

Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site

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Abstract

Dendritic cells (DC) are highly motile antigen-presenting cells that are recruited to sites of infection and inflammation to antigen uptake and processing. Then, to initiate T cell-dependent immune responses, they migrate from non-lymphoid organs to lymph nodes and the spleen. Since chemokines have been involved in human DC recruitment, we investigated the role of chemokines on mouse DC migration using the mouse growth factor-dependent immature DC line (D1). In this study, we characterized receptor expression, responsiveness to chemoattractants and chemokine expression of D1 cells during the maturation process induced by lipopolysaccharide (LPS). MIP-1 α and MIP-5 were found to be the most effective chemoattractants, CCR1 was the main receptor expressed and modulated during LPS treatment, and MIP-2, RANTES, IP-10 and MCP-1 were the chemokines modulated during DC maturation. Thus, murine DC respond to a unique set of CC and CXC chemokines, and the maturational stage determines the program of chemokine receptors and chemokines that are expressed. Since CCR1 is modulated during the early phases of DC maturation, our results indicate that the CCR1 receptor may participate in the recruitment and maintenance of DC at the inflammatory site.

Introduction

Dendritic cells (DC) are a specific subset of antigen-presenting cell (APC) capable of initiating primary T cell-mediated immune responses with high efficiency (1). They are generated from bone marrow precursors and seeded through the blood into non-lymphoid tissues, where they remain in a state which is defined as immature. This state is characterized by high efficiency in antigen sampling and processing, and a poor T cell stimulatory function. During the early phases of the acute inflammatory response or after the administration of cytokines and lipopolysaccharide (LPS) (2–8), DC undergo maturation, with subsequent trafficking from non-lymphoid to

lymphoid tissues where they may encounter and stimulate naive T cells (9). The maturation process coincides, indeed, with profound functional changes. That is, the loss of ability to capture antigen and the acquisition of the ability to stimulate T cells. Although DC migration is a well-established phenomenon, little is known about the factors which regulate it.

The exit of leukocytes from the vasculature is a complex process. Leukocytes migrate into extravascular tissue via a three-step mechanism involving selectins, chemoattractants and integrins (10). Chemoattractants play a central role in this process by directing leukocytes to inflammatory sites (11).

Chemokines comprise a growing family of proteins able to promote chemotaxis on different cell types. Member of the CXC subfamily (including IL-8 and NAP2) are mainly active on neutrophils; whereas the CC subfamily members (including MCP-1, MCP-2, MCP-3, MIP-1 α , MIP-1 β and RANTES) are monocyte and lymphocyte chemoattractants. The specific effects of chemokines on target cell types are mediated by a family of G-protein-coupled seven TM receptors. Sixteen chemokine receptors have been identified so far; five of which are specific for CXC chemokines (CXCR1–5) (12–14) the others being specific for CC chemokines (CCR1–9) (15–19). Most of these receptors bind more than one chemokine at high affinity with the exception of CXCR1, which is specific for IL-8, and CXCR4, which so far appears to be specific for SDF-1.

In this study we investigated the role of chemokines in the migration of DC during the maturation process. For this, we used the immature, splenic D1 DC line generated in our laboratory and fully characterized (20). The D1 cells are maintained in an immature state by the addition of a conditional medium containing granulocyte macrophage colony stimulating factor (GM-CSF) (20). They can be induced to maturation by adding inflammatory stimuli or bacterial products. Here we show that murine DC respond to a determined set of CC and CXC chemokines, and that migration in response to the chemokines tested is inhibited upon maturation. Moreover, the expression of chemokine receptors and chemokine production are influenced by LPS activation at early and late time points.

Methods

DC and culture medium

The D1 culture medium was IMDM (Sigma, St Louis, MO) containing 10% heat-inactivated FBS (Gibco), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (all from Sigma) and 50 mM 2-mercaptoethanol (complete IMDM) with 30% conditioned medium (CM) from GM-CSF-producing NIH 3T3 cells (R1).

RNase protection assay

Total RNA was extracted from D1 cells using TRIZOL reagent (Gibco). Multiprobe template sets mCK5 (containing DNA templates for Linfotactin, RANTES, Eotaxin, MIP-1 β , MIP-1 α , MIP-2, IP-10, MCP-1, TCA3, L32 and GAPDH) and mCR5 (containing DNA templates for CCR1, CCR1b, CCR3, CCR4, CCR5, CCR2, and the housekeeping gene products L32 and GAPDH) were purchased from PharMingen (San Diego, CA). The DNA templates were used to synthesize the [α - 32 P]UTP (3000 Ci/mmol, 10 mCi/ml; Amersham, Little Chalfont, UK)-labeled probes in the presence of a GACU pool using a T7 RNA polymerase (Promega, Madison, WI). Hybridization with 5–15 μ g of each target RNA was performed.

Cytokines

Human recombinant IL-8 was from Daiippon (Osaka, Japan) and human SDF-1 was from R & D System (Minneapolis, MN). Synthetic MIP-5/HCC2 and MDC were kind gifts of Dr T. Wells (Serono Pharmaceuticals Research Institute,

Geneva, Switzerland) and Dr M. Sassano (Technogen, Caserta, Italy). Human recombinant MCP-3, MIP-1 α , MIP-1 β and MIP-3 α , and murine recombinant RANTES, lymphotactin and eotaxin were from Peprotec (Rocky Hill, NJ). Cytokines were endotoxin-free as assessed by the limulus amebocyte assay. *N*-formyl methionyl leucylphenylalanine (fMLP), C5a and lipopolysaccharide (LPS; *Escherichia coli* 026:B6) were from Sigma.

Migration assay

Cell migration was evaluated using a chemotaxis micro-chamber technique as previously described (21). Twenty-seven microliters of chemoattractant solution or control medium (RPMI 1640 with 1% FCS) were added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA). A polycarbonate filter (5 μ m pore size; Neuroprobe) was layered onto the wells, and covered with a silicon gasket and a top plate. Fifty microliters of cell suspension (1.5×10^6 /ml) were seeded in the upper chamber. The chamber was incubated at 37°C in humidified atmosphere in the presence of 5% CO₂ for 90 min. At the end of the incubation, filters were removed, stained with Diff-Quik (Baxter, Rome, Italy) and high-power fields ($\times 100$) were counted. Results are expressed as the mean number of migrated cells in five high-power fields or as a chemotactic index (number of cell migrated in response to an agonist/basal migration). Each experiment was performed in triplicate.

Calcium mobilization assay

Changes in calcium concentrations were monitored using the fluorescent probe Fura-2 as previously described (22) following the technique reported by Grynkiewicz *et al.* (23). Briefly, DC (10^7 /ml) were resuspended in RPMI 1640 and incubated with 1 μ M Fura-2 acetoxymethyl ester (Calbiochem, San Diego, CA) at 37°C for 20 min. After incubation, cells were washed and resuspended in HBSS (Biochrom) containing 1.2 mM CaCl₂ and kept at room temperature until used. The untreated cells were loaded with Fura-2 and immediately challenged with MIP-1 α . The cells treated with LPS were first stimulated for 30 min, and 1, 2, 3 and 24 h. In the last 20 min the cells were collected labeled with Fura-2 and challenged with MIP-1 α . Fura-2 fluorescence was measured in a Perkin-Elmer LS 50B spectrophotometer (Perkin-Elmer, Norwalk, CT) at 37°C with cells ($3\text{--}5 \times 10^6$ /ml) continuously stirred. Samples were excited at 340 and 380 nm, and emission was continuously recorded at 487 nm.

Results

Chemotactic response during DC maturation induced by LPS

D1 cells were derived from mouse spleens and maintained as immature by using a CM containing GM-CSF. The cells can be induced to maturation by adding bacterial products such as LPS or bacteria (20,24).

The addition of LPS leads to the typical phenotypical and functional changes observed during DC maturation: D1 cells up-regulate MHC class II and co-stimulatory molecules, increase their capacity to stimulate T cells, and lose the function of antigen uptake and processing. Since it has been

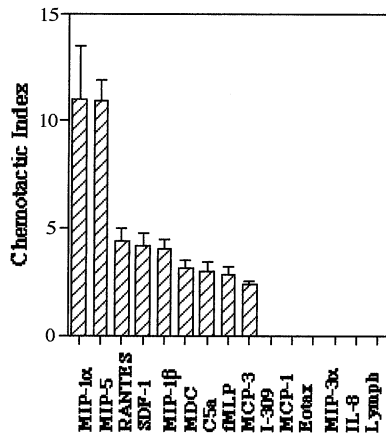


Fig. 1. Chemotaxis of D1 cells in response to chemokines and 'classic' chemotactic agonists. D1 (1×10^6 /ml) were tested for their ability to migrate across a 5 μ m pore-sized polycarbonate filter in response to different chemotactic agonists. At the end of the incubation (90 min), the number of cells in five high-power microscope fields was evaluated. Chemokines were used at a concentration of 100 ng/ml, with the exception of MDC used at 1 ng/ml. fMLP was used at a concentration of 10^{-7} M. Results are expressed as a chemotactic index (CI; stimulated/basal migration) and are the average \pm SE of three to five independent experiments.

proposed that inflammatory stimuli and bacterial products allow DC to migrate and that chemokines are implicated in the regulation of this phenomenon in human DC (11), we have investigated the chemotactic response of immature and mature mouse D1 cells using a microwell chemotaxis chamber assay. Figure 1 shows the results obtained with immature D1 cells using the chemotactic agonists, CXC, CC and C chemokines. MIP-1 α and MIP-5, two CC chemokines, were the most effective chemotactic signals, with respective chemotaxis indexes of 11.0 and 10.9. A second group of agonists, including the CC chemokine MIP-1 β , RANTES, MCP-3 and MDC, SDF-1 (CXC) and the two 'classic' chemotactic agonists, fMLP and C5a, were also effective in inducing dose-dependent D1 cell migration. However, their efficacy (number of cells migrating at the optimal concentration) was at most 40% of that observed with MIP-1 α .

IL-8 (CXC), MCP-1, I-309, eotaxin and MIP-3 α (CC), and lymphotactin (C) were inactive both in immature (Fig. 1) and mature DC (data not shown). These results indicate that CC chemokines and classical chemotactic factors are the main chemotaxanes for immature D1 cells *in vitro*. Similar results have been previously obtained with human DC (11,25,26).

We next investigated the chemotactic response in mature D1 cells. For this purpose they were stimulated with 10 μ g/ml LPS for 24 h and then used in a chemotactic assay (Fig. 2). D1 cells did not change their basal migration. However, they completely (>90%) lost the ability to migrate in response of CCR1 and CCR5 ligands such as MIP-1 α , MIP-5, MIP-1 β and RANTES. Chemotactic response to SDF-1 (CXCR4) and to MDC was also completely inhibited. Cell migration to fMLP, a chemotactic agonist of bacterial origin that interacts with an unrelated receptors, was completely inhibited by LPS treatment (data not shown).

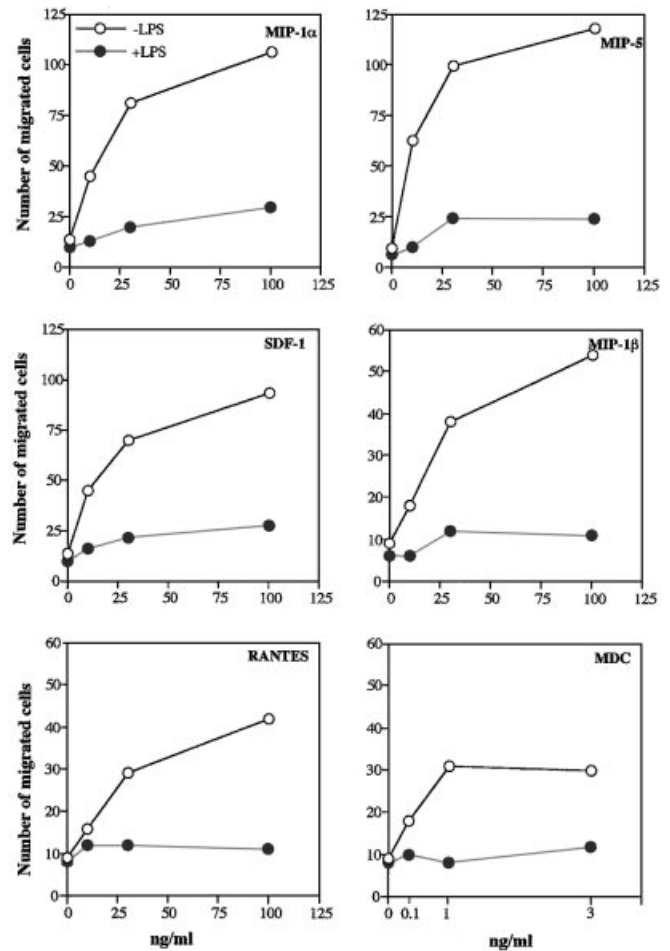


Fig. 2. Effect of cell maturation on D1 chemotactic responses. D1 cultured for 24 h in the presence of 10 μ g/ml of LPS and then tested for their ability to migrate in response to chemokines. Results of one experiment representative of two are shown.

Chemokine receptor expression

To assess the impact of inflammatory stimuli on chemokine receptor expression, we treated D1 cells with LPS at 10 μ g/ml. We performed a kinetic study by measuring the level of chemokine receptor mRNA expression by RNase protection assay at times of 30 min, and 1, 2, 3 and 24 h of LPS activation as described in Methods. Immature D1 cells expressed a high mRNA level for CCR1 receptor, and a very low level for CCR1b, CCR3, CCR4 and CCR5 (Fig. 3A). The pattern of CCR1b, CCR3, CCR4 and CCR5 expression did not increase after LPS treatment, although CCR4 seemed to be up-regulated at 24 h. However, the level of expression was low compared to CCR1 mRNA.

Interestingly, LPS was able to modulate CCR1 mRNA expression; we observed that the CCR1 mRNA was expressed in immature DC (time 0), up-regulated within 30 min and 1 h of LPS treatment, and slowly down-modulated after 3 h (Fig. 3A). Moreover, we found a strong down-regulation of CCR1 mRNA at 24 h when the cells were fully mature. Therefore, CC chemokine receptors expressed by D1 cells

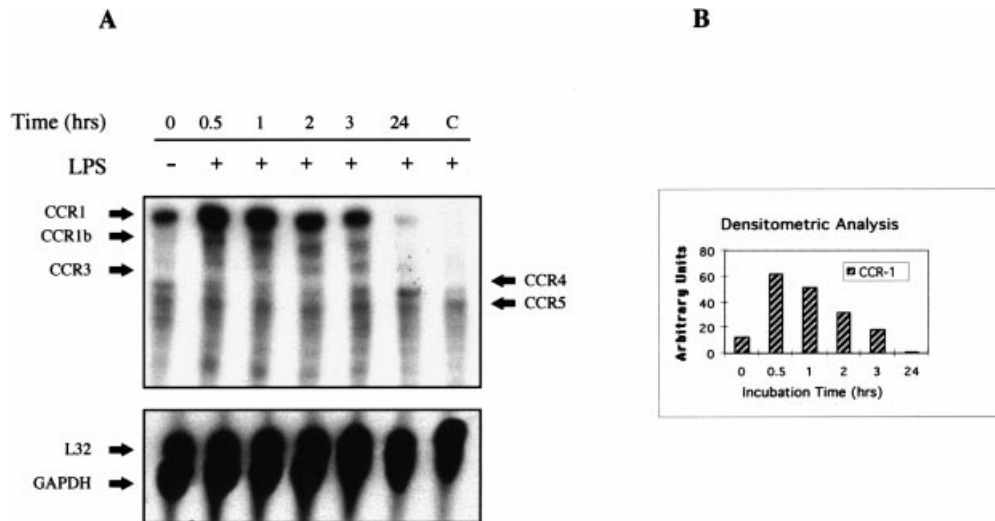


Fig. 3. Chemokine receptor expression. (A) Chemokine receptor message was determined by RNase protection assay using the multiprobe template set mCR5 (see Methods). CCR1 is expressed on immature D1 cells (time 0) and is up-regulated by LPS after 30 min. CCR1b CCR3, CCR4 and CCR5 have also been detected. (B) The graph shows the quantification by densitometric analysis of the CCR1 receptor.

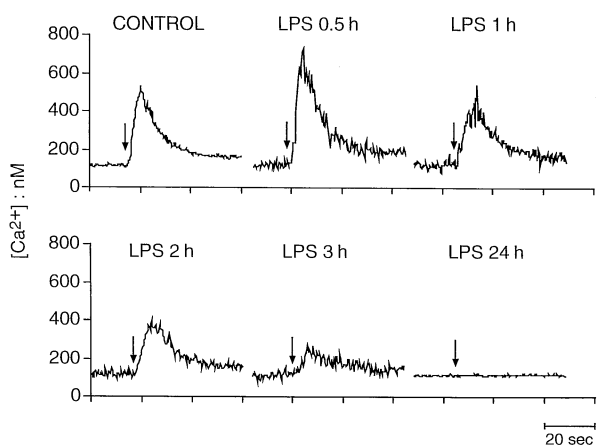


Fig. 4. Calcium influx measurement. DC (10^7 /ml) loaded with $1 \mu\text{M}$ Fura-2 D1 cells were stimulated with 100 ng/ml MIP- 1α . Results are expressed as a ratio of fluorescence of two excitation wavelengths (340 and 380 nm) and emission at 487 nm.

correlated well with the biological activity observed with the different ligands tested.

Densitometric analysis of the RNase protection assay was performed (Fig. 3B). Maximal expression of the CCR1 gene is detected within 30 min and 1 h; the mRNA level at 30 min showed an increase of ~6-fold compared to the untreated cells. At time of 1 and 2 h we observed an increase of ~5- and 3-fold respectively till reaching a 1.5-fold increase in relation to the untreated cells (time 0).

Calcium mobilization assay

We next investigated whether CCR1 chemokine receptor was functional on DC by monitoring increases in cytosolic calcium in response to chemokines. The results are summarized in Fig. 4. Figure 4 shows calcium flux changes upon stimulation of the CCR1 receptor with the main agonist MIP- 1α . The

maximal increase in the intracellular calcium response was observed at 30 min after LPS treatment. The calcium level ranged from ~500 to 700 nM. Interestingly we measured a decrease in the calcium concentration from 700 to 500 nM at 1 h when the receptor message is still highly expressed.

The down-modulation of the calcium levels was more evident at times of 2 and 3 h where the concentration measured reached the value of 200 nM. We were unable to detect any calcium flux at 24 h.

The very early up-regulation of CCR1 receptor activity suggests that during inflammatory responses this molecule is probably crucial for recruiting and maintaining DC at the inflammatory site. Thus, immature DC, which reside in peripheral tissues, modulate the CCR1 receptor immediately after contact with inflammatory stimuli to remain at the site of inflammation in order to sample and process antigens. It is likely that DC acquire the ability to leave peripheral tissues when they down-modulate the CCR1 receptor.

Chemokine expression kinetic induced by LPS

The modulation of the functional activity of the CCR1 receptor prompted us to investigate molecular events underlying this phenomenon. In previous studies it has been shown that rapid successive exposure to the same agonist desensitizes the signaling capacity of G-protein-coupled receptors (27). Since we observed a decrease in CCR1 activity starting 1 h after LPS activation when CCR1 mRNA is still highly expressed (Fig. 3A), we characterized the endogenously chemokines produced by DC during maturation. We performed another series of experiments in which we looked at chemokine gene expression during LPS treatment. Again, we performed a RNase protection assay on D1 cells with induction of maturation at different time points. As shown in Fig. 5A, immature D1 cells expressed RANTES and MIP- 1α mRNAs, whereas mature DC also expressed detectable levels of IP-10 and MCP-1 mRNA which were not present in any of the maturation stages tested. The addition of LPS induced an early change

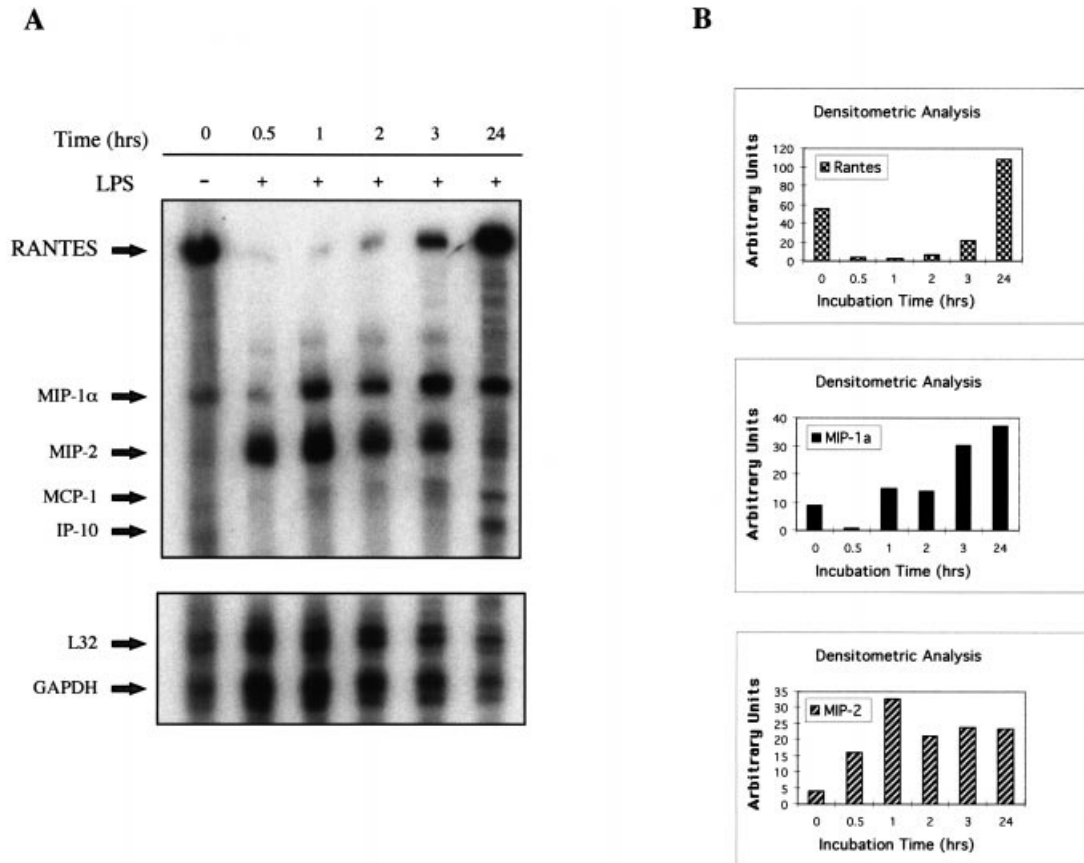


Fig. 5. D1 expression of chemokines. (A) D1 cells were cultured in the absence (time 0) or presence of LPS for the indicated time points. The chemokine message was determined by RNase protection assay using the multiprobe template set mCK5. The mRNAs for RANTES, MIP-2, IP-10 and MCP-1 are modulated during LPS treatment. (B) Quantification by densitometric analysis of RANTES (top panel), MIP-1 α (middle panel) and MIP-2 (bottom panel). The data have been normalized against the housekeeping genes. These results are representatives of three independent experiments.

of chemokine expression; RANTES mRNA expression was strongly down-regulated within 30 min and 1 h (Fig. 5A). A detectable level of RANTES mRNA have been observed after 3 h and reached the maximum level of expression after 24 h. In contrast, MIP-2 mRNA was undetectable in the immature cells and expressed at a low level in the mature D1 cells treated for 24 h, but its expression was strongly up-regulated within 30 min and 3 h. Densitometric analysis of this gel is shown in Fig. 5B. Interestingly, after 30 min of LPS treatment, we observed both a strong down-regulation of RANTES and MIP-1 α expression (the main CCR1 agonists) (Fig. 5A) and the highest level of CCR1 activity as detected by calcium flux experiment (Fig. 4). The inverse correlation between chemokine expression and receptor activity was striking. After 1 h of activation, MIP-1 α is expressed at a high level; this is paralleled by the concomitant expression of the CCR1 receptor whose functionality is rapidly modulated. This result suggests that a desensitization process may occur, likely due to endogenous chemokine production.

MIP-1 α has been purified from a LPS-treated monocyte cell line, and it has been shown to be constitutively expressed in LC (28,29) and in DC treated with CD40 (30). Nevertheless, kinetic studies have not been performed. Here we show that

indeed there is a down-regulation of MIP-1 α mRNA at time of 30 min after LPS treatment in D1 cells.

Taken together, these data suggest that DC are attracted to the site of inflammation very rapidly, and that they are triggered to produce a large amount of chemokines—probably to attract other DC (in the case of MIP-1 α) or neutrophils (in the case of MIP-2). It is tempting to speculate that the chemokines produced so early during the process of maturation act as an autocrine loop to arrest the DC that are needed in the inflammatory site and to recall other DC or other cells types to the site of inflammation to amplify the cascade of immune responses.

Discussion

Among the APC population, DC appear to have the capacity to activate naive helper and cytotoxic T cells with maximal efficiency (1). In the last decade DC have attracted the interest for their potential use as therapeutic agents. They normally reside in peripheral tissues where they function as 'sentinels' for the immune system. They take up and process antigens, migrate to lymphoid organs, and express co-stimulatory molecules that mediate T cell binding and T cell

activation. The migratory pattern of DC has been extensively studied (31–34) and although there is information available regarding the pathways of DC migration, the molecular mechanism controlling this phenomenon is less defined. We have used the immature growth factor-dependent DC line, D1, functionally characterized (20), to investigate the role of different chemokines and chemokine receptors in DC migration during the maturation process.

In this study we show that, among the CC chemokines tested, MIP-1 α and MIP-5, which are the main CCR1 agonists, were the most effective chemotactic signals. MIP-1 β , RANTES, MDC and SDF-1 (CXC) also elicited chemotactic migration in D1 cells. In contrast, the CXC chemokine IL-8 was not effective, and neither were the CC chemokines I-309, MCP-1, Eotaxin, MIP-3 α and Linfotactin. These results are similar to those obtained with human DC, although with some exceptions. DC obtained from different sources show a different pattern of responsiveness. CD34⁺ cell derived DC respond to MCP-1, MCP-2, MIP-1 α , MIP-1 β and RANTES (25), whereas monocyte-derived DC respond to the same chemokines but not to MCP-1 and MCP-2 (11,21). This discrepancy is likely to be related to the cell source and to the degree of DC maturation. All these factors may contribute to the different responsiveness of the cells to a given chemokine.

Chemokines are known to bind to G-protein-coupled receptor, and it has been shown that LPS can modulate chemokine and chemokine receptor expression (35). Thus we measured the expression of these genes during early and late time points of LPS stimulation. Immature D1 cells express a high level of the CCR1 chemokine receptor mRNA, whereas mRNA for CCR1b, CCR3, CCR4 and CCR5 was hardly detectable. The CCR1 mRNA is rapidly up-regulated within 3 h and it is down-regulated at 24 h, when the DC are supposed to be fully mature. We showed that the receptor is functional at an early time point of LPS treatment—as demonstrated from the calcium flux experiment—but not at 24 h. These data indicate that DC modulate the expression of CCR1 receptor in the early maturation stage. Immature DC are rapidly recruited to the sites of inflammation and are highly proficient at antigen uptake. Subsequently, they are again mobilized and migrate to draining lymph nodes. During this process of maturation, they lose their ability to process and present soluble antigens, and become potent stimulators of T lymphocytes (36–39). Since we have observed CCR1 down-regulation at 3 h of stimulation, we believe that the antigen-loaded DC down-modulate the receptor expression to leave the site of inflammation.

It is important to point out that there are fundamental differences between the structure and function of the chemokine system in mice and humans. For example, the mouse has an orthologue of the human IL-8 receptor subtype CXCR2 (40), but counterparts of IL-8 itself and another human IL-8 receptor subtype CXCR1 have not been identified. Also mouse and human neutrophils appear to differ in their use of CCR1. They both express this receptor and respond to its agonist with calcium flux. However, MIP-1 α does not attract human neutrophils *in vitro* (41), whereas it is a potent chemoattractant for mouse neutrophils (42). It may be that the CCR1

receptor in the murine cellular system is more important to compensate the absence of the CXCR1 on the mouse cells.

We have also characterized the chemokine profile produced by murine DC during maturation. Immature DC express mRNA for RANTES and MIP-1 α . During the process of maturation induced by LPS, D1 express a large amount of MIP-2 mRNA after as early as 30 min of LPS treatment. MIP-2 gene expression is known to be activated by LPS (43,44) and it has been described as one of the most potent chemoattractants for neutrophils (45). MIP-1 α is constitutively expressed in the immature and mature cells, but its expression is down-regulated at 30 min and again up-regulated at the other time points tested. We show that immature DC are able to migrate in response to exogenous MIP-1 α . It is likely that the amount of the chemokine produced by D1 cells is lower than the threshold level needed for the activity. In the untreated cells this chemokine is indeed produced and it may affect the calcium response we observed at time 0. At 30 min, both RANTES and MIP-1 α are down-regulated, and at that time receptor activity is maximal. This effect was almost due to the production of MIP-1 α message because pretreatment with MIP-1 α desensitizes the receptor if challenged with RANTES, the reverse is not true: pretreatment with RANTES does not abrogate calcium signaling in response to a second challenge with MIP-1 α (46–48). The data on RANTES should not surprise, because several observations suggested that RANTES interacts differently with CCR1, than does MIP-1 α . RANTES was shown to be a much less potent agonist than MIP-1 α , having a threshold for calcium mobilization at concentrations >100 nM (46).

Although MIP-1 α has been described as attracting and activating human monocytes, T lymphocytes and other human cell types, in the mouse system MIP-1 α may be an important neutrophil chemoattractant (41). Because MIP-1 α is highly expressed by DC and is also chemotactic for them, it may play an autocrine role in their accumulation at sites of inflammation. Thus, chemokines are likely to play a central role in the initial influx of immature DC. In contrast, RANTES, which is known to attract T lymphocytes (49), is down-regulated in the initial phase of inflammation but it is strongly up-regulated at 24 h when the cell are fully mature and are supposed to be in the lymphoid organs. Mature DC also produce the transcripts for MCP-1 and IP-10, chemokines that are known to be active on monocytes and T cells (50). These data are consistent with the idea that mature DC produce chemokines locally to attract T lymphocytes for antigen presentation and therefore the late activation of the adaptive immune response. These results suggest that DC not only up-regulate their chemokine receptor in order to be immediately recruited to the site of inflammation, but they also release chemotactic factors to attract other cell types, namely neutrophils and maybe other DC, to the site of inflammation. Thus DC undergo to a flexible program in the regulation of chemokine and chemokine receptors. This program may have a different impact depending on the site of DC localization and the stage of DC maturation. Thus, we propose that DC represent an important link between innate and acquired immunity.

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Abbreviations

APC	antigen-presenting cell
CM	conditioned medium
DC	dendritic cell
fMLP	<i>N</i> -formyl methionyl leucylphenylalanine
GM-CSF	granulocyte macrophage colony stimulating factor
LPS	lipopolysaccharide

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