CC-Chemokine Ligand 16 Induces a Novel Maturation Program in Human Immature Monocyte-Derived Dendritic Cells

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Dendritic cells (DCs) are indispensable for initiation of primary T cell responses and a host’s defense against infection. Many proinflammatory stimuli induce DCs to mature (mDCs), but little is known about the ability of chemokines to modulate their maturation. In the present study, we report that CCL16 is a potent maturation factor for monocyte-derived DCs (MoDCs) through differential use of its four receptors and an indirect regulator of Th cell differentiation. MoDCs induced to mature by CCL16 are characterized by increased expression of CD80 and CD86, MHC class II molecules, and ex novo expression of CD83 and CCR7. They produce many chemokines to attract monocytes and T cells and are also strong stimulators in activating allogeneic T cells to skew toward Th1 differentiation. Interestingly, they are still able to take up Ag and express chemokine receptors usually bound by inflammatory ligands and can be induced to migrate to different sites where they capture Ags. Our findings indicate that induction of MoDC maturation is an important property of CCL16 and suggest that chemokines may not only organize the migration of MoDCs, but also directly regulate their ability to prime T cell responses. The Journal of Immunology, 2006, 177: 6143–6151.

Dendritic cells (DCs) are the most important link between the innate and the acquired immune response and are involved in the initiation of both types of immunity (1). Immature DC (iDC) precursors exit from the bone marrow, circulate via the bloodstream to reach their target tissues, and take up residence at sites of potential pathogen entry in a physiological stage that is specialized for Ag capture (1). Tissue injury and inflammation cause dramatic changes throughout the microenvironment, including the release of inflammatory mediators such as cytokines and chemokines that shape protective immune responses and culminate in pathogen elimination. Many stimuli are involved in DC maturation (2). DCs receive information directly from pathogens via “pattern-recognition receptors” such as the TLRs (3) and from cytokines released by T cells during their differentiation toward Th1 or Th2 in peripheral tissues or lymph nodes (LNs) (4).

Furthermore, DCs are affected by endogenous mediators released by infected, damaged, or inflamed tissues, such as proinflammatory cytokines, prostaglandin, or catabolites from dying cells (5, 6). Chemokine receptors have also been shown to be directly involved in induction of DC maturation. Specifically, activation through CCR5 by Toxoplasma gondii-derived cyclophilin leads to production of high levels of IL-12 in DCs (7, 8). Little is known, on the other hand, about chemokines and DC maturation. We focused our attention on CCL16/liver-expressed chemokine’s effects because we had demonstrated its potent antitumor activity associated with recruitment of leukocytes (9). CCL16 is chemotactic for monocytes, lymphocytes, eosinophils, and DCs (for a review, see Zlotnik et al. (10)). It binds CCR1, CCR2, CCR5, and CCR8 (11, 12) and has been used to treat established murine tumors. CCL16 expressed by adenoviruses (13, 14) or as recombinant fusion protein (15) induces accumulation of T cells and DCs at the tumor site and in draining LNs.

Our previous data have shown that CCL16 costimulates macrophage cytotoxic and Ag-presenting functions in the mouse (16). CCL16 released by lamina propria-infiltrating macrophages elicits massive recruitment of immune reactive cells in ulcerative colitis (17). These results led us to study the effects of CCL16 on human DCs derived from monocytes (MoDCs) in the presence of GM-CSF and IL-4. We report here that human CCL16 is a potent maturation factor for MoDCs through differential use of its four receptors and an indirect regulator of Th cell differentiation. We analyzed the expression of costimulatory molecules and chemokine receptors and confirmed their functional efficiency. We also evaluated the ability of MoDCs induced to mature by CCL16 (CCL16/mDCs) to induce alloactivation of T cells and take up Ag. Our findings suggest that chemokines may not only organize the migration of DCs but also directly regulate their ability to prime T cell responses.

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¶Abbreviations used in this paper: DC, dendritic cell; CCL16/mDC, MoDC induced to mature in the presence of CCL16; iDC, immature DC; LN, lymph node; mDC, DC induced to mature in the presence of TNF-α plus IL-1β; MFI, mean fluorescence intensity; MoDC, monocyte-derived DC; PLC, phospholipase C; PTX, pertussis toxin.

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Materials and Methods

CCL16 source

The human recombinant CCL16 used in these experiments (PeproTech) is a 11.2-kDa protein of 97-aa residues produced in Escherichia coli from a DNA sequence encoding the mature human CCL16 protein sequence (Q26-Q120). Its endotoxin level is <0.1 ng/μg (1 EU/μg) as determined by the Limulus Amebocyte Lysate assay.

Generation and maturation of MoDCs

Human PBMCs were isolated from venous blood of voluntary healthy donors by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech). Monocytes were enriched with an isolation kit (Miltenyi Biotec). The resulting preparations were consistently >90% CD14 as determined by FACScalibur (BD Biosciences). To prepare iDCs, the enriched monocytes were incubated in 6-well culture plates (5 × 10⁶ cells/3 ml/well) in AIM V medium (Invitrogen Life Technologies) supplemented with 100 ng/ml GM-CSF and 50 ng/ml IL-4 (both PeproTech) for 6 days. On days 2 and 4, two-thirds of the culture medium was replaced by fresh medium containing GM-CSF and IL-4. iDCs were resuspended as 1 × 10⁷ cells/ml in AIM V supplemented or not with CCL3, CCL4, CCL14, and CCL16 (all from PeproTech) at 1000 and 100 ng/ml, as indicated, or human TNF-α (50 ng/ml) plus IL-1β (50 ng/ml) (both from PeproTech). Their endotoxin level is <0.1 ng/μg as determined by the Limulus Amebocyte Lysate assay. After 24 or 48 h, as indicated, cells were harvested and analyzed.

Immunophenotypic analysis

After 24 h in the presence of medium only, CCL16 (at both 100 and 1000 ng/ml), or TNF-α and IL-1β, cells were washed and resuspended in PBS (Sigma-Aldrich) supplemented with 0.2% BSA and 0.01% sodium azide, and incubated with fluorochrome-conjugated mAbs and isotype-matched negative controls (DakoCytomation) after blocking nonspecific sites with rabbit IgG (Sigma-Aldrich) for 30 min at 4°C. The following mAbs were used: anti-CD14 (BD Biosciences); anti-CD1a, anti-CD80, anti-CD83, anti-CD86, anti-HLA-DR, -DP, -DQ, and -DR (Ancell); and anti-CCR5, anti-CCR6, anti-CCR7, and anti-CXCR4 (R&D Systems). Samples were collected and analyzed using a FACScalibur CellQuest (BD Biosciences). Cells were electronically gated according to their light-scatter properties to exclude cell debris and contaminating lymphocytes. To determine which CCL16 receptor is involved in the CCL16-induced maturation of MoDCs, iDCs were pretreated with mAb to CCR1 (MBL International), CCR2 (R&D Systems), CCR5 (BD Biosciences), or CCR8 (R&D Systems), or an isotype control IgG (DakoCytomation) for 30 min at 4°C and, without washing, incubated in the presence or not of CCL16. To determine the signaling pathway effective in the CCL16-induced maturation of MoDCs, iDCs were pretreated with inhibitors, namely, pertussis toxin (PTX) from Bordetella pertussis (100 ng/ml; Sigma-Aldrich), LY290042 (25 μM; Sigma-Aldrich), wortmannin from Penicillium funiculosum (200 nM; Sigma-Aldrich), SB203580 (20 μM; Sigma-Aldrich), U73122 (1 μM; Sigma-Aldrich), or PD98058 (50 μM; Calbiochem) for 45 min at 37°C and, without washing, incubated in the presence or absence of CCL16. After 24 h, cells were processed as indicated above. The percent inhibition was calculated as (100 – (mAb treatment + CCL16/IgG + CCL16) × 100) or (100 – (inhibitor + CCL16/IgG, 1% DMSO + CCL16) × 100).

Chemotaxis

Boyden chambers (NeuroProbe) were filled with stimulus (CCL1, CCL2, CCL4, CCL14, CCL16, CCL20, CXCL12, or CCL19; all from PeproTech) diluted in RPMI 1640 (BioWhittaker Europe) 1% FCS certified (Invitrogen Life Technologies) at reported concentrations, and only medium as control, and covered with polycarbonate filters (pore size 5 μm; Nucleopore). After 24 h of stimulation in the presence or absence of CCL16 or TNF-α and IL-1β, 50 μl of MoDC suspension in RPMI 1640–1% FCS at a concentration of 1 × 10⁵ cells/ml was added to each chamber. After 2 h at 37°C, cells that had migrated to the lower part of the filter were stained with Giemsa-May-Grunwald (Diff-Quik kit; Dade Behring) and counted under a microscope (objective immersion oil, ×100; Olympus). To determine which receptor is involved in the CCL16-induced migration of iDCs, they were pretreated with 10 μg/ml neutralizing mAb against CCR1, CCR2, CCR5, CCR8 or isotype control IgG for 30 min at 4°C and then resuspended by centrifugation. These cells were incubated in a Boyden chamber in the presence or absence of 1000 ng/ml CCL16 for 2 h at 37°C, as described above. CCL1, CCL2, CCL4, and CCL14 were used as positive controls.

Mixed lymphocyte reaction

T cells were purified on a nylon wool column (Robbins Scientific) and placed in 96-well plates at 1 × 10⁶ cells/well with allogeneic iDCs and differentially matured DCs, after 24 h of stimulation with CCL16 or TNF-α and IL-1β, in increasing concentrations (150–10,000). After 4 days, 1 μCi of [³H]Thiouracil (Amersham Biosciences) was added to each well and incubation was prolonged for an additional 18 h. Cells were directly collected with a CellHarvester (Packard Instrument) in UNIfilter plates (Packard Instrument) and [³H]Thiouracil uptake was quantitated (TopCount microplates; scintillation counter; Packard Instrument). All tests were performed in triplicate.

Supernatants from T cells cultured with iDCs, CCL16/mDCs, or mDCs at ratio 20:1 T:DC were harvested after 48 h and analyzed by ELISA for IFN-γ and IL-4 production (both R&D Systems) in accordance with the manufacturer’s protocol.

Cytokine detection

Production of cytokines and chemokines in the supernatants was evaluated with a commercial RayBio Human Cytokine Array V (Tebu-bio) in accordance with the manufacturer’s protocol. The membranes were exposed to x-ray films and signals were detected with a film developer (Kodak Hyperfilm; Amershams Biosciences). Significance was increased by using pools of supernatants from single donors of iDCs, CCL16/mDCs and mDCs. The signal intensities were quantitated by densitometry with UTHSCSA ImageTool, a free image processing and analysis program, and positive controls present on the membranes were used to normalize the results for comparison. An increase or decrease of 30% was considered significantly different.

Supernatants from iDCs, CCL16/mDCs, and mDCs from 12 donors were analyzed by ELISA for IL-10 and CXCL10 (both from R&D Systems) secretion in accordance with the manufacturer’s protocol.

Ag uptake assay

After 24 h of stimulation with CCL16 or TNF-α and IL-1β, iDCs and differentially matured DCs were incubated at 1 × 10⁵ cells/ml in AIM V for 15 min at 4 or 37°C. FITC-labeled dextran (M₀, 70,000; Sigma-Aldrich) was added at the final concentration of 1 mg/ml and the cells were incubated for 15, 30, 60, and 120 min to allow capture of Ag. After thorough washing with cold PBS, fluorescence was measured by FACScalibur CellQuest analysis to reveal dextran uptake.

Statistical analysis

The significance of differences in counts per minute obtained from the [³H]Thiouracil uptake assay, in migration in the presence of stimuli, and in cytokine secretion was evaluated with an unpaired Student’s t test (GraphPad Prism 3.1; GraphPad Software).

Results

CCL16 induces migration of MoDCs by binding its four receptors

CCL16 is chemotactic for human DCs (10), especially at 1000 ng/ml. We therefore set out to determine which receptor is involved in this migration by pretreating (or not) iDCs with neutralizing Abs to CCR1, CCR2, CCR5, and CCR8 and assessing their ability to migrate in response to CCL16 at 1000 ng/ml. In each of three independent experiments we used the specific ligands for each CCR as the positive control. As shown in Fig. 1, CCL16 significantly induced migration of iDCs, while addition of the neutralizing Abs to CCR5 (100%) and CCR8 (73%) and (to a lesser extent) CCR1 and CCR2 (36–63%) inhibited this migration. The presence of all four mAbs inhibited iDC migration less (94%) than anti-CCR5 alone. Addition of an unrelated mAb had no effect on this migration. CCL16, therefore, uses all its receptors to induce iDC migration, probably with different affinity.

CCL16 induces maturation of human MoDCs

After only 24 h of stimulation with 100 and 1000 ng/ml chemokine concentrations, CCL16/mDCs displayed a higher expression of CCR1, CCR2, CCR4, and CCR14 and significantly different mature DCs, after 24 h of stimulation with CCL16 or TNF-α and IL-1β, in increasing concentrations (150–10,000). After 4 days, 1 μCi of [³H]Thiouracil (Amersham Biosciences) was added to each well and incubation was prolonged for an additional 18 h. Cells were directly collected with a CellHarvester (Packard Instrument) in UNIfilter plates (Packard Instrument) and [³H]Thiouracil uptake was quantitated (TopCount microplates; scintillation counter; Packard Instrument). All tests were performed in triplicate.

Supernatants from T cells cultured with iDCs, CCL16/mDCs, or mDCs at ratio 20:1 T:DC were harvested after 48 h and analyzed by ELISA for IFN-γ and IL-4 production (both R&D Systems) in accordance with the manufacturer’s protocol.
was expressed by a substantial percentage of CCL16/mDCs but was undetectable in iDCs. As shown in Fig. 2, both CCL16 concentrations exerted similar effects, although for some markers, the higher concentration more efficiently induced their increased expression as shown by the mean fluorescence intensity (MFI) and was therefore used in all the other experiments.

Because DC maturation is associated with differential regulation of chemokine receptors (18), we determined whether CCL16 modifies their expression pattern on MoDCs. CCL16/mDCs, in fact, up-regulated receptors CXCR4 and CCR7 but still maintained the expression of CCR5 (Fig. 2).

Interestingly, the CCR5 receptor was found on iDCs generated in vitro (Fig. 2), whereas mDCs down-modulated CCR5 and primarily expressed CXCR4 and CCR7. CCL16/mDCs differ from mDCs in their persistence of CCR5 expression and ex novo CCR6 expression, which is usually found on CD34+ -derived DCs and Langerhans DCs and is important for the recruitment of iDCs to an injury site.

CCL3, CCL4, and CCL14 share the same putative receptors as CCL16. When they were used as maturation stimuli, only CCL14, a CCR1 agonist, displayed a similar ability to induce CD83 and CCR7 maturation markers (Table I). The influence of trace amounts of endotoxin in the chemokine preparations can be ruled out because all are claimed to contain <0.1 ng/μg endotoxin. Even so, only CCL16 and CCL14 induced maturation markers on iDCs. Moreover, boiled CCL16 did not stimulate phenotypic changes in iDCs (data not shown).

To find out which receptor is involved in the CCL16-induced maturation of human MoDCs, we pretreated iDCs with neutralizing mAbs against CCR1, CCR2, CCR5, CCR8, or all together, or with an isotype control Ig for 30 min at 4°C were incubated in a Boyden chamber in the presence or not of CCL16 (1000 ng/ml) for 2 h at 37°C. The results are the mean ± SD of migrated cells/field from three experiments with three independent donors. *p < 0.05, values from mAbs pretreated cells compared with untreated cells that migrated in the presence of CCL16; **p < 0.05, values from cells that migrated in the presence of CCL16 compared with cells migrated in the absence of stimuli.

**FIGURE 1.** CCL16 induces migration of MoDCs. iDCs pretreated or not with neutralizing Abs against CCR1, CCR2, CCR5, CCR8, or all together, or with an isotype control Ig for 30 min at 4°C were incubated in a Boyden chamber in the presence or not of CCL16 (1000 ng/ml) for 2 h at 37°C. The results are the mean ± SD of migrated cells/field from three experiments with three independent donors. *p < 0.05, values from mAbs pretreated cells compared with untreated cells that migrated in the presence of CCL16; **p < 0.05, values from cells that migrated in the presence of CCL16 compared with cells migrated in the absence of stimuli.

**FIGURE 2.** CCL16 induces maturation of MoDCs. iDCs (first and fourth rows) were cultured for an additional 24 h with CCL16 at 100 and 1000 ng/ml (CCL16/mDCs, second and fifth rows, gray peak and thick solid line, respectively) or TNF-α and IL-1β (mDCs, third and sixth rows). CCL16/mDCs acquire a mature DC phenotype (HLA-DRhigh, CD83−, CD80high, CD86high, CXCR4+, and CCR7+) but maintain CCR5 and express ex novo CCR6. The thin solid line shows the isotype control. One of five independent experiments with different donors is shown. A, HLA-II, CD83, CD80, CD86 and B, chemokine receptors surface expression.
The isotype control IgG was used as negative control. We evaluated the expression of CD83, CD80, CD86, CXCR4, and CCR6, mainly increased or induced by CCL16 (Table II). Blockade of the CCRs by their specific mAbs differentially affected the activity of CCL16 on iDCs. The expression of CCR6 seems to be controlled mainly by CCR1, CCR8, and CCR5 because their blocking abrogated or strongly inhibited the effect of CCL16. Each CCR is equally involved in the induction of the ex novo CXCR4 expression, whereas CCR8 seems more involved in that of CD83. Up-modulation of CD80 and CD86 was slightly inhibited by the presence of mAbs. All CCL16’s CCRs would thus seem responsible for the increased expression of these molecules.

To determine which signaling transducing pathway is involved in CCL16-induced maturation of MoDCs, we pretreated iDCs with inhibitors for G protein, PI3K, Erk, p38 MAPK, and phospholipase C (PLC) pathways at 37°C, followed by incubation in the presence or absence of CCL16 for an additional 24 h. Medium-0.1% DMSO was used as background of stimulation. We evaluated the expression of CD83, CD86, CCR6, and CXCR4 (Table III). Inhibition of the signaling pathways variously affected the activity of CCL16, with the exception of the Erk pathway. PD98059, in fact, did not inhibit CCR expression nor CD83, and only weakly decreased the CCL16-induced CD86 expression. This means that the Erk pathway is not directly involved in the CCL16-induced maturation of MoDCs. CCR6 expression was abrogated or strongly inhibited by PTX, wortmannin, SB203580, U73122, and IL-1β. CCL16-induced chemokine receptors elicited migration to chemokines expressed by LNs or inflamed peripheral tissues, respectively.

### Table II. CCR involvement in the CCL16-induced maturation of human MoDCs

| mAb to   | CD83 | CD80 | CD86 | CCR6 | CXCR4 |
|----------|------|------|------|------|-------|
| CCR1     | 25   | 6    | 19   | 100  | 36    |
| CCR2     | 14   | 2    | 24   | 33   | 28    |
| CCR5     | 35   | 13   | 22   | 60   | 50    |
| CCR8     | 78   | 15   | 0    | 100  | 46    |

Percentage of inhibition is calculated for each marker increased or induced by CCL16. One of three experiments is represented.

### Table III. Signal pathways involved in the CCL16-induced maturation of human MoDCs

| Inhibitor  | CD83 | CD86 | CXCR4 | CCR6 |
|------------|------|------|-------|------|
| PTX        | 0    | 30   | 100   | 0    |
| LY294002   | 31   | 90   | 85    | 100  |
| Wortmannin | 0    | 42   | 0     | 100  |
| PD98059    | 0    | 17   | 0     | 0    |
| SB203580   | 53   | 51   | 100   | 84   |
| U73122     | 34   | 66   | 85    | 84   |

Percentage of inhibition is calculated for each marker increased or induced by CCL16. One of two experiments is represented.

### Table I. CD83 and CCR7 expression by stimulated iDCs

| Stimuli  | CD83 | CCR7 |
|----------|------|------|
| CCL3     | 8    | 1    |
| CCL4     | 7    | 24   |
| CCL14    | 36   | 34   |
| CCL16    | 54   | 57   |
| TNF-α plus IL-1β | 80   | 65   |

Chemokines were added to iDCs at 1000 ng/ml, TNF-α and IL-1β at 50 ng/ml, and the cells were harvested and analyzed by flow cytometry after 24 h (see Materials and Methods).

Percentage of positive cells. One of three independent experiments with different donors is shown.

Protein G and wortmannin (PI3K) pathways thus control the CCL16-dependent induction of CCR6 and moderately that of CCR8; the PI3K pathway controls CCL16-dependent induction of CD86; and the p38 MAPK and PLC pathways control CXCR4 and CCR6 and partially CD83 and CD86.

### MoDC migration

We evaluated the ability of CCL16-induced CCR to respond to specific chemokines (Fig. 3), namely CCL4 (Fig. 3A), CCL20 (Fig. 3B), CXCL12 (Fig. 3C), and CCL19 (Fig. 3D). iDCs migrated in response to CCL4, like CCL16/mDCs, in agreement with their membrane expression of CCR5. As expected, mDCs did not migrate in response to this chemokine. iDCs were not sensitive to CXCL12 (a CXCR4 agonist) and CCL19 (a CCR7 agonist), which induced migration by both mDCs and CCL16/mDCs. Both types of mDCs, in fact, express CXCR4 and CCR7 on their surface, whereas no expression was observed on the iDC membrane. Only CCL16/mDCs were sensitive to CCL20 (a CCR6 agonist), a finding consistent with their membrane expression of CCR6. Thus, all CCL16-induced chemokine receptors elicited migration to chemokines expressed by LNs or inflamed peripheral tissues, respectively.

### Alloactivation by mDCs

To check the ability of CCL16/mDCs to present Ag, we analyzed their allostimulatory function because mDCs present alloantigens and induce proliferation in allogeneic T cells. As shown in Fig. 4A, CCL16/mDCs and mDCs displayed a similar ability to induce a significant proliferative response by alloactivated T cells, which was higher than that induced by iDCs. Moreover, alloactivated T cells generated with CCL16/mDCs or mDCs showed a similar ability to produce IFN-γ. By contrast, IFN-γ production by alloactivated T cells generated in the presence of iDCs was significantly lower (Fig. 4B). IL-4 was similarly produced by alloactivated T cells, irrespective of their generation in the presence of iDCs, CCL16/mDCs, or mDCs (Fig. 4C). These data indicate a similar ability of CCL16/mDCs and mDCs to promote a Th1-deflected response.

### Differential cytokine and chemokine secretion by iDCs, CCL16/mDCs, and mDCs

Pools of supernatants of MoDCs from three donors after 48 h of incubation in the absence or presence of CCL16 or TNF-α and IL-1β were analyzed by the RayBio Human Cytokine Array V to simultaneously detect the secretion of 79 factors. After 24 h of culture (data not shown), supernatants from CCL16/mDCs presented a limited difference in factor secretion compared with that
by iDCs. They showed a decrease of CCL18 and an increase of CCL20 secretion (data not shown) in agreement with the observations of other groups (19). After 48 h, several cytokines were increased (Fig. 5A, H18554) by CCL16, including chemokines macrophage migration inhibitory factor, CCL15, CXCL10, CXCL5, CCL4, and fractalkine. By contrast, another seven factors, GRO, IL-4, CXCL8, IL-10, CCL18, CCL17, and CCL7, were reduced (Fig. 5A, H18554). Six factors, CCL20, IL-1β, IL-12, CCL23, and IL-2, were induced ex novo by CCL16 and TNF-α and IL-1β (Fig. 5B).

This array provides relative densitometry signals and not absolute concentration values. However, image analysis (UTHSCSA Image Tool for Windows) software, which linearizes OD to spot volume, revealed marked differences in protein secretion between supernatants from CCL16/mDCs and iDCs (Fig. 5, A and B). For almost all chemokines and cytokines secreted, however, there were no significant differences between supernatants from CCL16/mDCs and mDCs, although GRO, CXCL8, IL-10, CCL2, CXCL5, and IL-6 were decreased by the presence of CCL16.

To validate these results, we performed an ELISA for IL-10 and CXCL10, which are inhibited and induced, respectively, by the presence of CCL16, with supernatants from iDCs, CCL16/mDCs, and mDCs from different donors (Fig. 5, C and D). Secretion of both proteins reflected the results obtained with the array. The presence of CCL16 during MoDC maturation, in fact, always decreased IL-10 secretion (20–454 pg/ml compared with 211–774 pg/ml CCL16/mDCs and iDCs, respectively; Fig. 5C) and increased CXCL10 secretion (90–398 pg/ml compared with 17–56 pg/ml CCL16/mDCs and iDCs, respectively; Fig. 5D) in a donor-dependent manner. These data suggest that CCL16/mDCs are not "exhausted." They secrete many more factors after 48 h of stimulation and use them to attract different types of leukocytes.

**Ag uptake by CCL16/mDCs**

iDCs take up Ag efficiently but lose this ability on maturation (20, 21). Therefore, we measured Ag uptake by iDCs, CCL16/mDCs, and mDCs by flow cytometry (Fig. 6). At 4°C, cell metabolism is inhibited and such MoDCs are incapable of capturing Ag. However, at 37°C, the control iDCs showed a significant uptake of FITC-labeled dextran (Fig. 6A) after only 15 min and this progressively increased after 30 min (Fig. 6, A and D). As expected,
mDCs displayed very little ability to take up FITC-labeled dextran (Fig. 6, C and D). Surprisingly, CCL16/mDCs displayed a high Ag uptake (Fig. 6B), similar to that of control iDCs after 15 and 30 min (Fig. 6D). The uptake ability of CCL16/mDCs was maintained over 60 min (data not shown).

Discussion

This study demonstrates that CCL16 induces a novel functional subset of human MoDCs (CCL16/mDCs), which display a full maturation phenotype and maintain their ability to take up Ag. We evaluated their phenotype, downstream production of chemokines and cytokines, and ability to stimulate alloactivated T cells and take up Ag. CCL16 induces increased levels of MHC class II molecules and CD80 and CD86 costimulatory molecules and up-regulation of the marker CD83 at 100 and 1000 ng/ml. Both concentrations induced phenotypic changes in iDCs, but the higher one showed greater effects and was used in all the other experiments. In a previous report (22), on the other hand, we demonstrated that CCL16 secreted by Cos or tumor cells affected endothelial cells much more efficiently than recombinant protein. The membranes were exposed to x-ray films, analyzed by densitometry, and normalized using the positive controls present in the membranes. Percentage of decrease or increase (A) and the ex novo induction (B) in protein secretion was evaluated by comparing supernatants from CCL16/mDCs (■) and mDCs (□) to those from iDCs. N.D., not detected; N.E., not evaluable because present in medium culture. To validate the array results, IL-10 (C) and CXCL10 (D) secretion, inhibited and induced, respectively, by CCL16, were analyzed by ELISA in 12 supernatants from iDCs (■), CCL16/mDCs (○), and from mDCs (□). The horizontal bars mark the mean values. *p < 0.05 values from protein secretion by CCL16/mDCs compared with that by iDCs; **p < 0.05 values from protein secretion by CCL16/mDCs compared with that by mDCs. I.D., intensity of density, as calculated by UTHSCSA image tool for Windows.

FIGURE 5. Effect of CCL16 during MoDC maturation on cytokine and chemokine production. Pools of three supernatants from iDCs incubated in the absence or presence of CCL16 or TNF-α and IL-1β for 48 h were analyzed on a RayBio Human Cytokine Array V performed for 79 proteins. The membranes were exposed to x-ray films, analyzed by densitometry, and normalized using the positive controls present in the membranes. Percentage of decrease or increase (A) and the ex novo induction (B) in protein secretion was evaluated by comparing supernatants from CCL16/mDCs (■) and mDCs (□) to those from iDCs. N.D., not detected; N.E., not evaluative because present in medium culture. To validate the array results, IL-10 (C) and CXCL10 (D) secretion, inhibited and induced, respectively, by CCL16, were analyzed by ELISA in 12 supernatants from iDCs (■), CCL16/mDCs (○), and from mDCs (□). The horizontal bars mark the mean values. *p < 0.05 values from protein secretion by CCL16/mDCs compared with that by iDCs; **p < 0.05 values from protein secretion by CCL16/mDCs compared with that by mDCs. I.D., intensity of density, as calculated by UTHSCSA image tool for Windows.
Only CCL14, a CCR1 agonist, elicited CD83 and CCR7 expression, although to a lesser extent than CCL16. We have demonstrated that CCL16 induces human monocyte activation especially through binding with CCR1 (28). Chen and collaborators showed that even MoDCs could be affected by CCL16-CCR1 binding. However, neutralizing mAbs to CCL16 or CCR1 receptor, when added to iDCs during a chemotaxis assay, inhibited iDC migration, suggesting that they are functional receptors mediating CCL16’s effects on iDC migration. Interestingly, combined treatment of iDCs with neutralizing mAbs did not further inhibit their migration in response to CCL16 compared with the single mAb alone. These data suggest that all CCRs are used by CCL16, even if cooperatively, to stimulate iDC migration. We cannot determine from these experiments whether the receptors interact physically or simply cooperate. When we investigated which receptor is used by CCL16 in iDC maturation by pretreatment of iDCs with neutralizing mAbs, we found that all receptors are differently involved. The expression of CCR6 is induced mainly by CCL16 binding to CCR1, CCR8, and CCR5 because their blocking by specific mAbs abrogated or strongly inhibited CCL16 effects. Each CCR is equally involved in the induction of ex novo CXCR4 expression, whereas CCR8 is more involved in that of CD83. Costimulatory molecule expression was only slightly inhibited by each of the mAbs to CCRs. This result suggests that all receptors cooperate to induce their expression or that other chemokines induced by CCL16 can cooperate with it. Altogether, these data indicate that CCL16 induces different signaling cascades leading to a “new” maturation program by binding different receptors. Kim et al. (29) demonstrated a differential CCR1-mediated chemotaxis signaling induced by CCL16 in HOS cells in comparison to other CCR1-dependent chemokines, including CCL3, CCL5, and CCL15, which involves G<sub>i</sub>/G<sub>q</sub> protein, PLC, protein kinase C<sub>γ</sub>, and p38 MAPK. Studies to better define CCL16 cell transduction in iDCs were performed with several signaling pathway inhibitors. Our preliminary results demonstrate an early and persistent phosphorylation of Akt and Erk1–2, even after 2 h of stimulation (data not shown). The phosphorylation of Akt and Erk1–2 in iDCs after only 10 min of stimulation with CCL16 underlies a direct effect of chemokines on iDCs. However, the Erk pathway seems not to be directly involved in CCL16-induced MoDC maturation since PD98059, a specific MEK inhibitor, did not affect the CD83, CXCRR4, and CCR6 expression and only weakly inhibited the up-regulation of CD86. p38 MAPK and PLC are mostly involved as their blockade strongly inhibited the expression of all markers evaluated. Other groups demonstrated that p38 MAPK is preferentially involved during the DC maturation induced by LPS or proinflammatory cytokines (30–33). In our case, p38 MAPK pathway is activated by a chemokine. Erk and PI3K have been demonstrated to be involved essentially in the DC survival or TNF secretion by LPS-stimulated DCs (30–32). CCL16, however, also involves PI3K, even if the two inhibitors used displayed differential effects on protein expression by CCL16-stimulated MoDCs. G proteins, instead, are mainly involved in CCR6 ex novo expression. CCL16 seems able to trigger different pathway signaling to induce DC maturation. Other groups have reported chemokines as inducers of DC activation (34, 35). However, while we show that CCL16 affects immature not activated MoDCs, Marsland et al. (35) report that CCL19 and CCL21 are adjuvants for terminal activation of “licensed” or already activated DCs by binding CCR7 expressed only by matured DCs.

In tumors, a major problem is induction of T cell tolerance by iDCs that present Ag inefficiently. CCL16/mDCs could be very efficient in Ag presentation at the tumor site. There are costimulatory molecules on their surface and they secrete proteins that favor T cell attraction and activation, such as CCL4 (36), CXCL10 (37), and CCL13 (38). Molon et al. (39) have demonstrated recently that chemokines secreted by APCs during Ag presentation are sufficient to recruit chemokine receptors on the T cell membrane at the immunological synapse. This trapping enhances T cell proliferation and cytokine production (39). CCL16/mDCs show the same ability as classic mDCs to prime T cells to mismatched MHC. CCL16/mDC-activated T cells produced increased levels of IFN-γ compared with those activated in the presence of iDCs. CCL16/mDCs thus prime and activate Th1 cells.

The ability of CCL16/mDCs to take up Ag, compared with the inability of classic mDCs, is their third interesting feature. Usually iDCs are less potent initiators of immunity but specialized in capturing and processing Ags to form MHC peptide complexes. DCs thus perform their two key functions at different times and in different locations: they first handle Ags as iDCs, and then, as mDCs, stimulate T cells in LNs (40). This prolonged ability to take up Ag could be important to offer Ag for T cell recognition and activation by subsequent challenge of their TCR (41) at both the tumor and inflamed sites, or in LNs.

CCL16/mDCs are still sensitive to further maturation stimuli. In the presence of TNF-α and IL-1β, they increase CD83 expression and are no longer able to take up FITC-dextran (data not shown). This suggests that CCL16 prevents what has been called “DC exhaustion”: after activation by LPS, DCs produce cytokines, especially IL-12, only transiently and became refractory to further stimulation, including CD40L (42). Transient cytokine production affects T cell polarization. A comparison between supernatants...
from CCL16/mDCs after 24 (data not shown) and 48 h of culture demonstrated that CCL16 does not impair protein secretion. The cytokine array referred to the supernatant collected after 48 h showed many more spots, namely secreted proteins. In a series of studies, Luft et al. (43) have suggested that, depending on the signal encountered or its strength and persistence, DCs, in particular MoDCs, acquire the mutually exclusive abilities to either produce high levels of inflammatory cytokines or migrate to the draining LN (43, 44). In the light of these results, CCL16 seems to be a good stimulus to maintain the ability of mDCs to produce inflammatory proteins and migrate to LNs.

Monocytes provide a large pool of potential myeloid DC precursors in vivo. Therefore, agents that can quickly mobilize this pool and induce its differentiation into DCs and their maturation could be of assistance in the treatment of malignancies and infectious diseases. Although GM-CSF plus IL-4 have been effectively used to generate DCs from monocytes, and proinflammatory cytokines plus PGE2 to induce their maturation in vitro (45), their potential for use in vivo is quite limited. Thus, identifying novel agents that can reproducibly trigger monocyte differentiation in vivo may help to elucidate the mechanisms contributing to DC maturation and at the same time provide novel therapeutic opportunities. We think that activation of the CCL16 system is an important mechanism involved in the maturation/induction of the MoDC response to pathogens and tumors. Furthermore, CCL16 is present in healthy blood but has not yet been associated with any biological function (12). The present study suggests that serum CCL16 could accelerate the maturation and induction of MoDCs at an inflamed site without waiting for the production of proinflammatory cytokines and “license” peripheral DCs that acquire a complete mature phenotype when they reach the LNs (35). Additional studies are needed to elucidate this point. The results of this study, in addition to profiling a biological role for CCL16, show that it is a powerful natural adjuvant of potential use for novel immunotherapeutic strategies against infections and tumors.

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Disclosures

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