype match controls. Results are representative of three independent experiments.

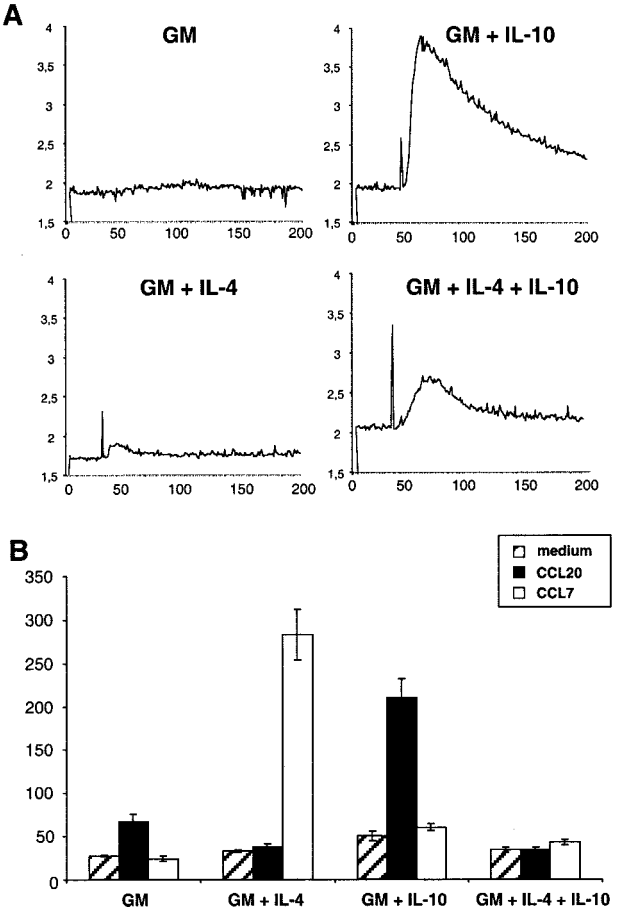

FIGURE 6. IL-10 positively regulates CCL20 responsiveness on differentiated monocytes. Monocytes were cultured for 7 days in presence of GM-CSF, in presence or absence of IL-4, and with or without IL-10. At the end of the culture, cells were recovered, and the chemotactic activity of CCL20 was determined by calcium flux analysis (*A*) and by chemotaxis assay (*B*). Migration assays were performed in Boyden microchambers, and results are expressed as number of migrating cells per two low power fields (magnification $\times$20). Results are representative of three independent experiments.

up-regulation on monocyte-derived DC (44, 57). However, blocking endogenous TGF-β prevents LC development (29) and CCR6 expression (not shown), suggesting that TGF-β is involved in CCR6 expression, as a part of a program of LC differentiation from CD34$^+$ progenitors or monocytes. The down-regulation of CCR6 by TGF-β on CCR6$^+$ LC precursors might reflect the terminal differentiation into epithelial LC induced by TGF-β. Thus, the control of the epithelial colonization by LC might be the result of combined action of TGF-β and CCL20 expressed at a very low level in normal skin (16, 52).

CCL20 has been reported to be expressed in airway (42, 58, 59) and intestinal (19, 60, 61) mucosa. As both TGF-β and IL-10 are key regulators of mucosal immunity, one might speculate that they cooperate to increase the efficiency of LC recruitment at epithelial surfaces. IL-10 maintains CCR6 expression on LC precursors, expanding the window of CCL20 action and thus the efficiency of recruitment. When reaching the epithelium, the local TGF-β will then drive their final differentiation and arrest them in the epithelial layers by down-regulating CCR6 expression. In this context, TGF-β has been shown to freeze in vitro generated LC into an epithelial state (62).

In addition, CCL20 is highly expressed by numerous tumors and cell lines: pancreas, papillary, renal, and breast carcinoma and metastasis (Refs. 63–65 and our unpublished observations), in which the production of both IL-10 and TGF-β has been documented. Thus, IL-10 and TGF-β produced in the tumor microenvironment

may have an important role not only in the differentiation/maturation of DC, but also in modulating the recruitment of selective DC precursors. In this context, IL-10-treated DC have been shown to induce tolerance (32).

CCR6 has been shown to be preferentially expressed on memory T cells with both skin- and gut-homing properties (17, 18). In addition, CCL20 has been shown to mediate the migration of intestinal epithelium $\gamma\delta$ T cells (19). We have reported the up-regulation of CCL20 in lesional psoriatic skin, in close vicinity of LC and memory T cells (15, 52). Altogether, these observations suggest that CCL20 may have specific role in regulating epithelial immunity through both the recruitment of LC precursors and memory T cells with epithelial tropism.

Acting on T lymphocytes, IL-4, IFN-γ, IL-10, and TGF-β are involved in cross-regulation between Th-1, Th-2, and regulatory immune responses. In the present study, we add a further level of complexity with cytokines that differentially regulate the recruitment of LC at epithelial surface through the regulation of CCR6 expression by those cells. While Th1 (i.e., IFN-γ) and Th2 cytokines (i.e., IL-4 and IL-13), by down-regulating CCR6 expression, may attenuate ongoing immune responses, regulatory cytokines (i.e., IL-10 and TGF-β) have the opposite effect, and thus may amplify those responses. Along with this hypothesis, we recently obtained evidence that $CD34^+$-derived LC allow the development of $CD4^+$ T cells producing IL-10, while the other populations ($CD14^+$-derived and monocyte-derived DC) lack this capacity (our unpublished observations). These IL-10-secreting $CD4^+$ T cells might be related to the described regulatory T cells. Collectively, these observations suggest that through their actions on LC, TGF-β, IL-10, and CCL20 contribute to the regulatory immune responses at epithelial surfaces.

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