

# Mammalian and Viral IL-10 Enhance C-C Chemokine Receptor 5 but Down-Regulate C-C Chemokine Receptor 7 Expression by Myeloid Dendritic Cells: Impact on Chemotactic Responses and In Vivo Homing Ability<sup>1</sup>

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The immunosuppressive and anti-inflammatory cytokine IL-10 inhibits the phenotypic and functional maturation of dendritic cells (DC) and has been reported to confer tolerogenic properties on these important professional APC. Here, we exposed murine bone marrow-derived myeloid DC to either mouse (m) or viral (v) IL-10 early during their in vitro generation in response to GM-CSF and IL-4. Both mIL-10 and vIL-10 down-regulated the expression of CCR7 mRNA determined by RT-PCR, while mIL-10 up-regulated the expression of CCR5 transcripts. These changes in CCR7 and CCR5 expression were associated with inhibition and augmentation, respectively, of DC chemotaxis toward their respective agonists, macrophage inflammatory proteins 3 $\beta$  and 1 $\alpha$ , while in vivo homing of DC from peripheral s.c. sites to secondary lymphoid tissue of syngeneic or allogeneic recipients was significantly impaired. Anti-mIL-10R mAb reversed the effects of mIL-10 on CCR expression and restored DC homing ability. Retroviral transduction of mIL-10- and vIL-10-treated DC to overexpress transgenic CCR7 partially restored the cells' lymphoid tissue homing ability in allogeneic recipients. However, CCR7 gene transfer did not reinstate the capacity of IL-10-treated DC to prime host naive T cells for ex vivo proliferative responses or Th1 cytokine (IFN- $\gamma$ ) production in response to rechallenge with (donor) alloantigen. These findings suggest that in addition to their capacity to subvert DC maturation/function and confer tolerogenic potential on these cells, mIL-10 and vIL-10 regulate DC migratory responses via modulation of CCR expression. *The Journal of Immunology*, 2001, 166: 7136–7143.

Dendritic cells (DC)<sup>3</sup> are highly specialized APC that play critical roles as instigators and regulators of immune reactivity (1–4). Their progenitors in the bone marrow (BM) give rise to circulating precursors that home to peripheral tissues. Following Ag capture in the periphery, DC migrate to secondary lymphoid organs where they activate Ag-specific naive T cells and initiate primary immune responses. These events are influenced by microenvironmental factors, in particular cytokines that affect DC growth and differentiation (5–8) and the ability of DC to process and present Ag (7), traffic from nonlymphoid tissue (9), and induce naive T cell proliferation.

Mammalian IL-10 is mainly produced by activated lymphocytes, monocytes/macrophages, and other cell types (10). IL-10

down-regulates MHC class II Ag and costimulatory molecule expression (11–13), IL-12 and proinflammatory cytokine secretion (14, 15), and T cell stimulatory function of several APC (i.e., monocytes, macrophages, and DC). It confers tolerogenic properties on DC (16), induces long-term, Ag-specific anergy in human CD4<sup>+</sup> T cells (17), and drives the generation of mouse CD4<sup>+</sup> T regulatory 1 cells (18). IL-10 can also exhibit T cell stimulatory properties, including enhancement of thymocyte proliferation and generation of CD8<sup>+</sup> T cells (19, 20), exacerbation of organ allograft rejection (21), and graft-vs-host disease (22).

Viral IL-10 (vIL-10), encoded by EBV, is highly homologous to mammalian IL-10 and shares many of its anti-inflammatory properties (23). In addition, it may act directly on T cells to inhibit costimulatory signals mediated via CD28 or CTLA4 (24). It lacks the immunostimulatory properties ascribed to mammalian IL-10, such as augmentation of CTL proliferation and B cell expansion, and can induce local anergy to syngeneic or allogeneic tumors (10). Cardiac allografts transduced with vIL-10 show prolonged survival (25). Recently, we demonstrated that exposure of myeloid DC to vIL-10 protein or retroviral transduction of DC to overexpress the vIL-10 gene impaired their T cell stimulatory capacity and promoted Ag-specific T cell anergy (26). Although there are reports that IL-10 modulates leukocyte migration in vivo, e.g., to suppress leukocyte infiltration into specific tissues (27) or heart allografts (28), there is little information on the influence of IL-10 on chemokine receptor expression by DC or on the migratory capacity of DC either in vitro or in vivo (29).

Chemokines are a group of structurally related polypeptides that have been recognized recently to have critical roles in the selective recruitment of leukocyte subsets to secondary lymphoid organs

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cells; BM, bone marrow; vIL-10, viral IL-10; mIL-10, mouse IL-10; MIP, macrophage-inflammatory protein; MFI, mean fluorescence intensity.

and to sites of inflammation (30). There are two major groups: the CXC chemokines, in which the two N-terminal cysteines are separated by a single amino acid, and the CC chemokines, in which the two terminal cysteines are adjacent (30). All chemokines exert their activity by binding to single-chain, seven-helix transmembrane G-protein-coupled receptors that are expressed on a variety of leukocytes. Five of the receptors are specific for CXC chemokines (CXCR1–5), 11 are specific for CC chemokines (CCR1–11), and the Duffy Ag receptor binds both CXC and CC chemokines (30–32). Numerous chemokine receptors have been demonstrated on DC and their progenitors at the transcriptional level in mice and humans and at the protein level in humans (33–38). Upon maturation, DC undergo a chemokine receptor switch, with down-regulation of inflammatory chemokine receptors (such as CCR1, CCR2, and CCR5), followed by induction of CCR7 (29, 39).

In this study we assessed the impact of mouse IL-10 (mIL-10) and vIL-10 on myeloid DC CCR1, CCR5, and CCR7 expression and on the chemotactic responses of these cells to specific CC chemokines. In addition, we determined the influence of CCR7 expression and IL-10 on the *in vivo* migratory and T cell stimulatory ability of the DC. We report that exposure of myeloid DC to mIL-10 enhances CCR1 and CCR5, but down-regulates CCR7 gene expression, with consequent effects on chemotactic responses to appropriate agonist ligands; in addition, mIL-10 and vIL-10 inhibit the homing ability of these DC *in vivo*. vIL-10 exerted similar effects, but its overall activity was less pronounced. Studies conducted with CCR7-transduced DC revealed that overexpression of CCR7 could reverse IL-10-induced inhibition of migration, but not impairment of the capacity of the cells to prime host T cells.

## Materials and Methods

### Animals

C57BL/10 (H2<sup>b</sup>; B10) and C3H/HeJ (H2<sup>k</sup> C3H) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in the specific pathogen-free facility of the University of Pittsburgh Central Animal Facility, received standard laboratory chow and tap water *ad libitum*, and were used for all experiments at 8–12 wk of age.

### Propagation and purification of DC

BM-derived DC were propagated using procedures similar to those reported initially by Inaba et al. (5), with some modifications. In brief, freshly isolated BM cells from femurs and tibias of normal B10 mice were depleted of T and B lymphocytes and granulocytes with an mAb mixture comprising rat IgG anti-B220 (RA3-3A1/6.1), anti-Lyt 2 (2.43), and anti-L3T4 (GK1.5; all generated from hybridomas provided by American Type Culture Collection, Manassas, VA) and anti-mouse Gr-1 mAb (PharMingen, San Diego, CA), plus rabbit complement (Accurate Chemical and Scientific, Westbury, NY). The mixture-depleted BM cells were then cultured overnight in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with antibiotics, and 10% (v/v) heat-inactivated FCS. Twenty-four hours later (day 1), nonadherent cells were removed carefully and replated at  $5 \times 10^5$ /ml with 1000 U/ml recombinant mouse GM-CSF and 1000 U/ml recombinant murine IL-4 (gifts from Schering Plough, Kenilworth, NJ) together with 1–500 ng/ml either recombinant mIL-10 or recombinant vIL-10 (provided by K. W. Moore, DNAX Research Institute, Palo Alto, CA). The cells were harvested on day 6. CD11c<sup>+</sup> cells were immunobead-sorted by labeling with a bead-conjugated anti-mouse CD11c mAb (Miltenyi Biotec, Auburn, CA) followed by passage through a positive selection paramagnetic column (Miltenyi Biotec).

### Endocytosis assay

To determine their endocytic activity, untreated DC or DC exposed to IL-10 were cultured for 2 h at 37°C with FITC-dextran (Sigma, St. Louis, MO) at a final concentration of 1 mg/ml. After incubation the cells were washed three times with ice-cold PBS, then analyzed immediately by flow cytometry.

### Mixed leukocyte reaction

The ability of IL-10-exposed DC to stimulate freshly isolated, naive, allogeneic T cells was determined in primary MLR. Normal C3H splenic T lymphocytes were purified by passage through nylon wool columns (30 min, 37°C), then cultured with  $\gamma$ -irradiated DC that had been pre-exposed to different concentrations of mIL-10 or vIL-10 protein in 96-well microculture plates for 72 h. For the final 18 h of culture, 1  $\mu$ Ci [<sup>3</sup>H]TdR was added to each well. The cultures were harvested onto glass-fiber filter disks using a multiple cell harvester, and thymidine incorporation was quantified using a liquid scintillation counter. The ability of CCR7 gene-transduced DC (see below) to stimulate freshly isolated, naive, allogeneic T cells in primary MLR was determined in the same manner.

### Flow cytometry

B10 DC were characterized by cytofluorography, as previously described (40, 41), using a panel of mAb including those directed against the mouse DC-restricted Ag CD11c, MHC class II (I-A<sup>b</sup>), CD80, or CD86 (all mAbs were from PharMingen and were used at saturating concentrations). Two-color analysis was performed on gated CD11c<sup>+</sup> cells, using a Coulter EPICS Elite (Coulter, Hialeah, FL) (38).

### Chemotaxis assay

*In vitro* chemotaxis analysis was performed as described previously (42). Recombinant macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , or MIP-3 $\beta$  (R&D Systems, Minneapolis, MN) were diluted in assay medium without FCS, and 600- $\mu$ l aliquots were placed in 24-well culture plates (Costar, Cambridge, MA). Transwell culture inserts of 6.5-mm diameter and 5- $\mu$ m pore size (Costar) were placed in each well, and  $5 \times 10^5$  DC in 100  $\mu$ l were added to the upper chamber. After incubation at 37°C in 5% CO<sub>2</sub> for 2 h, the cells that had migrated to the bottom chamber were harvested and counted by light microscopy.

### RT-PCR

Total RNA was isolated from cultured, immunobead-sorted CD11c<sup>+</sup> DC using RNeasy (Life Technologies, Grand Island, NY) and used for cDNA synthesis. The cDNAs were used as templates for PCR (94°C for 1 min, 57°C for 1 min, and 74°C for 1 min), and 26 cycles were performed using primers specific for mCCR1 (forward, 5'-TCTAGTGTTCATCATTTGGAGTGGTG; reverse, 5'-GACGCACGGCTTTGACCTTCTTCTC), mCCR5 (forward, 5'-ACTTGGGTGGTGGCTGTGTGT; reverse, 5'-TTGTCTTGTGGAAAAT TGAA), and mCCR7 (forward, 5'-ACAGCGGCCTCCAGAAGAA GAGCGC; reverse, 5'-TGACGTCATAGGCAATGTTGAGCTG). To ensure the quality of the procedure, RT-PCR was also performed using primers specific for  $\beta$ -actin.

### *In vivo* DC migration

Immediately before their injection, DC were labeled with the red fluorescence marker PKH-26 Red (Sigma) according to the manufacturer's instructions. In brief, the DC were incubated with  $10^{-6}$  M PKH-26 at room temperature for 5 min, then rinsed extensively with HBSS. Viability was determined by trypan blue exclusion. Mice were injected s.c. in the hind footpad with  $10^6$  PKH-26-labeled DC. After 48 h, animals were killed, and the draining popliteal lymph nodes were removed. The nodes were gently disrupted, and  $2 \times 10^6$  cells/ml were suspended in PBS and 1% paraformaldehyde. Aliquots (200  $\mu$ l) of this suspension were centrifuged onto glass slides at 800 rpm for 3 min using a cytocentrifuge (Shandon, Pittsburgh, PA). The slides were then examined by fluorescence microscopy (Olympus BH-2 and Olympus Provis AX-70; Tokyo, Japan), and the number of PKH-26-labeled cells was determined. The total number of migrated cells was determined as the product of fluorescent cells per ml and the total volume (in ml) of cell suspension.

### Blocking of the mIL-10R

Binding of mIL-10 to its cognate receptor was inhibited using blocking rat anti-mIL-10R mAb (1  $\mu$ g/ml) (43) provided by Kevin W. Moore (DNAX). The mAb was added to the DC culture medium in the presence or the absence of mIL-10 protein on day 1. On day 6 the cells were harvested, and CD11c cells were sorted as described above.

### CCR7 gene transfer

MFG-Zeo and MFG-CCR7 plasmid vectors were transfected into the BOSC 23 ecotropic packaging cell line to obtain supernatant containing recombinant retrovirus encoding Zeo and CCR7, respectively (44). Transduction efficiency was determined by flow cytometry using MFG-enhanced

green fluorescent protein. Retroviral transduction of DC was performed by the centrifugal enhancement method (45). Briefly, BM-derived cells were harvested on day 2;  $10^6$  cells were suspended in 1 ml retroviral supernatant with 1000 U/ml GM-CSF, 1000 U/ml IL-4, either mIL-10 (100 ng/ml) or vIL-10 (100 ng/ml), and 8 mg/ml Polybrene (Sigma), then centrifuged at  $2500 \times g$  for 2 h. The transduction process was repeated on days 3 and 4. During this treatment period the DC were exposed continuously to either mIL-10 or vIL-10. The cells were harvested for study on day 6.

#### *Ex vivo analysis of the influence of gene-modified DC on allogeneic T cell function*

The influence of CCR7-transduced DC on host anti-donor T cell responses was determined ex vivo by MLR. CCR7 gene-modified DC ( $1 \times 10^6$ ) were injected s.c. into one hind footpad of allogeneic C3H (H2<sup>b</sup>) mice. The animals were killed 7 days later. Popliteal lymph node lymphocytes were isolated and restimulated in secondary MLR with various numbers of  $\gamma$ -irradiated B10 splenocytes for 72 h. For the final 18 h of culture, 1  $\mu$ Ci of [<sup>3</sup>H]TdR was added to each well. The cultures were harvested, and thymidine incorporation was quantified as described above. Cytokine levels in 48-h cultures (stimulator-responder ratio = 1:10) were also determined.

#### *ELISA*

ELISA for mouse IFN- $\gamma$ , IL-4, and IL-10 in culture supernatants of restimulated T cells was performed using reagents purchased from Pharmingen and following the recommended procedures. The limit of sensitivity for detection of each cytokine was 19 pg/ml.

#### *Statistics*

Comparisons were made using the two-tailed unpaired Student's *t* test and ANOVA, as appropriate. Values of *p* < 0.05 were regarded as significant.

## **Results**

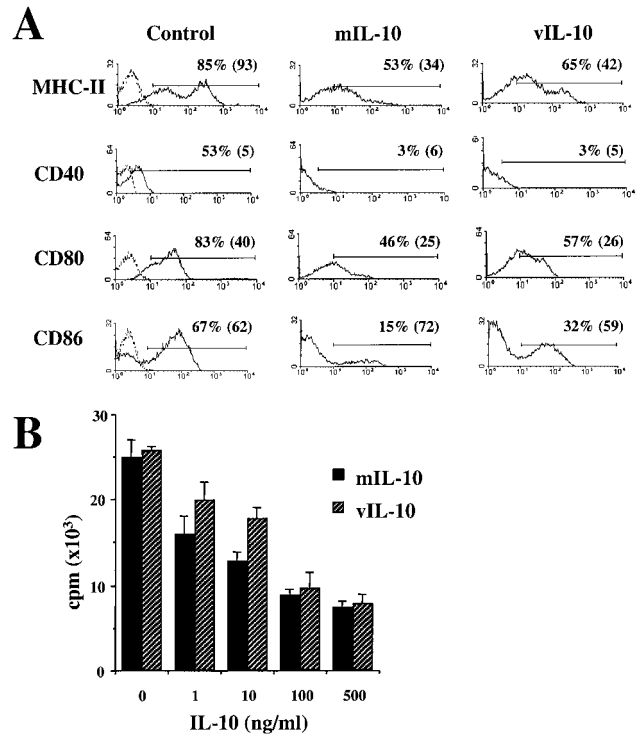
#### *Exposure to either mammalian or vIL-10 promotes development of immature DC*

MHC class II Ag and costimulatory molecule (CD40, CD80, and CD86) expression was examined on mIL-10- or vIL-10-treated, CD11c<sup>+</sup>-gated DC by two-color staining as described in *Materials and Methods*. Exposure to either mIL-10 (100 ng/ml) or vIL-10 (100 ng/ml) from day 1 of culture reduced the percentage of CD11c<sup>+</sup> DC positive for MHC class II molecules (IA<sup>b</sup>), CD40, CD80, and CD86 compared with untreated DC (Fig. 1A). These data showed that the phenotypic maturation of the DC was inhibited by exposure to mIL-10 or vIL-10 early during culture. When mIL-10 or vIL-10 was added from day 4 of culture (instead of day 1), no inhibitory effect on phenotypic maturation was observed. Thus, as reported for human DC (16), more mature mouse DC appear to be refractory to IL-10.

Immature myeloid DC display avid endocytic activity that is diminished progressively with cell maturation (1, 3). The endocytic activity of mIL-10- or vIL-10-treated DC was assessed using FITC-dextran, a tracer that is internalized mainly via the mannose receptor. Day 6 cultured, mIL-10- or vIL-10-treated or control CD11c<sup>+</sup>-sorted DC were exposed for 2 h to FITC-dextran and washed extensively, then fluorescence intensity was determined by flow cytometry. IL-10-treated DC exhibited more intense fluorescence staining than control cells. mIL-10- or vIL-10-treated DC displayed 42% (mean fluorescence intensity (MFI) = 411.8) and 26% (MFI = 364.4) increases, respectively, above control values (MFI = 289.8). Thus, using the criterion of endocytic activity, both cytokines inhibited DC maturation.

#### *Exposure to IL-10 markedly impairs DC allostimulatory activity*

To ascertain whether the reduction in surface MHC and costimulatory molecule expression and the increase in endocytic activity were associated with impaired T cell stimulatory activity, the capacity of IL-10-treated, immunobead-sorted DC to induce proliferation of naive allogeneic T cells was determined. Compared with control DC, DC pre-exposed to mIL-10 or vIL-10 exhibited sig-



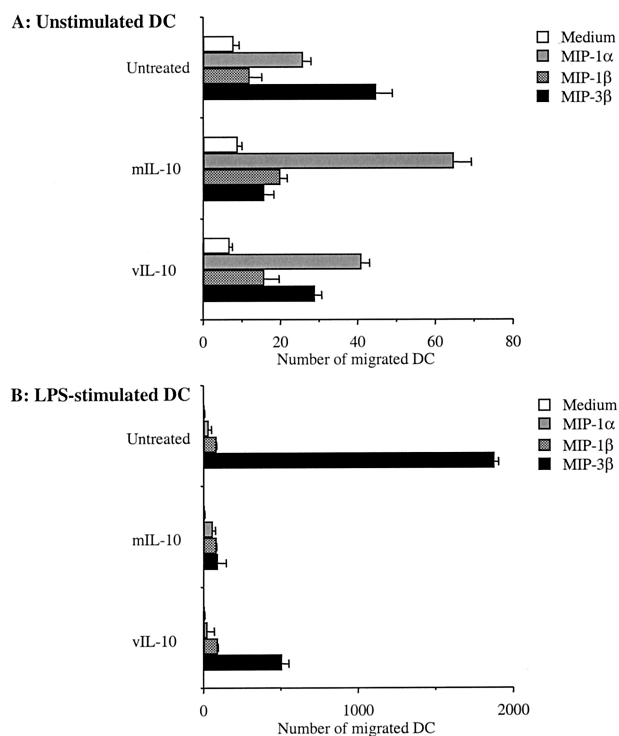
**FIGURE 1.** A, Addition of m- or vIL-10 to cultures of mouse BM-derived DC from day 1 of culture decreased the level of expression of MHC-II Ag and costimulatory molecules (CD40, CD80, and CD86) of CD11c<sup>+</sup> DC. DC were propagated from normal B10 mouse as described in *Materials and Methods*. On day 1 of culture, either m- or vIL-10 (100 ng/ml) was added; on day 6, low buoyant density cells were harvested. Following two-color immunofluorescence staining, surface expression of molecules of interest was determined by flow cytometric analyses of gated, CD11c<sup>+</sup> cells. The results are representative of three separate experiments. B, Addition of m- or vIL-10 to cultures of mouse (B10; H2<sup>b</sup>) BM-derived DC from day 1 of culture inhibits the capacity of CD11c<sup>+</sup> DC to induce allogeneic T cell proliferation. DC were harvested on day 6, as described in *Materials and Methods*, and CD11c<sup>+</sup> cells were sorted using immunomagnetic beads.  $\gamma$ -Irradiated DC exposed to various concentrations of IL-10 were used as stimulators (S/R ratio = 1:10) of naive allogeneic splenic T cells (C3H; H2<sup>b</sup>). MLR cultures were maintained for 72 h, and incorporation of [<sup>3</sup>H]TdR was determined over the final 18 h. The results are the mean  $\pm$  SD of triplicate cultures and are representative of three separate experiments.

nificantly reduced allostimulatory activity in primary MLR. Significant inhibition (*p* < 0.01) was achieved with as little as 1 ng/ml, whereas maximal inhibition was achieved for each protein at 100 ng/ml (Fig. 1B). This latter concentration of IL-10 was used in all subsequent experiments.

#### *Exposure to IL-10 differentially affects the chemotactic responses of DC to specific CC chemokines*

We next evaluated the chemotactic responses of mIL-10- or vIL-10-treated DC to MIP-1 $\alpha$  (ligand for CCR1 and CCR5), MIP-1 $\beta$  (ligand for CCR5), and MIP-3 $\beta$  (ligand for CCR7; Fig. 2A). These CC chemokines were chosen because there is evidence that IL-10 inhibits MIP-1 $\alpha$  and MIP-3 $\beta$  expression by human monocytes/macrophages and DC (29, 46). It has also been reported that IL-10 selectively up-regulates the expression of CCR1, -2, and -5 in human monocytes (47), and that CCR1, -5, and -7 play critical roles in DC migration in mice (48). Untreated DC exhibited the most marked migratory responses to MIP-3 $\beta$  and responded to a lesser extent to MIP-1 $\alpha$  and MIP-1 $\beta$ . Both IL-10 proteins enhanced the





**FIGURE 2.** Exposure of B10 BM-derived DC to m- or vIL-10 (100 ng/ml) from day 1 of culture differentially affects the chemotactic responsiveness of unstimulated (A) and LPS-activated (B; 10  $\mu$ g/ml, 6 h) CD11c<sup>+</sup> DC to specific CC chemokines. Immunobead-sorted CD11c<sup>+</sup> DC were placed in the upper chambers of Transwell culture inserts, and DC migration in response to individual chemokines (100 ng/ml) was assessed after 2-h incubation by determining the number of cells in the bottom chamber. Pre-exposure to IL-10 augmented the migratory responses of unstimulated DC to MIP-1 $\alpha$  ( $p < 0.01$ ), but diminished migration induced by MIP-3 $\beta$  ( $p < 0.01$ ). Pre-exposure to IL-10 diminished migratory responses of LPS-stimulated DC to MIP-3 $\beta$  ( $p < 0.001$ ). The results are the mean  $\pm$  SD obtained from triplicate cultures and are representative of two separate experiments.

cells' migratory capacity in response to MIP-1 $\alpha$ , but reduced their chemotactic response to MIP-3 $\beta$ . IL-10-treated DC exhibited a more pronounced enhancement of migration in response to MIP-1 $\alpha$ . Given that, unlike MIP-3 $\beta$ , MIP-1 $\alpha$  binds to both CCR1 and CCR5, the effect of IL-10 on DC chemotaxis may have been caused through the up-regulation/activation of either CCR1 or CCR5.

The effect of IL-10 on chemotaxis to CC chemokines was also investigated in DC terminally differentiated after 6-h incubation with the maturation-enhancing factor LPS. As expected, LPS-matured DC exhibited enhanced chemotaxis in response to MIP-3 $\beta$  and almost no migration in response to MIP-1 $\alpha$  and MIP-1 $\beta$  (Fig. 2B). LPS-stimulated DC exposed to IL-10 showed strikingly diminished chemotactic responses to MIP-3 $\beta$ . In the case of mIL-10, migration was almost totally inhibited. These findings are consistent with a suppressive effect of both IL-10 molecules on DC maturation.

#### mIL-10 enhances CCR5, but down-regulates CCR7, expression on DC

Chemokine receptor (CCR5/CCR7) expression on murine DC has been shown previously to correlate with the cells' state of maturation and in vivo homing ability (38). To assess the impact of exposure to mIL-10 or vIL-10 on CCR expression by DC, we performed RT-PCR to evaluate the transcription of mRNA encod-

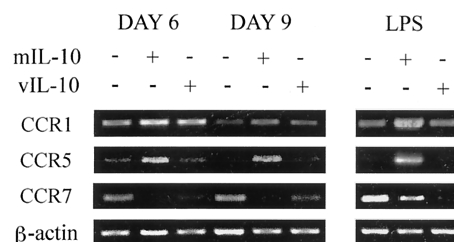
ing the MIP-1 $\alpha$  ligands CCR1 and CCR5 and the MIP-3 $\beta$  ligand CCR7 (Fig. 3). Messenger RNA was extracted from immunobead-sorted CD11c<sup>+</sup> DC that had been cultured in the absence or the presence of mIL-10 or vIL-10 for 6 or 9 days as described above. RT-PCR analysis revealed that expression of CCR1 and CCR5 mRNA was up-regulated by mIL-10 at both time points, whereas CCR7 mRNA expression was inhibited compared with that in untreated DC. These changes were also evident in DC exposed to mIL-10 from days 1–6 of culture, then stimulated for 6 h with LPS (10  $\mu$ g/ml) in the continued presence of mIL-10. vIL-10 showed similar, but less pronounced, effects (Fig. 3). The absence of migration of LPS- plus IL-10-treated DC in response to MIP-1 $\alpha$  or MIP-1 $\beta$  (Fig. 2B) despite the increased levels of CCR1 and CCR5 mRNA (Fig. 3) appears contradictory. However, the observation is in agreement with the recent finding that mIL-10 generates functional CCR1, CCR2, and CCR5 decoy receptors on the surface of DC exposed to microbial Ag (LPS) or to an inflammatory environment (29).

#### Exposure to IL-10 impairs the in vivo homing ability of cultured DC

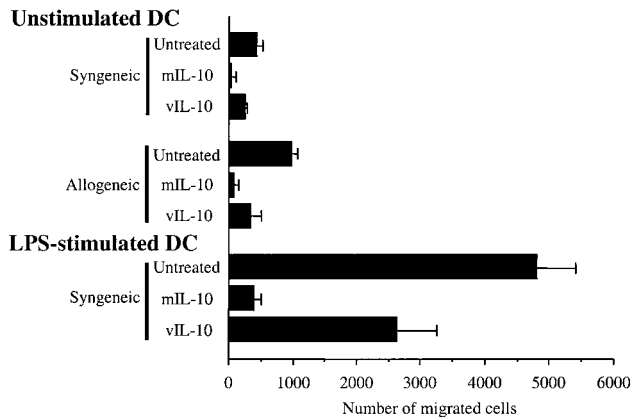
We next addressed the impact of exposure to IL-10 on the subsequent capacity of DC to migrate in vivo from the periphery to secondary lymphoid tissue. We determined the capacity of IL-10-treated unstimulated or LPS-stimulated immunobead-sorted CD11c<sup>+</sup> DC labeled with PKH-26 and inoculated s.c. in the hind footpad to migrate to the draining popliteal lymph node. Migrated cells were counted by fluorescence microscopy in cytospin preparations of lymph node cell suspensions. Both mIL-10- and vIL-10-treated DC showed significantly diminished capacity to migrate to the draining lymph node in syngeneic ( $p < 0.05$ ) and allogeneic recipients ( $p < 0.01$ ; Fig. 4). In vitro stimulation of untreated DC with LPS (as described above) enhanced their migration in syngeneic hosts, but this augmented activity was also inhibited by exposure to IL-10, in particular mIL-10, during their propagation (Fig. 4).

#### Blocking of the IL-10R reverses the impact of mIL-10 on CCR expression and the in vivo homing ability of DC

To confirm the specificity of the effects of mIL-10, we blocked the IL-10R on cultured DC before their exposure to IL-10 using anti-mIL-10R mAb (1  $\mu$ g/ml). Blocking of the IL-10R not only prevented the effects of mIL-10 on CCR5 and CCR7 gene expression by DC (Fig. 5A), but also substantially restored the capacity of these cells to home to secondary lymphoid tissue of both syngeneic and allogeneic recipients (Fig. 5B).



**FIGURE 3.** Exposure of mouse B10 BM-derived DC to m- or vIL-10 (100 ng/ml) from day 1 of culture differentially affects CCR expression on unstimulated and LPS (10  $\mu$ g/ml, 6 h)-terminally differentiated DC. RNA was extracted from immunobead-sorted CD11c<sup>+</sup> DC after 6 or 9 days culture, as described in *Materials and Methods*, and the expression of CCR1, CCR5, and CCR7 mRNA was determined by RT-PCR. The results of RT-PCR for  $\beta$ -actin demonstrate equal loading of DNA on the gel. The results are representative of three experiments with similar results.

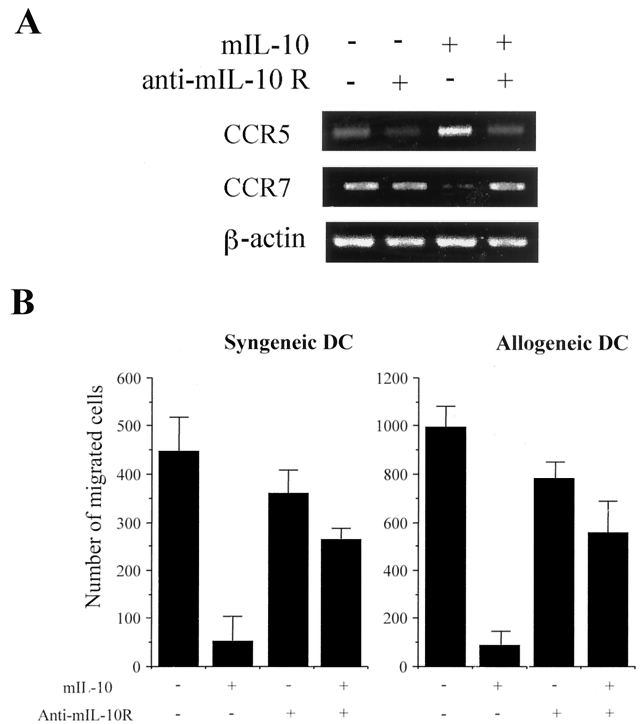


**FIGURE 4.** Exposure of B10 BM-derived DC to m- or vIL-10 (100 ng/ml) from day 1 of culture inhibits the *in vivo* homing of unstimulated and LPS-activated CD11c<sup>+</sup> DC. Immunobead-sorted DC were labeled with the fluorescence marker PKH-26, then injected ( $10^6$ , s.c.) in the hind footpad of syngeneic or allogeneic (C3H) recipients. After 48 h the animals were killed, and the total number of migrated cells in the popliteal lymph node was determined by fluorescence microscopy of cytospin preparations. Results are the mean  $\pm$  SD and are representative of two experiments with similar results.

*CCR7 gene transfer partially restores the chemotactic response to MIP-3 $\beta$  and the *in vivo* homing capacity of IL-10-treated DC*

We next determined the influence of CCR7 expression on the *in vitro* chemotaxis and *in vivo* trafficking of IL-10-treated DC. DC were retrovirally transduced to express CCR7 by the centrifugal transduction method, then were exposed to mIL-10 or vIL-10 as described above. As shown in Fig. 6A, CCR7 was successfully transduced to both untreated and IL-10-treated DC. The transduction efficiency was the same (43%) for IL-10-treated and control DC, as determined using the MFG-enhanced green fluorescent protein vector and flow cytometric analysis. CCR7 transduction did not alter MHC or costimulatory molecule expression (data not shown) or the allostimulatory capacity of DC in primary MLR (Fig. 7A). Due to the fact that on day 6 of culture BM-derived DC generated with GM-CSF and IL-4 were composed of a mixed population of immature (MHC-II<sup>low</sup>, CD86<sup>low</sup>; Fig. 1A) and mature (MHC-II<sup>high</sup>, CD86<sup>high</sup>; Fig. 1A) DC, it was not surprising that a subpopulation of control (nontreated) DC migrated in response to MIP-3 $\beta$  (Fig. 6B). Treatment of DC with mIL-10 or vIL-10 diminished their chemotactic response to MIP-3 $\beta$ , a result that agrees with the observation that both cytokines reduce the expression of CCR7 mRNA in DC (Fig. 3). The phenomenon was more pronounced with mIL-10. Retroviral CCR7 transduction partially restored the response of mIL-10 treated-DC to MIP-3 $\beta$  and fully restored the chemotactic response of vIL-10-treated DC (Fig. 6B).

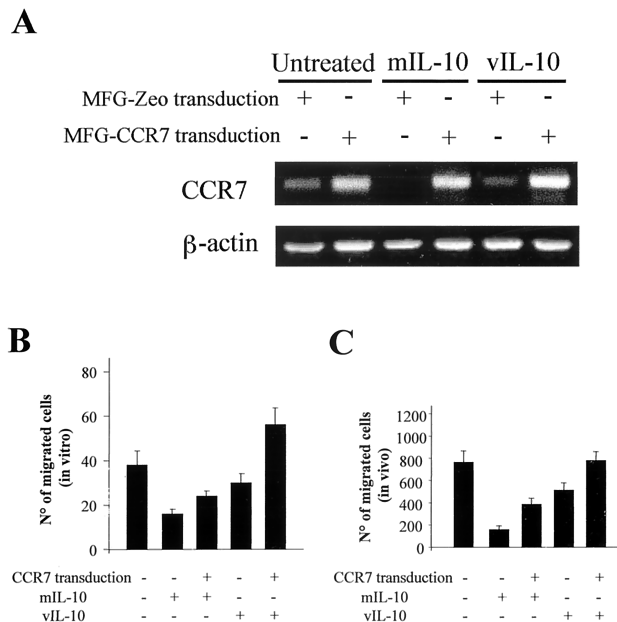
Following s.c. injection in the hind footpad, a subpopulation of control DC homed in the ipsilateral popliteal lymph node (Fig. 6C). The number of donor DC detected in recipient lymphoid tissue was fewer when DC generated in the presence of mIL-10 or vIL-10 were administered (Fig. 6C). Compared with nontransduced control DC, CCR7 gene transfer partially restored the homing ability of mIL-10-treated DC and fully restored that of vIL-10-transduced DC (Fig. 6C). As expected, and as a control for CCR7 transduction efficiency, CCR7-transduced control DC exhibited an increased homing capacity to the popliteal lymph node in relation to nontransduced control DC (data not shown).



**FIGURE 5.** Anti-mIL-10R mAb prevents the differential effect of mIL-10 on CCR5 and CCR7 mRNA expression by BM-derived DC and partially restores their homing capacity *in vivo*. Mouse B10 BM-derived DC were exposed to mIL-10 (100 ng/ml) from day 1 of culture in the absence or the presence of mAb (1  $\mu$ g/ml) added concomitantly on day 1. **A**, Cells were harvested on day 6, and RNA was extracted from immunobead-sorted CD11c<sup>+</sup> DC as described in *Materials and Methods*. Expression of CCR5 and CCR7 mRNA was determined by RT-PCR. PCR products for CCR5, CCR7, and  $\beta$ -actin are shown. Results for  $\beta$ -actin demonstrate equal loading of DNA on the gel. Results are representative of three experiments with similar results. **B**, Untreated or mIL-10-treated, immunobead-sorted CD11c<sup>+</sup> DC with or without addition of mAb were labeled with PKH-26 and injected ( $10^6$ , s.c.) into the hind footpad of either syngeneic or allogeneic (C3H) recipients. Animals were killed after 48 h, and the migration of DC to the popliteal lymph node was determined as described in Fig. 4.

*CCR7 gene transfer indirectly augments the *in vivo* allostimulatory ability of normal, but not IL-10-treated, DC*

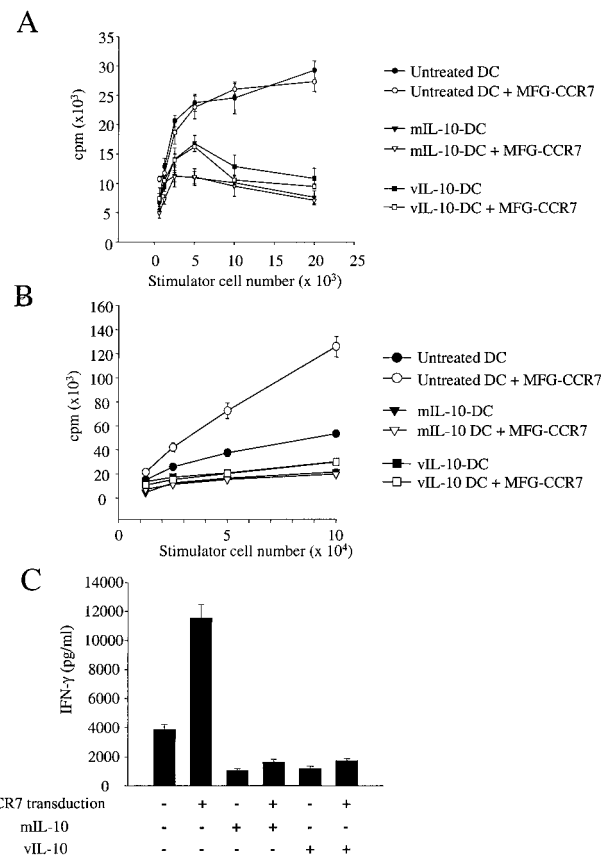
To evaluate the influence of CCR7 overexpression on the *in vivo* allostimulatory activity of control or IL-10-treated DC, sorted B10 CD11c<sup>+</sup> control or gene-transduced DC pre-exposed to IL-10 were injected s.c. ( $10^6$ ) into the hind footpad of C3H recipients. *Ex vivo* responses of draining lymph node cells to rechallenge with donor Ag were determined 7 days later. CCR7 transduction of control DC significantly increased their capacity to prime host T cells for *ex vivo* responsiveness to donor alloantigen (Fig. 7B). mIL-10- or vIL-10-treated, CCR7-transduced DC failed to induce enhanced responses, even though transgene expression significantly increased the migration of these cells to host lymphoid tissue (Fig. 6C). IFN- $\gamma$  production by draining lymph node cells restimulated with allogeneic B10 splenocytes *in vitro* was markedly increased in response to that by CCR7-transduced control DC (Fig. 7C). By contrast, CCR7 transduction of IL-10-treated DC only slightly increased their capacity to prime host cells for subsequent IFN- $\gamma$  responses (Fig. 7C). Neither IL-4 nor IL-10 secretion by restimulated splenocytes was detected (data not shown).



**FIGURE 6.** CCR7 gene transfer partially restores the in vivo homing capacity of mIL-10-treated DC and fully restores that of vIL-10-treated DC. Mouse B10 BM-derived DC were retrovirally transduced with either MFG-Zeo or MFG-CCR7 using the centrifugal transduction method on days 2–4 of culture. IL-10 was added from day 2, and the cultures were harvested on day 6. CD11c<sup>+</sup> DC were sorted using immunomagnetic beads. **A**, RNA was extracted, and the expression of CCR7 mRNA was determined by RT-PCR. PCR products for CCR7 and  $\beta$ -actin are shown. The results for  $\beta$ -actin demonstrate equal loading of DNA. **B**, Immunobead sorted CD11c<sup>+</sup> DC were placed in the upper chambers of Transwell culture plates, and DC migration in response to MIP-3 $\beta$  (100 ng/ml) was assessed by determining the number of cells in the bottom chamber after 2-h incubation. Pre-exposure to IL-10 reduced the migratory responses to MIP-3 $\beta$ . Retroviral CCR7 transduction partially and completely restored the response of mIL-10- and vIL-10-treated DC, respectively. The results are the mean  $\pm$  SD obtained from triplicate cultures and are representative of two separate experiments. **C**, DC were labeled with PKH-26 and injected ( $10^6$ , s.c.) in the hind footpad of allogeneic (C3H) recipients. Twenty-four hours later the popliteal lymph node was removed, and the total number of migrated cells was determined by fluorescence microscopy of cytospin preparations. The results are representative of three experiments.

## Discussion

Localization in tissues and migration to lymphoid organs are essential steps in DC immunobiology that are linked to the differentiation and T cell stimulatory function of these cells. Chemokines play an important role in regulation of DC trafficking, which is also affected by differential regulation of the repertoire of chemokine receptors expressed by immature and mature DC (39). Upon exposure to maturation signals (LPS, IL-1 $\beta$ , TNF- $\alpha$ , or CD40 cross-linking) DC down-regulate inflammatory chemokine receptors (CCR1, CCR2, and CCR5) followed by induction of CCR7. The purpose of this study was to evaluate and compare the influence of mIL-10 and vIL-10, cytokines known to inhibit DC maturation and function, on chemotaxis and tissue homing of murine BM-derived myeloid DC generated in vitro in response to GM-CSF and IL-4. In parallel studies we sought to ascertain how exposure to IL-10 might affect CCR5 and CCR7 expression, shown previously to be modulated during DC maturation, and the chemotactic responses of the DC to appropriate agonist ligands (MIP-1 $\alpha$  and MIP-3 $\beta$ ). Our investigations revealed that both m- and vIL-10 differentially affected the expression of transcripts for CCR5 and CCR7. Whereas CCR5 mRNA expression was in-



**FIGURE 7.** CCR7 gene transfer indirectly augments the capacity of untreated, but not IL-10-treated, DC to prime alloreactive T cells in vivo. Mouse B10 BM-derived DC were retrovirally transduced with either MFG-Zeo or MFG-CCR7 using the centrifugal transduction method on days 2–4 of culture. mIL-10 or vIL-10 was added from day 2, and the cultures were harvested on day 6. Immunobead-sorted CD11c<sup>+</sup> DC were used either as stimulators of allogeneic (C3H) T cells in primary MLR (**A**) or to prime allogeneic mice for subsequent ex vivo T cell proliferative responses to donor alloantigen. **B** and **C**, the B10 DC were injected ( $10^6$ , s.c.) into the hind footpad of recipients (C3H), and the animals were killed 7 days later. **B**, Ex vivo proliferative responses of recipient T cells from the popliteal lymph node to restimulation with B10 (allogeneic) gamma-irradiated spleen cells in 72-h MLR. [ $^3$ H]TdR was added for the final 18 h of culture. **C**, Levels of IFN- $\gamma$  determined by ELISA in 48-h supernatants of cultures established, as described in **B**.

creased, CCR7 mRNA levels were decreased by exposure of BM-derived DC to either IL-10 molecule. This was associated with augmentation and inhibition, respectively, of chemotactic responses to their respective agonists, MIP-1 $\alpha$  and MIP-3 $\beta$ . These results suggest that the IL-10-induced modulation of chemotactic responses of murine myeloid DC to CC chemokines may be mediated by changes in the expression of their respective receptors.

The findings are in keeping with the well-recognized ability of IL-10 to inhibit the maturation of myeloid DC, as reported in this and previous studies (10–13). They are also consistent with reports of selective CCR expression and polarized responses to chemokines by immature compared with mature DC of different species (48–50). Thus, others have observed recently that immature monkey DC express CCR5 and migrate in response to MIP-1 $\alpha$ , whereas CD40 ligand-matured DC switch expression to CCR7 and respond exclusively to MIP-3 $\beta$  and 6-Ckine (49). In the mouse, mature or cytokine-activated, but not quiescent, skin, Langerhans cells express CCR7 mRNA (50), whereas maturation stimuli, such



as TNF- $\alpha$ , down-regulate CCR1, CCR2, and CCR5 and up-regulate CCR7 mRNA in BM-derived DC (48). The CCR7 ligands, MIP-3 $\beta$  and 6-Ckine, are potent and selective chemoattractants of mature murine BM-derived DC (51) and may play an important role in homing of DC to lymphoid tissues.

The present observations are also consistent with recent reports that IL-10 increases CCR5 expression in human monocytes by increasing the half-life of the mRNA (37). Another immunosuppressive cytokine, TGF- $\beta$ 1, that, like IL-10, suppresses DC maturation and function (8) inhibits the expression of CCR7 transcripts in DC and their precursors derived from mouse hemopoietic progenitor cells (52). In addition, the migration of these cells in response to MIP-3 $\beta$  is suppressed. Furthermore, TGF $\beta$ 1 enhances CCR5 expression by human monocyte-derived DC, concomitant with enhanced chemotactic migratory responses to its inflammatory ligand, RANTES (53). In keeping with the present findings concerning the capacity of IL-10 to down-modulate LPS-induced expression of CCR7 and chemotactic activity in response to MIP-3 $\beta$ , TGF- $\beta$ 1 has been shown to exert similar effects on TNF- $\alpha$ -stimulated human DC (52).

Our findings clearly show that in vivo homing to secondary lymphoid tissue of myeloid DC pre-exposed to IL-10 and thus retaining an immature phenotype was markedly inhibited compared with that of untreated DC. Although the role of IL-10 in leukocyte trafficking in vivo remains unclear, insights provided by IL-10 knockout mice suggest that it suppresses T cell and macrophage accumulation in transplanted tissue and, therefore, may confer protection against rejection (28). Immature DC may be converted in vivo into mature DC by extracellular stimuli, including TNF- $\alpha$ , LPS, and CD40 ligation in sites of inflammation, such as skin, and may lose their responsiveness to proinflammatory chemokines (39). In turn, CCR7 may be induced in mature DC, and these cells may acquire responsiveness to MIP-3 $\beta$ , which is specifically expressed in T cell-rich areas of secondary lymphoid tissue where DC home to become interdigitating DC (38, 51). These properties may account for the accumulation of Ag-loaded mature DC in T-dependent areas of secondary lymphoid tissue. We showed that IL-10 enhanced MIP-1 $\alpha$ -mediated chemotaxis via up-regulation of CCR5 in DC, while the chemotactic migratory activity of DC for MIP-3 $\beta$  was inhibited by IL-10 via down-regulation of CCR7 expression. Collectively, these observations suggest that IL-10 is involved in the trafficking behavior of immature and mature DC via regulating chemotaxis to CC (and probably other) chemokines.

To directly examine the influence of CCR7 expression on control and IL-10-treated DC migration in vivo, we examined the functions of DC genetically modified to express high levels of CCR7. Our results show that CCR7 transduction enhanced the ability of the control and, to a lesser extent, the IL-10-treated DC to migrate to draining lymph nodes within 24 h of injection. Its expression appeared to play an important role in promoting the migration of these cells from peripheral sites to secondary lymphoid tissue. Moreover, we were able to demonstrate that enhanced migration of CCR7-transduced control DC, but not CCR7-transduced IL-10-treated DC, was associated with enhanced priming of recipient T cells manifested by augmented ex vivo proliferative and IFN- $\gamma$  responses upon rechallenge with alloantigen. Because retroviral transduction did not affect the expression of maturation markers on the DC, it is possible that CCR7-transduced, IL-10-treated DC retained an immature APC phenotype (low levels of surface costimulatory molecules) after homing to T cell areas of lymphoid tissue. This may explain the lack of T cell allostimulatory potential of these APC.

We and others have demonstrated previously that exposure of myeloid DC to m- or vIL-10 impairs their maturation and T cell stimulatory capacity (14, 26) and that either treatment of DC with IL-10 or retroviral delivery of vIL-10 to DC induces allospecific T cell hyporesponsiveness (16, 26). In the present study we have extended these observations to show that inhibition of DC maturation by v- or mIL-10 is associated with up-regulation of mRNA-encoding receptors for inflammatory chemokines (CCR1, CCR5) and down-regulation of CCR7 mRNA expression. The impaired homing capacity of IL-10-treated DC to secondary lymphoid tissue, probably linked to decreased CCR7 expression, may be another component of the immunosuppressive effect of IL-10.

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