

Distinct patterns and kinetics of chemokine production regulate dendritic cell function

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Dendritic cells (DC) have been showed to both produce and respond to chemokines. To understand how this may impact on DC function, we analyzed the kinetics of chemokine production and responsiveness during DC maturation. After stimulation with LPS, TNF- α or CD40 ligand, the inflammatory chemokines MIP-1 α , MIP-1 β and IL-8 were produced rapidly and at high levels, but only for a few hours, while RANTES and MCP-1 were produced in a sustained fashion. The constitutive chemokines TARC, MDC and PARC were expressed in immature DC and were up-regulated following maturation, while ELC was produced only at late time points. Activated macrophages produced a similar spectrum of chemokines, but did not produce TARC and ELC. In maturing DC chemokine production had different impact on chemokine receptor function. While CCR1 and CCR5 were down-regulated by endogenous or exogenous chemokines, CCR7 levels gradually increased in maturing DC and showed a striking resistance to ligand-induced down-regulation, explaining how DC can sustain the response to SLC and ELC throughout the maturation process. The time-ordered production of inflammatory and constitutive chemokines provides DC with the capacity to self-regulate their migratory behavior as well as to recruit other cells for the afferent and efferent limb of the immune response.

Key words: Dendritic cell / Chemokine / Chemokine receptor / Migration

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1 Introduction

There is growing evidence that chemokines and their G protein-coupled receptors play an important role in positioning leukocytes for the afferent and efferent limbs of the immune response [1, 2]. Chemokines can be broadly divided into two categories. Firstly, inflammatory chemokines, induced or up-regulated by inflammatory stimuli, are responsible for recruiting cells involved in inflammatory reactions. Secondly, constitutive chemokines, produced in bone marrow, thymus and secondary lymphoid organs, are responsible for the homeostatic control of

leukocyte traffic and for driving the encounter of cells that need to interact to generate an immune response [3]. Dendritic cells (DC) behave as the sentinels of the immune system [4, 5]. Their function is to sample antigens in inflamed tissue and migrate to the local lymph nodes, where they present these antigens to T cells. The capacity of DC to migrate first to sites of inflammation and then to the draining lymph nodes can be understood in terms of a switch in the expression of chemokine receptors [6–11]. Immature DC express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6 and CXCR1, which guide them to inflammatory sites where antigen sampling can take place and maturation can be induced. The maturation process, which is triggered by inflammatory cytokines, bacterial and viral products, leads to down-regulation of receptors for inflammatory chemokines and up-regulation of receptors for constitutive chemokines such as CXCR4 and CCR7. These receptors may drive the maturing DC first towards the lymphatics and then into T cell areas within the lymph nodes, where the CCR7 ligands secondary lymphoid tis-

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Abbreviations: SLC: Secondary lymphoid tissue chemokine ELC: EBI1-ligand chemokine PARC: Pulmonary and activation-regulated chemokine MIP: Macrophage inflammatory protein MCP: Monocyte chemoattractant protein MDC: Macrophage-derived chemokine RANTES: Regulated on activation of normal T cell expressed and secreted TARC: Thymus and activation-regulated chemokine

sue chemokine (SLC) and EBI1-ligand chemokine (ELC) are produced [12–14]. DC not only respond to, but also produce chemokines. DC have been reported to produce both constitutive chemokines such as DC-CK1/pulmonary and activation-regulated chemokine (PARC) [15], macrophage-derived chemokine (MDC) [16, 17], thymus and activation-regulated chemokine (TARC) and ELC [8, 14] as well as inflammatory chemokines such as macrophage inflammatory protein (MIP)-3 α [18], MIP-1 α and monocyte chemoattractant protein (MCP)-1 [8]. The relative role played by these different chemokines may underline the capacity of DC to recruit other cells as well as to regulate the function of their own chemokine receptors. We report here that, upon induction of maturation, DC produce inflammatory and constitutive chemokines in a time-ordered fashion. At early time points large amounts of inflammatory chemokines are produced, whereas at later time points constitutive chemokines are selectively up-regulated. These findings demonstrate that chemokine production is instrumental for DC to regulate their own migratory capacities and to organize recruitment of different cell types for both the afferent and efferent limb of the immune response.

2 Results

2.1 Inflammatory and constitutive chemokines are produced with distinct kinetics by maturing DC

Immature DC were generated from human peripheral blood monocytes using GM-CSF plus IL-4 [19] and were tested for their capacity to produce inflammatory chemokines in response to various maturation stimuli. MIP-1 α , MIP-1 β , and RANTES were measured in the culture supernatant at different time points after stimulation (Fig. 1). LPS was consistently the most effective stimulus, followed by CD40L, whereas TNF- α was less effective. MIP-1 α and MIP-1 β were produced very rapidly within 6 h after stimulation, while RANTES was produced in a more sustained fashion, since it continued to accumulate up to 24 h. Strikingly, a single DC was able to produce an extremely large amount of MIP-1 β following LPS stimulation (> 1 pg/h and 7 pg in 12 h).

As expected, these different kinetics of chemokine production were detected at the mRNA level (Fig. 2A). MIP-1 α and MIP-1 β mRNAs were strongly up-regulated at 3 h after LPS stimulation, but returned to basal levels at later time points. In contrast, RANTES mRNA was induced rapidly, but remained at high levels even at late time points. MIP-1 α , MIP-1 β and RANTES mRNA were also induced in monocytes and macrophages stimulated with LPS or *Streptococcus pyogenes*, respectively. In addi-

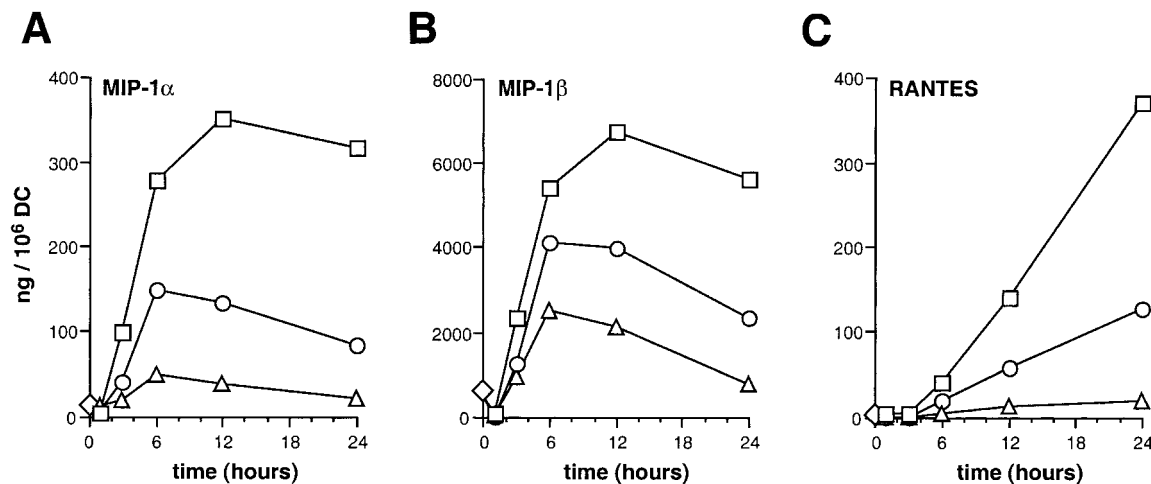


Figure 1. Production of inflammatory chemokines following induction of DC maturation. Immature DC (10^6 /ml) were stimulated with LPS (squares), TNF- α (triangles) or CD40L (circles) and the concentration of MIP-1 α (A), MIP-1 β (B) and RANTES (C) was measured at different times in the culture supernatant by ELISA. The diamond indicates chemokine levels in the 24-h culture supernatant of unstimulated cells. The data are from one representative experiment out of three.

tion, IP-10 was induced in a sustained fashion in DC only, whereas IL-8 was induced transiently in all three cell types (Fig. 2B).

The production of constitutive chemokines was analyzed by semiquantitative reverse transcription (RT)-PCR (Fig. 3), a method which detected the expected changes in mRNA for inflammatory chemokines. mRNA for the constitutive chemokines PARC, MDC and TARC were already present in immature DC and were up-regulated by LPS stimulation, but only at late time points. In contrast, ELC mRNA was absent in immature DC and was up-regulated 10–30 h after LPS stimulation. The comparison between DC, monocytes and macrophages revealed consistent differences in the production of the constitutive chemokines (Fig. 3). DC produced PARC, MDC and TARC, macrophages PARC and MDC, and monocytes only PARC. In addition, ELC was not detected in macrophages, even at late time points, but it was detected in monocytes at 30 h, although at low levels.

Taken together these results indicate that upon maturation DC produce both inflammatory and constitutive chemokines, each with distinctive kinetics. In addition, the production of the CCR7 ligand ELC and the CCR4 ligand TARC appears to be restricted to DC.

2.2 Chemokine production impacts on chemokine receptor function

It has been reported that DC maturation results in a switch in responsiveness from inflammatory to constitutive chemokines [7–11]. The kinetics of this process was analyzed by measuring the calcium response to two prototypic chemokines: MIP-1 α , which signals through CCR1 and CCR5, and ELC, which signals through CCR7. As shown in Fig. 4, immature DC were highly responsive to MIP-1 α , but lost responsiveness as early as 1 h after LPS stimulation. Immature DC, instead, did not respond to ELC, but gradually acquired responsiveness starting at 6 h after stimulation and remained so for up to 50 h (Fig. 4, and data not shown). These results

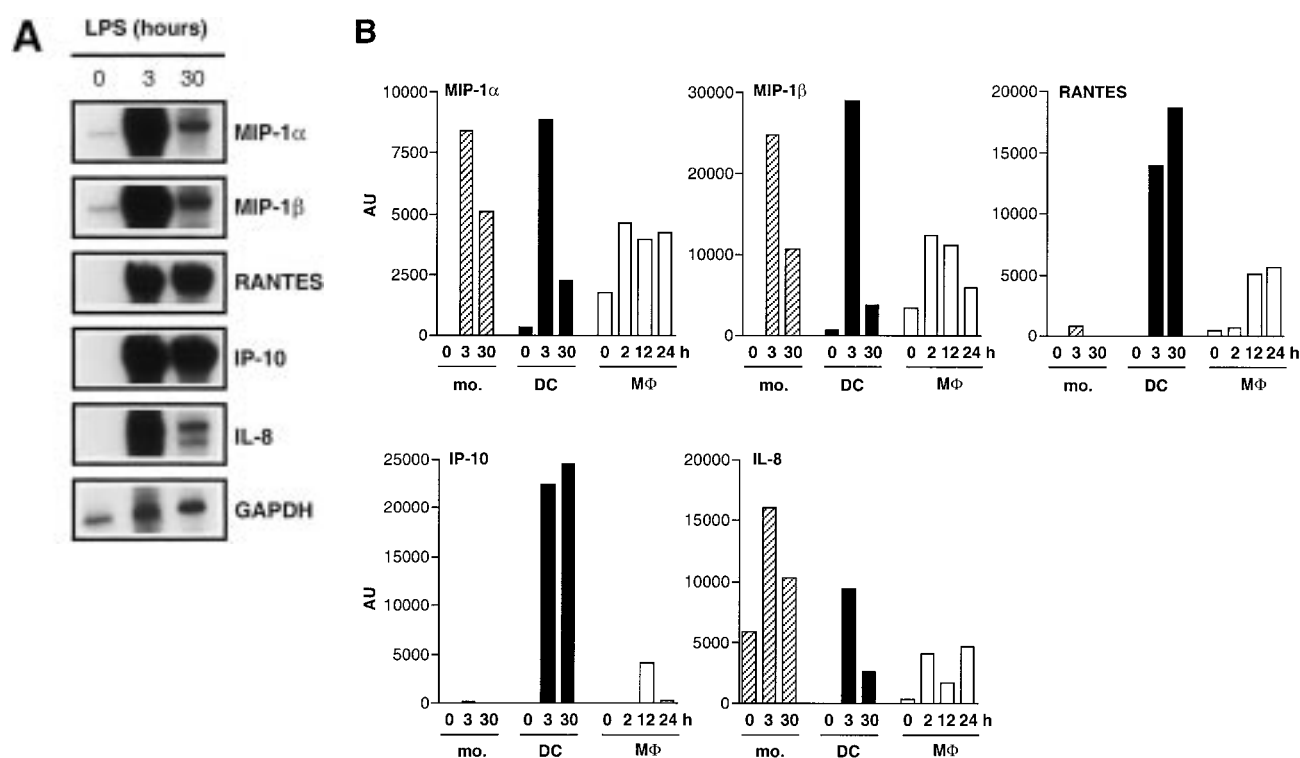


Figure 2. Inflammatory chemokine mRNA levels in DC, macrophages and monocytes following stimulation with LPS or bacteria. mRNA levels for six inflammatory chemokines were measured at different times by RNase protection assay on monocytes and DC stimulated with LPS or macrophages stimulated with *S. pyogenes*. The bands were quantified by phosphorimager and normalized to the level of GAPDH. *S. pyogenes* was superior to LPS in stimulating macrophages. The data are from one representative experiment out of two.

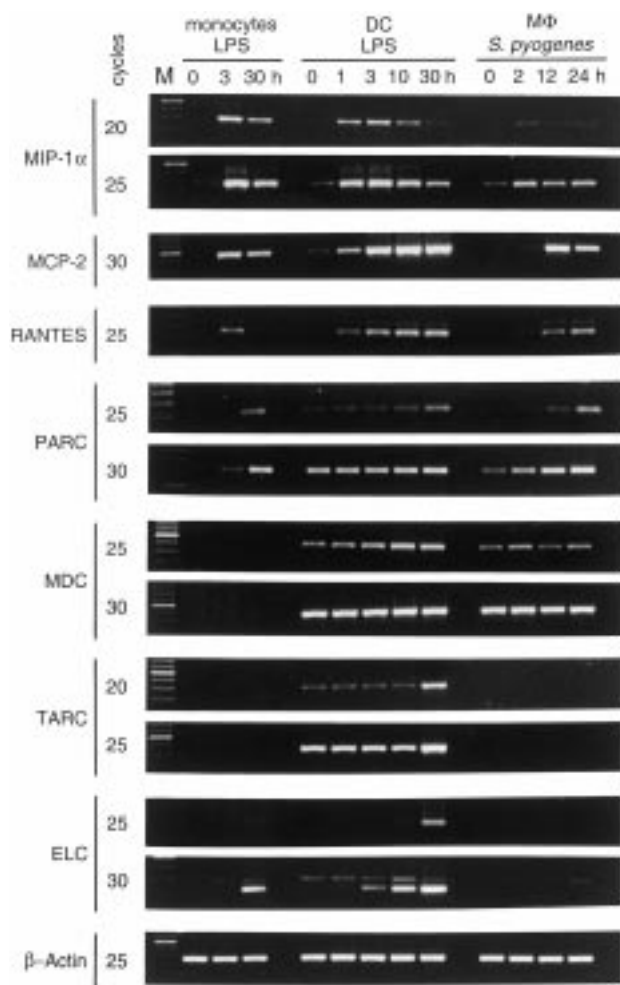


Figure 3. Patterns and kinetics of inflammatory and constitutive chemokines following stimulation with bacterial products in DC as compared to macrophages and monocytes. Immature DC and monocytes were stimulated with LPS, while macrophages were stimulated with *S. pyogenes*. Chemokine expression was measured before and at different times after stimulation by RT-PCR. The number of PCR cycles is indicated. One representative experiment out of five with DC and two with monocytes and macrophages is shown. ELC production was not detected in LPS-stimulated macrophages, even at late time points.

indicate that the function of CCR1 and CCR5 is rapidly lost, while the function of CCR7 is gradually acquired and maintained during the maturation process. We have previously shown that upon maturation CCR1 and CCR5 are rapidly down-regulated and this process is inhibited by brefeldin A or cycloheximide [8]. This suggested that, by producing chemokines, DC down-regulate the cognate receptors in an autocrine fashion. Because maturing DC produce chemokines which bind to CCR1, CCR5 and CCR7, it was important to assess how this produc-

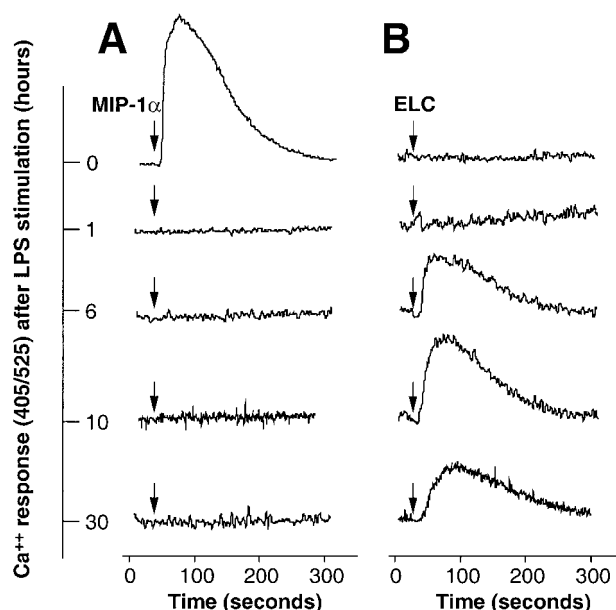


Figure 4. Switch in responsiveness to MIP-1 α and ELC in maturing DC. DC were tested before and at different times after LPS stimulation for their capacity to flux calcium in response to MIP-1 α (A) or ELC (B) both at 300 ng/ml.

tion might affect receptor function, and in particular how DC could stop responding to MIP-1 α , while continuing to respond to ELC.

The surface and total levels of CCR5 and CCR7 were measured at different times after stimulation with LPS on intact and permeabilized cells, respectively (Fig. 5). Surface CCR5 was rapidly down-regulated to ~30 % of the initial values 2 h after LPS stimulation and remained at low levels for up to 50 h. Total CCR5 levels, however, were not changed throughout the culture period, indicating that the receptor was redistributed from the cell surface to the endocytic compartment. In contrast, surface CCR7 levels steadily increased in maturing DC from 10 to 50 h, even at times when DC were producing ELC. Interestingly, CCR7 was present mainly on the cell surface, and its intracellular fraction actually decreased during the culture period, paralleling the redistribution of class II molecules from intracellular compartments to the cell surface [20].

The above results suggest that CCR1 and CCR5 may be more susceptible to ligand-induced down-regulation than CCR7. To test this possibility we challenged DC at 37 °C or 4 °C with different concentrations of chemokines and measured receptor levels after 1 h. As shown in Fig. 5, in immature DC, MIP-1 α induced a complete down-regulation of CCR1 at a concentration of 30 ng/ml. In contrast, in mature DC, ELC failed to induce CCR7

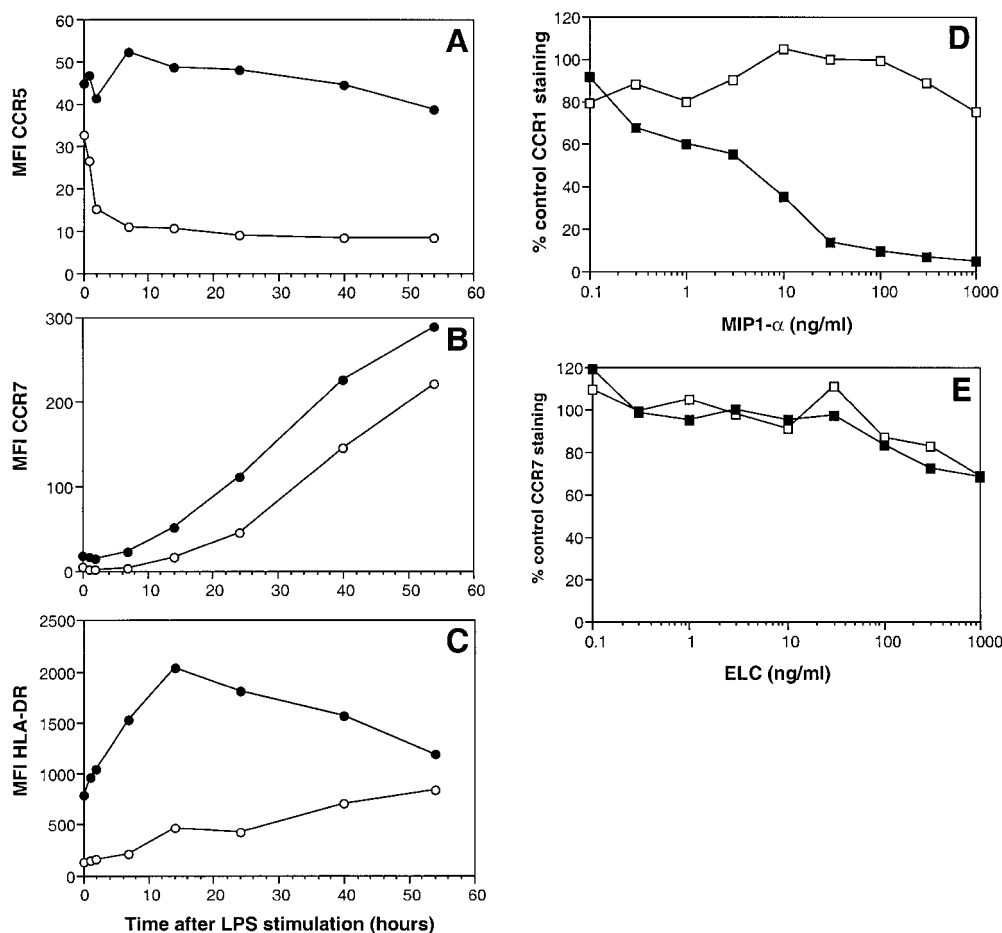


Figure 5. Differential distribution of CCR5 and CCR7 in maturing DC and different sensitivity to down-regulation. (A–C) Immature DC were stimulated with LPS and stained with antibodies to CCR5 (A), CCR7 (B) or MHC class II (C) before (empty circles) or after permeabilization (filled circle). (D) Surface CCR1 levels (expressed as % of control) in immature DC incubated for 1 h at 37 °C (filled squares) or 4 °C (empty squares) with different concentrations of MIP-1 α . (E) Surface CCR7 levels in LPS-matured DC incubated for 1 h at 37 °C (filled squares) or 4 °C (empty squares) with different concentrations of ELC. In control experiment a calcium signal was detected with less than 10 ng/ml of both MIP-1 α and ELC.

down-regulation, even at concentrations of 3000 ng/ml, since the staining was comparable in cells treated at 37 °C or 4 °C (Fig. 5). We conclude that CCR7, unlike other chemokine receptors, is resistant to ligand-induced down-regulation, thus explaining how DC can sustain the response to ELC.

3 Discussion

The time-ordered production of inflammatory chemokines early on and of constitutive chemokines at later time points, represents a key feature of DC physiology with implications for antigen sampling, migration and T cell priming.

We have shown that immature DC produce large amounts of inflammatory chemokines in response to maturation stimuli. MIP-1 α , MIP-1 β and IL-8 are expressed rapidly and at high levels but also transiently, whereas MCP-1, MCP-2, IP-10 and RANTES are up-regulated in a more sustained fashion. The amount of MIP-1 β produced can be as much as 1 pg/cell/h, indicating that chemokines are among the most abundant proteins produced. The production of inflammatory chemokines by maturing DC is likely to occur early on when DC are still in peripheral tissues and, thus, may contribute to recruitment of two cell types: (i) APC such as immature DC or their precursors, and (ii) effector cells such as macrophages, granulocytes and T cells. While the first may be important to enhance and sustain antigen sampling, the second may be important to initiate the effec-

tor phase of the immune response. The fact that whole bacteria or LPS are more potent than TNF- α in inducing production of inflammatory chemokines suggests that the nature of the maturation stimulus may determine the extent of leukocyte recruitment in peripheral tissues. It is possible that the production of inflammatory chemokines at late time points, when DC have already reached the lymph nodes, may enhance recruitment of particular cell types or co-stimulate T cell responses [21]. Like DC, macrophages also produce significant amounts of inflammatory chemokines that may contribute to the recruitment process.

ELC binds with high fidelity to CCR7 [22] and is produced by DC [14]. We have shown here that ELC is produced only by mature DC 10–30 h after induction of maturation. Thus, it is likely that DC will produce ELC only after homing to the T cell areas of secondary lymphoid organs [23]. Here, ELC production may allow mature DC to attract naive T and B cells, which constitutively express CCR7, as well as maturing DC, which also express CCR7. The constitutive chemokines TARC and MDC bind to CCR4 [24, 25] and PARC binds to a yet-unknown receptor expressed on naive T cells [15, 26]. These three chemokines have previously been identified as DC products [8, 15, 16]. We have confirmed these findings and shown that they are all produced by immature DC and up-regulated by the maturation process, but only at late time points. Moreover, only TARC was found to be DC-specific since MDC and PARC were also detected in macrophages or monocytes. The fact that these chemokines are up-regulated at late time points suggests that, like ELC, they may play an important role in secondary lymphoid organs by recruiting naive T cells which express the receptor for PARC, as well as subpopulations of memory or recently activated T cells which express or up-regulate CCR4 [27–29].

Chemokine production by DC has consequences not only for recruitment of other cell types, but also for the function of the maturing DC themselves, since it results in triggering and down-regulation of the cognate receptors ([8] and this study). Triggering of CCR1 and CCR5 may be important to reorganize the actin cytoskeleton and mobilize DC, while their desensitization may allow DC to exit from the inflammatory sites [30]. The down-regulation of chemokine receptors is known to be mediated through a COOH-terminal serine-rich domain which is phosphorylated by G-protein-coupled receptor kinases (GRKs), resulting in receptor internalization by interaction with β arrestins [31, 32]. This process results in extinction of the response to the ligand [33], as well as interference with HIV infection [34–37]. We have previously shown that in DC CCR1 and CCR5 are down-regulated within 2 h following induction of maturation

and that this process is prevented by brefeldin A and cycloheximide [8]. These results suggested an autocrine mechanism by which the chemokines produced by maturing DC down-regulate the receptors on the same cells. Here we have shown that CCR1 and CCR5 are very sensitive to ligand-induced down-regulation and, in maturing DC, are redistributed to an intracellular compartment. These results confirm the auto-desensitization model that accounts for the rapid loss of responsiveness to the ligand upon DC maturation.

Another key aspect of DC maturation is the up-regulation of CCR7. This chemokine receptor allows maturing DC to migrate from tissues into the lymphatic vessels, where the CCR7 ligand SLC is produced [12] and, once they have reached the lymph node, into T cell areas where the ELC is produced by resident DC [14]. Using a specific antibody we showed that CCR7 is progressively up-regulated and is mainly present on the cell surface, even at late time points when DC produce ELC. Furthermore, CCR7 is not down-regulated even by concentrations of ELC 100 times higher than those required to elicit a calcium response. The striking resistance of CCR7 to down-regulation as well as its steady up-regulation explain how maturing DC can sustain the response to ELC, which is required to complete their migration to the T cell areas of lymph nodes.

4 Materials and methods

4.1 Media and reagents

The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1 % nonessential amino acids, 1 % pyruvate, 50 μ g/ml kanamycin (Gibco, Grand Island, NY) 5×10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 10 % fetal calf serum (FCS, Hyclone Laboratories, Logan, UT). Human recombinant IL-4 and GM-CSF were produced in our laboratory by PCR cloning and expression in the myeloma expression system [38]. MIP-1 α , MIP-1 β , ELC were purchased from Peprotech (London, UK). SLC was kindly provided by G. Garotta, Bethesda, NJ.

4.2 Monocytes, DC, and macrophages

Monocytes were purified by positive sorting using anti-CD14 conjugated magnetic microbeads (Milteny I. Bergisch Gladbach, Germany). The recovered cells were > 99 % CD14 $^{+}$ as determined by flow cytometry with the anti-CD14 antibody TIB228 (IgG2b; American Type Culture Collection, Rockville, MD). DC were generated as previously described [19] by culturing monocytes in RPMI-10 % FCS supplemented with 50 ng/ml GM-CSF and 1000 U/ml IL-4 for 7 days. Macro-

phages were obtained as previously described [39] by culturing monocytes in macrophages SFM medium (Gibco) containing 10 ng/ml GM-CSF for 7 days. Cells were stimulated by addition of either 1 µg/ml LPS (from *Salmonella abortus equi*, Sigma Chemicals, St. Louis, MO), 100 ng/ml TNF-α (R&D System, Minneapolis, MN), or CD40L-transfected J558 cells (at 1:5 ratio, provided by P. Lane, Oxford, GB) or live *S. pyogenes* (serotype T1H32030 at 1:1 ratio).

4.3 FACS® analysis

Cell staining was performed using mAb followed by FITC- or PE-conjugated affinity purified, isotype specific goat anti-mouse or goat anti-rat antibodies (Southern Biotechnology Associates, Birmingham, AL). The following antibodies were used: anti-CCR1 (2D4, mouse IgG1) anti-CCR5 (2D7, mouse IgG2a) (all produced at LeukoSite, Cambridge, MA), anti-CCR7 (rat IgG2a; R. Burgstahler, manuscript in preparation). The samples were analyzed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using propidium iodide to exclude dead cells. In some experiments staining was performed on fixed and permeabilized cells.

4.4 ELISA

MIP-1α, MIP-1β and RANTES were measured in culture supernatants using commercially available kits (R&D System).

4.5 Ca²⁺ flux measurement

DC were loaded with Indo-1 (Sigma) and their response to various concentrations of chemokines was analyzed on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) [40].

4.6 RNase protection assay

Total RNA was extracted using RNeasy B (Tel-Test, Friendswood, TX). Multi-probe template set hCK5 (containing DNA templates for Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, I-309, L32, GAPDH) was purchased from PharMingen. The DNA templates were used to synthesize the [³²P]TUP (3000 Ci/mmol, 10 mCi/ml, Amersham Life Science, Amersham, GB) labeled probes in the presence of a GACU pool using a T7 RNA-polymerase (Promega, Madison, WI). Hybridization with 5–15 µg of each target RNA was performed overnight followed by digestion with RNase A (Boehringer Mannheim, Rotkreuz, Switzerland) and T1 (Calbiochem, La Jolla, CA) according to the PharMingen standard protocol. The samples were treated by proteinase K (Boehringer Mannheim)/SDS mixture and then extracted with chloroform and precipitated in the presence of ammonium

acetate. The samples were loaded on an acrylamide/urea sequencing gel next to labeled DNA molecular weight markers and to the labeled probes, and run at 50 W with 0.5 × TBE. The gel was absorbed to filter paper, dried under vacuum and exposed on Kodak X-AR film with intensifying screens at –70 °C. The bands were also quantified by phosphorimager.

4.7 RT-PCR

Single-strand cDNA was synthesized from 2 µg total RNA using AMV-RT (Promega). Primers used for PCR were: hMIP-1α: 5'-CGAGCCCACATTCCGTCACC-3' and 5'-CGCATGTTCCCAAGGCTCAGG-3' (annealing temperature 60 °C), amplifying a 390-bp product; hMCP-2: 5'-CAGCCAGATTCAGTTTCCATTC-3' and 5'-ACCCCAC-AACACTACAGACAGG-3' (annealing temperature 60 °C), amplifying a 555-bp product; hRANTES: 5'-CCCCGTGCCCCACATCAAGGAGT-3' and 5'-TCAAGGAGCGGG-TGGGGTAGGA-3' (annealing temperature 60 °C), amplifying a 257-bp product; hPARC: 5'-AGTTTCCAAGCCCCAGC-TCACTCT-3' and 5'-TGGGGGCTGGTTTCAGAATAGTCA-3' (annealing temperature 64 °C), amplifying a 208-bp product; hMDC: 5'-GCATGGCTCGCCTACAGACT-3' and 5'-GCA-GGGAGGGAGGCAGAGGA-3' (annealing temperature 64 °C), amplifying a 497-bp product; hTARC: 5'-CCT-CCTCCTGGGGGCTTCTCTG-3' and 5'-GACTTTAATCT-GGGCCCTTTGTGC-3' (annealing temperature 64 °C), amplifying a 445-bp product; hELC: 5'-CACCCCTCCAT-GGCCCTGCTACT-3' and 5'-TAACTGCTGCGGCGC-TTCATCT-3' (annealing temperature 60 °C), amplifying a 304-bp product; β-actin: 5'-ACACTGTGCCCATCT-ACGAGGGG-3' and 5'-ATGATGGAGTTGAAGGTAG-TTTCGTGGAT-3' (annealing temperature 60 °C), amplifying a 340-bp product. cDNA were amplified by PCR using the following conditions: 30 s at 94 °C, 20 s at 60 or 64 °C (as specified for each primer pair) and 30 s at 72 °C. PCR products were harvested after 20, 25 and 30 cycles and resolved on a 1.5 % agarose gel containing ethidium bromide.

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