Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells

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Abstract Upon exposure to immune or inflammatory stimuli, dendritic cells (DC) migrate from peripheral tissues to lymphoid organs, where they present antigen. The molecular basis for the peculiar trafficking properties of DC is largely unknown. In this study, mouse DC were generated from CD34$^+$ bone marrow precursors and cultured with granulocyte-macrophage-CSF and Flt3 ligand for 9 days. Chemokines active on immature DC include MIP1α, RANTES, MIP1β, MCP-1, MCP-3, and the constitutively expressed SDF1, MDC, and ELC. TNF-α-induced DC maturation caused reduction of migration to inducible chemokines (MIP1α, RANTES, MIP1β, MCP-1, and MCP-3) and increased migration to SDF1, MDC, and ELC. Similar results were obtained by CD40 ligation or culture in the presence of bacterial lipopolysaccharide. TNF-α down-regulated CC chemokine receptor (CCR)1, CCR2, and CCR5 and up-regulated CCR7 mRNA levels, in agreement with functional data. This study shows that selective responsiveness of mature and immature DC to inducible vs. constitutively produced chemokines can contribute to the regulated trafficking of DC. *J. Leukoc. Biol.* 66: 489–494; 1999.

Key Words: *CD34-derived DC · chemotaxis · transmigration · chemokine receptors*

INTRODUCTION

Dendritic cells (DC) are bone marrow (BM)-derived professional antigen-presenting cells (APCs). BM and blood DC progenitors seed nonlymphoid tissues, where they develop into immature DC with a high ability to capture antigens. The specific role of DC is to capture, process, and present antigens to T cells and to migrate through tissues to lymphoid compartments where immune responses initiate [1–4]. Immune and inflammatory signals have been shown to induce mobilization of DC from the periphery to lymph nodes or spleen T cell areas: these physiological processes cause a shift from a 'processing' to a 'presenting' stage, characterized by an increased capacity to stimulate T lymphocytes [5–11].

Chemotactic agonists are currently believed to be key effector molecules in the multistep process of leukocyte recruitment into tissues [12–14]. Chemokines are a superfamily of chemotactic proteins divided in four groups on the basis of a cysteine structural motif. The subfamilies of the α (or CXC) chemokines, mainly active on neutrophils and lymphocytes, and of the β (or CC) chemokines, active on multiple subsets of mononuclear cells, including DC, contain most of the chemotactic proteins. Lymphotactin (γ or C chemokines) and fractalkine (δ or CX3C chemokines) are two additional molecules in this superfamily [14, 15]. Most chemokines are inducible proteins that are secreted in response to inflammatory signals [e.g., interleukin 1 (IL-1), tumor necrosis factor (TNF), and endotoxin]. However, a subset of chemokines can also be secreted in an apparent constitutive way and may regulate trafficking of leukocytes under physiological conditions [14–16].

In previous studies it has been reported that a set of chemokines and bioactive lipids are able to induce chemotactic and transendothelial migration in human DC generated *in vitro* [17–28]. The migratory potential of mouse DC to chemotactic stimuli *in vitro* has not been studied.

We investigated the ability of a set of chemokines to induce migration of mouse DC generated *in vitro* from CD34$^+$ bone marrow cells. Moreover, we explored how immune and inflammatory signals, which stimulate the antigen presenting function of DC and concomitantly their trafficking to lymphoid organs, affect DC migration and chemokine receptor expression.

MATERIALS AND METHODS

Cytokines.

All the cytokine and chemokine used were recombinant proteins. Human monocyte chemoattractant protein 3 (MCP-3) was a kind gift from Dr. A. Minty (Sanofi Elf Bio Recherches, Labège, France); human microphage inflammatory protein 1α (MIP-1α), MIP-1β, SDF1, and murine RANTES were from PeproTech Inc. (Rocky Hill, NJ); mouse macrophage-derived chemokine (MDC) was a kind gift from Pat Gray (ICOS Corporation, Bothell, WA), mouse MCP-1 was a kind gift from B. Rollins (Dana Farber Cancer Institute, Boston MA), and EBI1 ligand chemokine (ELC) was a kind gift from Osamu Yoshie (Shionogi Institute for Medical Science, Osaka, Japan). Mouse granulocyte-macrophage-colony-stimulating factor (GM-CSF) and TNF-α were a generous gift from Sandoz (Basel, Switzerland) and BASF (Knoll, Germany), respectively.

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Human Flt3 ligand was a generous gift from Immunex (Seattle, WA). Cytokines were endotoxin free as assessed by Limulus amebocyte assay. Lipopolysaccharide (LPS; *E. coli* 055:B5) was from Sigma (St. Louis, MO).

DC cultures

$CD34^+$ bone marrow cells from femurs and tibias of DBA/2 mice were prepared by positive immunoselection using MACS microbeads coated with goat anti-rat IgG (Miltenyi Biotec Inc. Auburn, CA), using the rat mAb MEC14.7 [29] to mouse CD34 as selecting agent. Cells were separated following the manufacturer's instructions. $CD34^+$ positive cells (2×10^5/mL) were cultured in RPMI1640 medium with 10% fetal calf serum, 2×10^{-5} 2-ME, GM-CSF (40 ng/mL), and Flt3 ligand (100 ng/mL) [30]. Cells were diluted 1:2–1:3 every 2 or 3 days. Cultured cells were collected after 9 days and used in the different assays. DC were characterized in terms of membrane phenotype (expression of DEC205, MHC class II, CD11c, CD86), pinocytosis, and antigen presentation in allogeneic MLR.

Where specified, DC were cultured with 20 ng/mL TNF-α or 1 ng/mL LPS for the last 24 h of culture. J558L cells transfected with the gene encoding CD40L [31] (kindly provided by Dr. Peter Lane, Basel Institute for Immunology, Switzerland) were co-cultured with DC at 1:5 ratio for the last 48 h of culture.

Analysis of the chemokine receptors

For Northern blot analysis, total RNA was extracted by the guanidinium thiocyanate method, blotted, and hybridized as described [18]. Probes were labeled by Megaprime DNA labeling system (Amersham, Buckinghamshire, U.K.) with a^{32}P-dCTP (3000 Ci/mmol, Amersham). CC chemokine receptor 1 (CCR1) cDNA was kindly donated by Dr. Philip Murphy (LHD, NIAID, NIH, Bethesda, MD). CCR2 cDNA was generated by reverse transcriptase-polymerase chain reaction from elicited peritoneal macrophage total RNA. CCR5 and CCR7 cDNA were prepared as described [32, 33]. RNAse protection assays were performed using the mCR-5 and mCR-6 kits, following manufacturer's instructions (PharMingen, San Diego, CA).

Migration assay

Cell migration was evaluated using a chemotaxis chamber (Neuroprobe, Pleasanton, CA) and polycarbonate filter (5 μm pore size; Neuroprobe) as previously described [17]. Fifty microliters of cell suspensions (1.5×10^6/mL) were incubated at 37°C for 90 min. Results are expressed as the mean number of migrated cells in five high-power fields (100×). Each experiment was performed in triplicate.

Transmigration assay

Transendothelial migration was performed in polycarbonate transwell inserts (5 μm pore, Corning, Costar, Cambridge, MA) as previously described [22], with minor modifications. The microvascular mouse endothelial cell line 1G11 [34] was grown as monolayer on fibronectin-coated inserts. ^{51}Cr-labeled DC (5×10^4/well in 0.1 mL) were seeded in the upper compartment and chemoattractants were placed in the lower compartment. After 1 h of incubation at 37°C, the radioactivity in the lower compartment was evaluated. Results are reported as percentage of input, as in the following formula: (cpm in the lower compartment/cpm of the input) × 100.

RESULTS

Basal and maturation-induced chemotactic response of DC to chemokines

Immature DC efficiently migrated in response to the inducible chemokines MIP1α, RANTES, MIP1β, MCP-1, and MCP-3 in the dose range evaluated (10–100 ng/mL) (**Fig. 1**). Immature DC did not migrate in response to eotaxin. MIP1α was consistently the most efficient chemokine for immature DC, evaluated as number of migrated cells. Constitutively produced chemokines such as MDC, SDF1, and ELC were also chemotactic for immature DC (**Fig. 2**); MDC was the most potent stimulus: 1 ng/mL consistently was able to induce migration (1.3- to 1.8-fold the control value) of immature DC. Data reported in Figures 1 and 2 are from one representative experiment of the three to five performed with each chemokine.

Previous studies have shown that inflammatory cytokines (e.g., IL-1 and TNF-α), microbial products (e.g., LPS), and CD40 ligation induce DC maturation [1–4]. The effect of DC maturation induced by TNF-α exposure for 24 h was next evaluated. TNF-α caused a marked decrease in the chemotactic response of DC to MIP1α, RANTES, MIP1β, MCP-1, and MCP-3 (Fig. 1). TNF-α-exposed DC became weakly responsive to eotaxin (Fig. 1): the effect, though small, was consistently seen in all three experiments performed. TNF-α-induced matu-

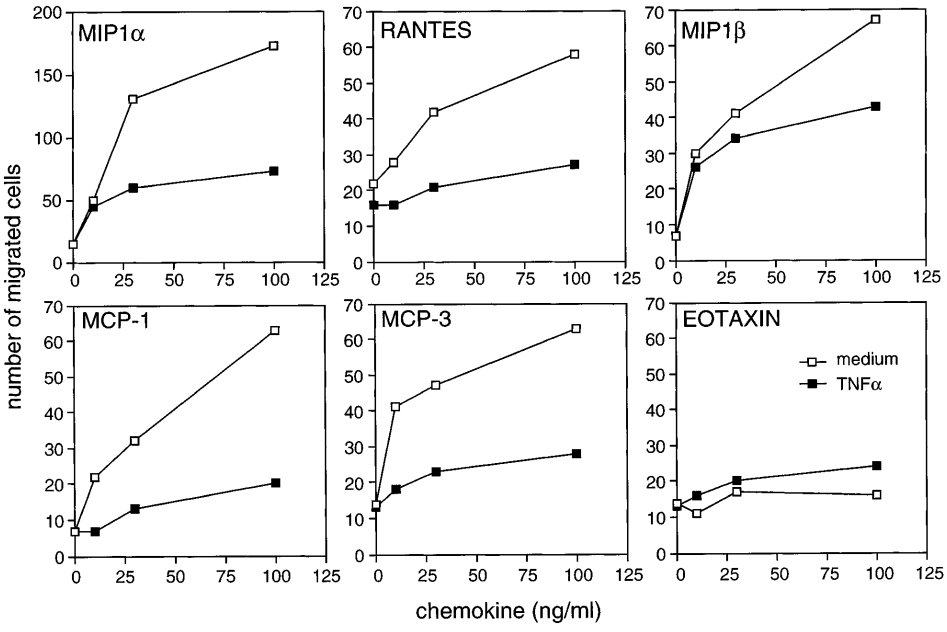

Fig. 1. Effect of inducible chemokines on chemotaxis of immature and mature DC. Migration of DC cultured with medium or TNF-α (20 ng/mL for 24 h) was evaluated using a polycarbonate filter in a chemotaxis chamber with different stimuli in the lower compartment and 50 μL of cell suspensions (1.5×10^6 cell/mL) in the upper compartment. Results are mean values of triplicates. Standard deviations were always ≤ 15% of the mean and are not reported. Data presented are from one representative experiment of the 3–5 performed for each cytokine.

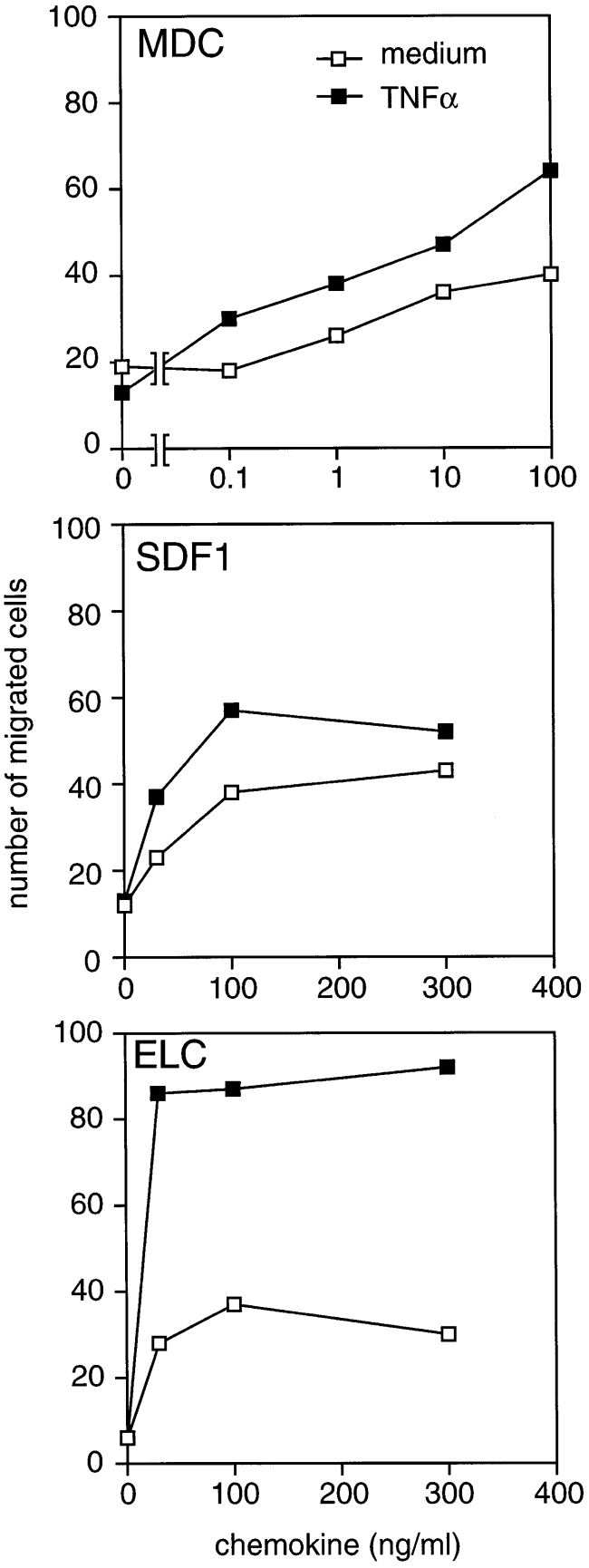

Fig. 2. Effect of constitutively produced chemokines on chemotaxis of immature and mature DC. Experimental conditions as in Fig. 1.

ration increased the DC chemotactic response to MDC and SDF1; at 100 ng/mL, migration was increased 167±15%, and 153±17%, respectively, in four experiments (Fig. 2). The sensitivity of DC to MDC was increased 10-fold by TNF-α-induced maturation: 0.1 ng/mL of MDC was consistently sufficient to induce DC migration. ELC was the most efficient chemokine on TNF-α-matured DC (Fig. 2), inducing a chemotactic response about threefold higher (251±25% in three experiments) than that of immature DC. In one experiment, the kinetics of the differential effect of TNF-α on the DC chemotactic response to inducible vs. constitutive chemokines was examined. Chemotaxis to RANTES (100 ng/mL) after 1 h exposure of DC to TNF-α was 60% of the control, whereas chemotaxis to ELC (100 ng/mL) was not modified. At 6 h, response to RANTES was further decreased (45% of control) and that to ELC started to increase (145% of control) to reach maximum at 24 h (342% of control). Maturation stimuli other than TNF-α (LPS and CD40) were then examined. Mouse DC were stimulated with CD40L for 72 h and TNF-α or LPS for 24 h. All the three maturation stimuli modified DC migration capacity; their responsiveness to MIP1? and RANTES was strongly decreased, but they migrated better in response to ELC and SDF1 (**Fig. 3**).

Transendothelial migration of DC

Transendothelial migration was then investigated using the 1G11 mouse endothelial cell line [34]. MIP1α, RANTES, and SDF1, but not ELC, tested at concentrations active in chemotaxis increased DC transmigration across the endothelial monolayer (**Fig. 4**). TNF-α-induced maturation decreased DC ability to transmigrate in response to MIP1? and RANTES and increased transmigration in response to SDF1 and ELC (Fig. 4). Experiments with DC matured by CD40 ligation gave similar results, with decreased transmigration to MIP1α and RANTES and increased transmigration to SDF1 and ELC, in agreement with the data on chemotaxis (data not shown).

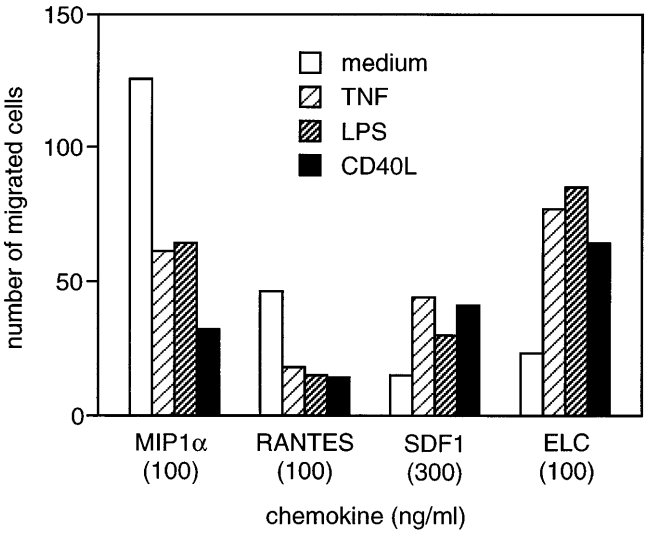

Fig. 3. Effect of different maturation stimuli on chemotaxis of immature and mature DC. DC were cultured with TNF-α (20 ng/mL) or LPS (1 ng/mL) for 24 h or with CD40L-transfected J558L cells for 48 h. Other experimental conditions were as in Fig. 1.

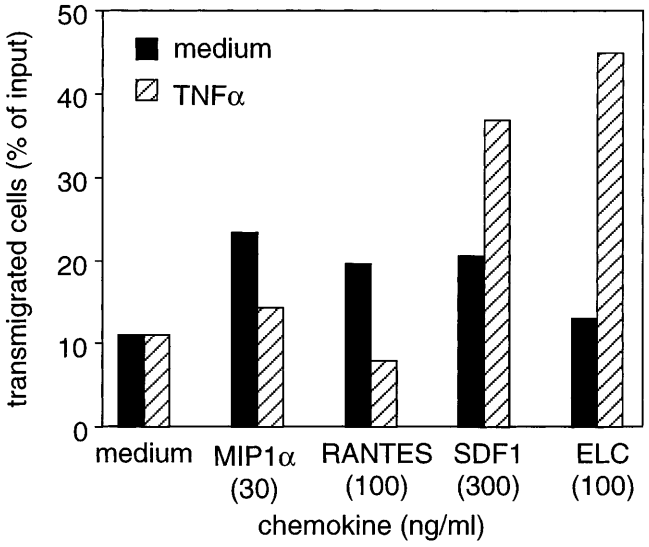

Fig. 4. Effect of chemokines on transendothelial migration of immature and mature DC. Polycarbonate transwell inserts were coated with a monolayer of mouse endothelial cells. DC were treated with 20 ng/mL of TNF-α for 24 h. 5×10^4 ^{51}Cr labeled DC were put in 0.1 mL in the upper part of the trans-well system and chemokines at the reported concentrations were put in 0.6 mL in the lower part. Transmigration is reported as percentage of the input, as detailed in Materials and Methods. Standard deviations were always ≤ 15% of the mean and are not reported. Data presented are from one representative experiment of the 3–5 performed for each cytokine.

Receptor expression on DC

Expression of the receptors CCR1, CCR2, CCR5, CCR4, CCR7, and CXC chemokine receptor (CXCR4), which bind the chemokines (MIP1α, RANTES, MCP-3, MCP-1, MIP1β, MDC, ELC, and SDF1) whose effects were modulated by maturation, was investigated at the mRNA level. CCR1, CCR2, CCR5, and CXCR4, as evaluated by both Northern blot assay and RNAse protection, were constitutively expressed in control DC (**Fig. 5**A, B). On the contrary, CCR7 was undetectable in control DC (Fig. 5A). Exposure of DC to TNF-α for 24 h decreased CCR1, CCR2, and CCR5 mRNA levels (Fig. 5A, B), whereas CCR7 was strongly induced (Fig. 5A). CXCR4 mRNA levels (corrected for the expression of the housekeeping genes) were unaffected (2/4 experiments) or slightly increased, as already reported in human DC [35]. The expression of CCR4, the only characterized receptor for MDC [36], was never detected in our preparations of immature or mature DC (Fig. 5B). This result, along with recent evidence [37, 38] strongly suggests that MDC can interact with a still uncharacterized receptor also expressed on DC.

CONCLUDING REMARKS

This study shows that mouse BM-derived DC respond to a defined set of chemokines and that maturation differentially affects responsiveness to constitutive vs. inducible chemokines. Results presented here represent the first characterization of the response of mouse BM-derived DC to chemokines and extend previous observations obtained with human DC [17, 18, 22, 28, 33, 35, 39]. Although we used human chemokines in some experiments, the characterization of mouse DC migration permits a direct comparison of *in vitro* chemotactic responsiveness to *in vivo* migration and provides the rationale for the design of new immune experimental strategies based on the selective recruitment of mature or immature DC.

Human immature DC showed little chemotaxis in response to MCP-1 [17, 18, 28] even though CCR2 was expressed as mRNA, and specific binding and calcium flux were observed [18, 35]. These *in vitro* results with human DC seem at odds with the finding that keratinocyte transgenic expression of MCP-1 resulted in increased accumulation of DC [40]. The finding that MCP-1 is an attractant for mouse DC now provides an *in vitro* correlate for these *in vivo* observations.

MCP-3 is a potent attractant for immature mouse and human DC [17, 18; present results]. It is of interest that human MCP-3 gene transfer in a mouse tumor resulted in perivascular accumulation of DC in peritumoral tissues [41], a finding consistent with the *in vivo* relevance of DC attraction by this chemokine.

Immune and inflammatory stimuli or microbial products (i.e., CD40L, IL-1, TNF-α) promote the production of a set of chemokines that includes MCPs, MIPs, and RANTES. The same signals also induce maturation [1–4] and trafficking of DC to lymph nodes or spleen, where they present antigen [5–11]. Recent observations on migration of human cells to ELC [33, 35, 39] and the present extensive analysis with mouse cells indicate that TNF-α, LPS, and CD40 ligation differentially regulate responsiveness of DC to inducible vs. constitutively produced chemokines and concomitantly modulate receptor mRNA expression.

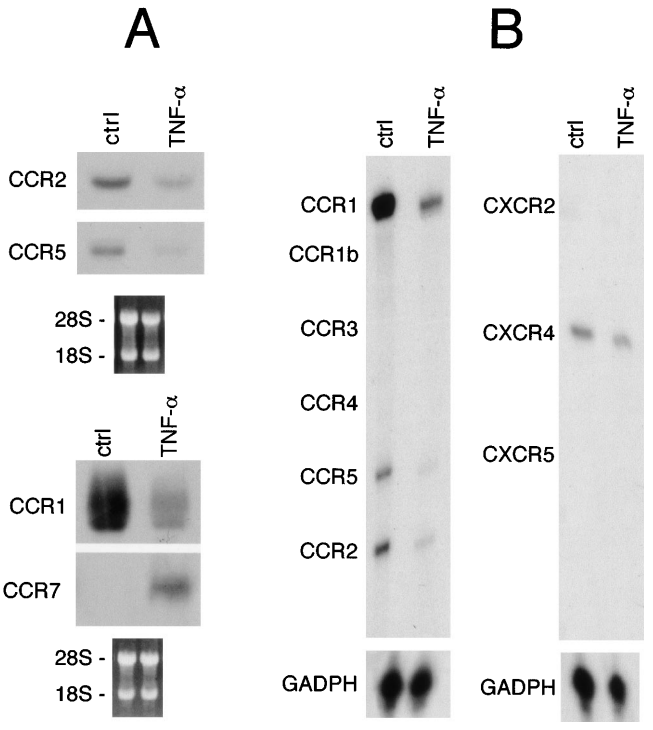

Fig. 5. Effect of TNF-α on chemokine receptor expression. DC were incubated with medium or TNF-α (20 ng/mL for 24 h). (A) Northern blot analysis: 15 μg of total RNA were purified and used in the analysis. Ethidium bromide staining of the membranes is reported below. (B) RNAse protection assay.

Activation of DC with TNF-α, LPS, and CD40L strongly augmented the chemotactic response to ELC, SDF1, and MDC, chemokines that are constitutively expressed in lymphoid organs and would be instrumental to recruit and arrest DC at these sites [42]. The same inflammatory stimuli or bacterial products inhibit responsiveness to inducible chemokines produced locally, and would allow antigen-loaded DC to leave the sites of infection and inflammation and reach lymphoid organs.

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