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Unique Regulation of CCL18 Production by Maturing Dendritic Cells

Marisa Vulcano,* Sofie Struyf,‡ Patrizia Scapini,§ Marco Cassatella,‡ Sergio Bernasconi,* Raffaella Bonecchi,*§ Angelica Calleri,* Giuseppe Penna,¶ Luciano Adorini,‖ Walter Luini,* Alberto Mantovani,*¶ Jo Van Damme,‡ and Silvano Sozzani‡*§

Dendritic cells (DC) orchestrate the trafficking of lymphocytes by secreting chemokines with different specificity and function. Chemokines are produced at higher levels by mature DC. This study shows that CCL18 is one of the most abundant chemokines produced by immature DC. In contrast to all other chemokines investigated to date, CCL18 was selectively down-regulated during the maturation process induced by LPS, TNF, CD40 ligand, Staphylococcus aureus Cowan I, Candida albicans, and influenza virus. IL-10 and vitamin D₃, two known inhibitors of DC differentiation and function, strongly promoted CCL18 secretion, whereas IFN-γ, a costimulator of DC function, inhibited its production. IL-10 also induced CCL18 secretion in blood myeloid DC. No CCL18 secretion was observed in blood plasmacytoid DC. The opposite pattern of regulation was observed for CCL20, a prototypic inflammatory chemokine. CCL18 was found to be a chemotactic factor for immature DC. Therefore, CCL18 may act as a chemotactic signal that promotes the colocalization of immature DC with naive T lymphocytes in an IL-10-dominated environment with the consequent generation of T regulatory cells. These characteristics suggest that CCL18 may be part of an inhibitory pathway devoted to limiting the generation of specific immune responses at peripheral sites. The Journal of Immunology, 2003, 170: 3843–3849.

Dendritic cells (DC) are potent APC with a unique ability to induce T and B cell responses (1–3). DC reside in an immature state in peripheral tissues where they exert a sentinel function for incoming Ags (2, 4). Following encounter with Ags, in the context of an inflammatory situation, or upon direct stimulation by specific pathogenic, DC undergo a process of maturation that enhances their APC functions and promotes their migration to the draining lymph nodes (5, 6). In the secondary lymphoid organs, mature DC prime naïve T cells (7–9). The proper localization of DC in secondary lymphoid organs and their recruitment at sites of inflammation in response to chemotactic stimuli are critical for an optimal immune response (10, 11).

DC are not only potent initiators of immune responses, they also play an important regulatory role. DC can induce either a Th1 cytotoxic response or favor a Th2 humoral-polarized response (12–14). Furthermore, DC can function as tolerogenic cells in response to self or environmental Ags (5, 15, 16). Recent evidence indicates that the microenvironment (e.g., type of Ag, pathogen, and cytokines) present at the site of DC activation will determine the quality of the T cell response generated (17–20).

Chemokines are a large family of chemotactic proteins that play a crucial role in regulating leukocyte composition in inflamed tissues (6, 8, 21–23). DC secrete high levels of several chemokines (24, 25). DC-derived chemokines are believed to contribute to the recruitment of precursor cells and immature DC at the peripheral sites of inflammation (6, 8, 22) and within the lymph nodes, where they play a role in T and B cell localization and the DC-T cell interaction (2, 8). Chemokine production is usually associated with DC maturation both in vitro and in vivo (24–26). However, a limited number of chemokines, such as macrophage-derived chemokine/CCL22, TARC/CCL17, and PARC/DC-CK1/macrophage inflammatory protein-4/CCL18, are secreted in a constitutive manner by immature monocyte-derived DC and blood myeloid DC (24, 25, 27–29) (M. Vulcano, unpublished observations). Whereas several studies have investigated the up-regulation of CCL22 and CCL17 in maturing DC, the regulation and the role of CCL18 in DC biology are poorly understood. CCL18 is a chemokine active on naïve T cells and B lymphocytes with no known receptor identified to date and no rodent homologues (28, 30–33). CCL18 mRNA expression was reported in monocytes, DC, normal lung, pneumo-myositis-affectted lungs, germinal centers of regional lymph nodes and tonsils, atherosclerotic plaques, inflamed liver, septic rheumatoid arthritis, and dermis of contact hypersensitivity patients (28, 30, 31, 33–38). Recent work has identified CCL18 as the major chemokine produced by tumor-associated macrophages in ovarian carcinoma (39). The aim of this study was to investigate the regulation of CCL18 production at both the mRNA and protein levels in human DC at different stages of maturation. In addition, CCL18 chemotactic activity on DC migration was investigated. The results reported here outline a unique pattern of regulation for CCL18 in maturing DC, and we propose that this chemokine is an inhibitory signal in the control of the immune response.

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3 Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; Dex, dexamethasone; M-DC, myeloid DC; P-DC, plasmacytoid DC; SAC, Staphylococcus aureus Cowan I; ViD₃, vitamin D₃.
Materials and Methods

Cell culture media and reagents

The following reagents were used for tissue culture: pyrogen-free saline (S.A.L.F., Bergamo, Italy), RPMI 1640 (Biochrom, Berlin, Germany), and aseptically collected FCS (HyClone Laboratories, Logan, UT). All reagents containing <0.125 endotoxin units/ml, as checked by the Limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD), LPS from Escherichia coli strain 055:B5 (LPS) was obtained from Difco (Detroit, MI), and Staphylococcus aureus Cowan I (SAC) was purchased from Calbiotech (San Diego, CA). Inactivated influenza virus strain A/Moscow/1999 was a gift from Dr. T. D. Magistris (Institute for Viral Pathogenesis, Rome, Italy). Candida albicans was obtained as previously described (40). Dexamethasone (Dex), PGE2, and 1,2-dihydroxyvitamin D3 (Vitamin D3) were obtained from Sigma (St. Louis, MO). Human rGM-CSF was a gift from Novartis (Milan, Italy). Human IL-13 was a gift from Dr. A. Minty (Elf Bio Recherches, Labege, France). Human TNF-α was obtained from BAS (Ludwighafen, Germany). Human rIL-10 was purchased from Schering-Plough (Milan, Italy), and human rIFN-γ was obtained from Rousell-UCLAF (Paris, France). Neutralizing mAb against TNF-α (B154,2) and an isotype-matched control were provided by Dr. G. Trinchieri (Scherin-Plough, Dardilly, France). Anti-IL-10 mAb (23738.11) was obtained from R&D Systems (Minneapolis, MN) and was used as previously described (41). Anti-TNF-α and anti-IL-10 mAbs had undetectable endotoxin levels by the Limulus amebocyte lysate assay.

Monocyte-derived DC preparation

DC were generated as previously described (24). Briefly, highly enriched blood monocytes (>95% CD14+) were obtained from buffy coats (through the courtesy of the Centro Trasfusionale, Ospedale Civil Formaroli, Magenta, Italy) by Ficoll (Biochrom) and Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Monocytes were cultured for 6 days at 1 × 10^6/ml in six-well tissue culture plates ( Falcon; BD Biosciences, Franklin Park, NJ) in RPMI 1640 supplemented with 10% FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-13. Where indicated, DC were further cultured in the presence of 100 ng/ml LPS and 20 ng/ml TNF for 48 h or as otherwise specified. CD40 ligand (CD40L)-transfected J558L cells or mock-transfected J558L control cells were cultured with DC at a 1:4 ratio. Incubation of DC with the J558L mock-transfected cells did not induce cell maturation or chemokine production (data not shown).

Peripheral blood DC purification and culture

PBMC were isolated from buffy coats by Ficoll gradient (Pharmacia Biotech, Uppsala, Sweden), and peripheral blood myeloid (M-DC) and plasmacytoid (P-DC) DC were magnetically sorted with BDCA-1 and BDCA-4 cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, as previously described (42) to a purity of 95–98%. Blood M-DC and P-DC (2 × 10^6 cells/well) were cultured in 96-well plates (Costar, Cambridge, MA) in RPMI 1640 culture medium supplemented with 5% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 1% nonessential amino acids plus 1000 U/ml GM-CSF and 10 ng/ml IL-4 (BD PharMingen, San Diego, CA) or 20 ng/ml IL-3 (BD PharMingen, respectively). Where indicated, cells were stimulated with 10 ng/ml IL-10 or 1 μg/ml LPS, SAC (1/5000), CD40L-transfected J558L cells at a ratio of 4:1, 6 μg/ml CpG oligonucleotides 2006 (MWG Biotech, Ebersberg, Germany), and 20 ng/ml hemagglutinin-inactivated influenza virus strain A/Moscow/10/99. After 24 h of culture, supernatants were collected, and chemokine concentrations were measured by ELISA.

Northern blot analysis

DC total RNA was extracted by the guanidinium thiocyanate or TRIzol method, blotted, and hybridized as previously described (43). Probes were obtained as previously described (80°C. The CCL20-speci.
CD40L and LPS stimulated CCL20 expression, with TNF-α being a marginal, if any, agonist for this chemokine. In contrast, all three maturation agents inhibited CCL18 production after 48 h of stimulation, although with different potencies: CD40L > LPS > TNF (71, 65, and 43% inhibition, respectively). mRNA levels, as evaluated by Northern blot analysis using specific probes, paralleled the chemokine protein expression (Fig. 2, top and bottom, lower panels). Down-regulation of CCL18 production during DC maturation was also observed when other pathogen-derived maturing agents (SAC, C. albicans, and influenza virus) were used (Table I). Maturation of DC is associated with TNF secretion (50). To assess the contribution of this potential autocrine loop in LPS- and CD40L-induced CCL18 inhibition, experiments were performed in the presence of a TNF-α-blocking mAb (51). Results obtained with three different donors showed no effect of the anti-TNF Ab on CCL18 production by both immature and mature DC (data not shown).

Divergent regulation of CCL18 production by IFN-γ and IL-10

The maturation and function of DC are controlled by pro- and anti-inflammatory cytokines. For instance, IFN-γ is known to promote IL-12 production by mature DC, whereas IL-10 inhibits DC maturation and IL-12 production (4, 12, 52). IL-12 production by DC is pivotal to orient T cells to a Th1-polarized phenotype, whereas in the presence of IL-10, T cells with Th2/T regulatory functions are generated (20). Fig. 3A shows that reduced levels of CCL18 are secreted by immature DC in the presence of IFN-γ (46 and 56% inhibition (p < 0.01) at 24 and 48 h, respectively). The degree of the inhibition was almost comparable to that observed following LPS-induced maturation. No additive effect was observed when LPS and IFN-γ were used together. In contrast, IL-10 strongly increased CCL18 secretion. The effect was already apparent after 24 h of stimulation (60% increase over control) and was increased further after 48 h (Fig. 3B). Despite the fact that IL-10 is known to inhibit LPS function in phagocytic cells, including DC, IL-10 could not reverse LPS-induced inhibition of CCL18 production (Fig. 3B). In the same experimental conditions CCL20 was increased further after 48 h (Fig. 3B).

Table I. **Down-regulation of CCL18 production by pathogen-derived maturing stimuli**

<table>
<thead>
<tr>
<th>DC Treatment</th>
<th>CCL18 (% inhibition)</th>
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<tbody>
<tr>
<td>LPS</td>
<td>46.2 ± 9.6b</td>
</tr>
<tr>
<td>SAC</td>
<td>56.5 ± 9.5b</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>47.2 ± 2.5b</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>19.6 ± 16</td>
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DC were treated with LPS (100 ng/ml), SAC (1/5000), *C. albicans* (100 μg/ml), and 20 ng/ml hemagglutinin-inactivated influenza virus strain A/Moscow/10/99. Culture supernatants were collected after 24 and 48 h of stimulation, and CCL18 levels were detected by ELISA. The data shown represent the percent inhibition of CCL18 production with respect to immature DC (100% inhibition corresponds to 170 ± 26 and 312 ± 47 ng/10⁶ DC CCL18 at 24 and 48 h, respectively). Results are expressed as the mean ± SD of three independent experiments. The ability of these stimuli to promote DC maturation was assessed in parallel as the expression of CD83 at 24 h of culture (% CD83⁺ DC, 89 ± 5, 78 ± 3, 82 ± 6, and 29 ± 8 for LPS, SAC, *C. albicans*, and virus, respectively).

b p < 0.05 vs iDC.
expression was induced by IFN-γ and inhibited by IL-10 (data not shown) as previously reported (48).

Recent evidence indicates that endogenous IL-10 production might regulate DC maturation and functions (41). To rule out a possible role of endogenous IL-10 in the constitutive CCL18 release by immature DC, experiments were performed in the presence of a blocking IL-10 Ab. Results obtained with three different DC cultures excluded a role of endogenous IL-10 in CCL18 production (data not shown).

**Regulation of CCL18 secretion by inhibitors of DC maturation**

The results obtained with IL-10 were extended to other drugs known to affect different aspects of DC maturation and function, such as VitD3, corticosteroids, and PGE2. VitD3 and Dex block DC maturation (e.g., CD83 expression and IL-12 production) and inhibit chemokine production (e.g., CCL22, CXCL8, and CCL2), whereas neither M-DC nor P-DC produced CCL18 in a constitutive manner; following in vitro culture with a number of maturing agonists, including LPS, SAC, influenza virus, CpG, and CD40L; or in the presence of VitD3 (data not shown). However, in agreement with the results obtained with monocyte-derived DC, IL-10 was able to selectively up-regulate CCL18 production in M-DC, with 19 ± 4 and 103 ± 17 ng/10⁶ DC after 24- and 48-h culture, respectively (n = 3). On the contrary, no CCL18 production was observed by IL-10-stimulated P-DC (data not shown).

**Production of CCL18 by blood M-DC and P-DC**

The studies on CCL18 production were extended to circulating DC subsets. Neither M-DC nor P-DC produced CCL18 in a constitutive manner; following in vitro culture with a number of maturing agonists, including LPS, SAC, influenza virus, CpG, and CD40L; or in the presence of VitD3 (data not shown). However, in agreement with the results obtained with monocyte-derived DC, IL-10 was able to selectively up-regulate CCL18 production in M-DC, with 19 ± 4 and 103 ± 17 ng/10⁶ M-DC after 24- and 48-h culture, respectively (n = 3). On the contrary, no CCL18 production was observed by IL-10-stimulated P-DC (data not shown).

**Migration of immature DC to CCL18**

Finally, the ability of CCL18 to induce chemotaxis of DC was investigated. Fig. 6 shows that CCL18 was able to induce chemotaxis of immature DC with a bell-shaped dose-response curve. At

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**FIGURE 3.** Regulation of CCL18 production by IFN-γ and IL-10. DC (10⁶/ml) were incubated in medium alone (control) or in the presence of VitD3 (10⁻⁷ M) for 4 h and then stimulated with LPS (100 ng/ml) for 48 h. Supernatants were tested by ELISA. Results are the average determinations (±SD) of three independent experiments. *, p < 0.01; #, p < 0.05 (vs control (-)).

**FIGURE 4.** Induction of CCL18 in immature DC by VitD3. A, DC (10⁶/ml) were incubated in medium alone (control) or in the presence of VitD3 (10⁻⁷ M) for 4 h and then stimulated with LPS (100 ng/ml) for 48 h. Supernatants were tested by ELISA. Results are the average determinations (±SD) of three to seven independent experiments. The average (100%) CCL18 (by immature DC) and CCL20 (by mature DC) productions were 566 ± 55 and 1.8 ± 0.6 ng/10⁶ DC, respectively. B, Total RNA was isolated from immature DC and mature DC that had been stimulated with LPS or CD40L for 48 h. The expression of VitD3 receptor (VD3R) mRNA was analyzed by RT-PCR as described in Materials and Methods. *, p < 0.01 vs control (-); #, p < 0.05 vs LPS.
the peak of chemotactic response the number of migrated DC was similar to that observed in the presence of an optimal concentration of CCL3 (43). Because the optimal CCL18 concentration slightly varied (between 100 and 300 ng/ml) using two different commercially available CCL18 preparations, results were confirmed using natural purified CCL18 (39). Fig. 6 shows that natural CCL18 was also active as a chemotactic agonist for immature DC, with an optimal concentration of 100 ng/ml. No migration of mature DC was induced by any of the three CCL18 preparations, indicating that the still unknown CCL18 receptor must be inactivated/down-regulated during the DC maturation process (data not shown).

Discussion
DC are located at the interface of innate and specific immunity. At their immature stage DC reside in nonlymphoid organs, and after the capture of an Ag within an inflammatory context, DC undergo maturation and migrate to secondary lymphoid organs (2–6, 8). The DC contribution to the onset and evolution of the immune response is represented by their ability to release large amounts of chemokines, some of them with a predominant proinflammatory function (e.g., CCL2 and CXCL8) (21, 56), and others more involved in leukocyte positioning and cell-cell interaction (e.g., CCL19 and CCL22) (10, 24, 57). In this study we investigated the regulation of CCL18 (a chemotactic factor for naïve T lymphocytes and CD38- B cells (28, 58)) in both immature and mature DC. The secretion of CCL20, an inflammatory chemokine active on memory T cells and B lymphocytes (47, 49), was examined in parallel studies for comparison. This study reports two main findings. First, CCL18 and CCL20 undergo opposite regulation during DC maturation. CCL18 is down-regulated during DC maturation, whereas the maturation process induces CCL20 as well as all other chemokines investigated to date (59). Second, CCL18 is a chemotactic agonist for immature DC.

CCL18 is released at high levels by immature DC (387 ± 66 ng/10^6 DC in 48 h). Therefore, CCL18, together with CCL22, CCL17, and CXCL8, represents one of the most abundantly secreted chemokines by DC (24, 25). CCL18 inhibition was detectable after 8 h and was observed with all maturation agonists used (i.e., LPS, TNF, CD40L, SAC, C. albicans, and influenza virus) and in the presence of IFN-γ, a costimulator of DC functions (12). Previous studies performed by RT-PCR reported a limited induction of CCL18 mRNA in 24-h LPS-stimulated DC (25). However, in the present study the decrease in CCL18 protein secretion was paralleled by the inhibition of CCL18 mRNA levels, as evaluated by Northern blot, a more quantitative analysis than RT-PCR. The reason for this discrepancy is at present uncertain. To our knowledge, this is a unique feature, since only one other chemokine, CXCL13, was shown to be selectively inhibited at the mRNA level by CD40L, but not by LPS or TNF stimulation (60). Incubation of immature DC with Dex and PGE2 also resulted in a partial decrease in CCL18 secretion. The blocked acquisition of the DC mature phenotype by these two agents did not reverse CCL18 inhibition. Conversely, up-regulation of CCL18 secretion by immature DC was observed in the presence of VitD3 and IL-10, two known suppressors of DC maturation and function (52, 53). VitD3 is an immunosuppressive agent active in autoimmune diseases and graft rejection models by inhibiting Ag-induced T cell proliferation, cytokine production, and Th1 development (61, 62). IL-10 is an anti-inflammatory cytokine produced in a constitutive way at the mucosal sites and involved in the inhibition of inflammatory and immune reactions in many pathological conditions, including tumors (63). It is interesting to note that Dex was reported to enhance IL-4-induced expression of CCL18 in human monocytes (31). In the same experimental system CCL18 was induced by IL-10. CCL18 was recently identified as the most abundant chemokine in human ovarian ascites. CCL18 was produced by tumor-associated macrophages, very likely in response to tumor- and macrophage-derived IL-10 (39). The regulation of CCL18 production in DC reported in this study strengthens the concept that CCL18 is a mediator of the alternative activated (or type II) APC response (64). Cytokines and drugs associated with polarized type II responses (e.g., IL-4, IL-10, corticosteroids, and VitD3) induce

![Figure 5](http://www.jimmunol.org)  
**FIGURE 5.** CCL18 and CCL 20 production is differentially regulated during alternative pathways of DC maturation. DC (10^6/ml) were incubated in the presence of Dex (10^{-8} M) or PGE2 (10^{-5}M) for 4 h and then stimulated with LPS (100 ng/ml), CD40L, or medium alone (control) for 48 h. Supernatants were tested by ELISA. Results are the average determinations (±SD) of three independent experiments. Protein production in supernatants (not shown).

![Figure 6](http://www.jimmunol.org)  
**FIGURE 6.** Migration of immature DC in response to CCL18. Immature DC (iDC) were assayed for their migratory capacity toward recombinant (R&D Systems or PeproTech) and purified natural CCL18 (39). The migration assay was performed as indicated in Materials and Methods. Results are expressed as the mean (±SD) of three replicates of a single experiment and are representative of at least three independent donors. Basal migration, against medium (12 ± 2 cells), was subtracted from the data. The dashed line indicates the number of migrated cells in response to 100 ng/ml CCL3. *, p < 0.05; **, p < 0.01 vs medium (no chemokine).
an alternative activation program with distinct functional properties. These functionally polarized cells play a key role in the subversion of adaptive immunity and in inflammatory circuits that promote tolerance and tumor progression (65).

There is evidence that stimulation of naive T lymphocytes with immature DC induces the generation of IL-10-producing T regulatory cells (20). Since CCL18 is chemotactic for both immature DC and naive T cells, CCL18 production by immature DC could promote the interaction of these two cell types. Finally, a recent study by Nibbs et al. (66) showed that CCL18, in addition to being a T cell chemotaxant, exhibits antagonistic activity for CCR3, a chemotactic receptor for eosinophils and basophils. Taken together, these findings strongly suggest that at nonlymphoid sites CCL18 may not act as a proinflammatory chemokine, but, rather, it may represent a negative mechanism to limit the onset of immune reactions. CCL18 antagonistic activity for CCR3 may serve to antagonize local production of proallergic chemokines. Furthermore, the recruitment of naive T cells by immature DC may favor a tolerogenic condition. However, in the presence of a severe inflammatory situation, DC will be induced to mature with the consequent down-regulation of CCL18 production. This CCL18 homoeostatic function might be increased in the presence of immunosuppressive, anti-inflammatory cytokines, such as IL-10, or during immunosuppressive pharmacological treatments, as in the case of VitD3 or corticosteroid administration. Therefore, CCL18 production by immature DC could be part of a default strategy to limit the uncontrolled generation of inflammatory reactions. Unfortunately, CCL18 was generated by the fusion of two macrophage inflammatory protein-1α-like genes, an event that happened late in evolution (32, 33). No rodent CCL18 exists, and this makes it impossible to test this hypothesis in an in vivo experimental model. In conclusion, chemokines, like other proinflammatory cytokines, possess strategies to limit their own inflammatory potential (67). This goal is exploited by alternative strategies, such as the production of chemokines with antagonist activity (66, 68, 69), the generation of structural decoy chemokine receptors (67), or the production of chemokines, such as CCL18, that are able to limit the onset of an immune response.

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