The murine chemokine receptor CXCR4 is tightly regulated during T cell development and activation

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Abstract: We have characterized the murine homolog of the HIV-co-receptor CXCR4 during T cell development and activation. Our data demonstrate that this chemokine receptor, although highly conserved between human and mouse, is differently expressed and regulated in both species. Mitogenic activation resulted in an increase of surface CXCR4 on murine T cells within 2 days, whereas the receptor was strongly down-regulated on human T cells during this period. Furthermore, intraperitoneal immunization of mice resulted in a strong increase of splenic and mesenteric cytotoxic T cells co-expressing CXCR4. It is interesting that, on thymocytes, expression of CXCR4 is restricted to $CD4^+CD8^+$ cells. Stromal cell-derived factor-1α, a natural ligand of CXCR4, induced chemotaxis of thymocytes and was found to counteract dexamethasone-induced apoptosis to a certain extent in these cells. Thus, our data show that expression of CXCR4 is tightly controlled on murine T cells and indicate that this highly conserved chemokine receptor might serve different functions in humans and mice. *J. Leukoc. Biol.* 66: 996–1004; 1999.

Key Words: *thymocytes · $CD4^+CD8^+$ cells · SDF-1α*

INTRODUCTION

The migration of lymphocytes through the body is a strictly regulated process directing distinct lymphocyte subsets to particular lymphoid organs such as spleen, lymph nodes, and thymus. Once having entered the lymphoid organs, cells have to migrate to defined microanatomic compartments such as B cell follicle, the T cell-rich periarteriolar lymphoid sheath (PALS), or to the germinal center (GC) in order to fulfill their function during immune surveillance. Several families of adhesion molecules, including selectins and integrins, have been identified to participate in this phenomenon and a multistep model of adhesion and transendothelial migration has been proposed [1–3]. However, molecular mechanisms underlying the localization of lymphoid cells within lymphoid organs are still poorly understood. Using gene targeted mice we recently identified the Burkitt's lymphoma receptor 1 (BLR1/CXCR5), a member of the chemokine receptor family, as the first molecule involved in this process by directing the migration of B cells from the PALS to the B cell follicles in Peyer's patches and the spleen [4]. Because the migration of lymphocytes to and within other organs was not affected in CXCR5-mutant mice, it seems likely that further chemokine receptors direct the migration of various lymphocyte subsets during maturation and activation to different lymphoid organs and specialized microenvironments.

Besides their role in leukocyte migration, some other members of the chemokine receptor family have been shown to function as major HIV co-receptors [reviewed in ref. 5]. Expression of human CXCR4, one of those receptors, has been observed on different cells of the immune system including B cells, monocytes, macrophages, Langerhans cells, neutrophils, and T cells [6–9]. In the thymus, expression of CXCR4 was found on all stages of T cell development including double-positive ($CD4^+CD8^+$) and single-positive ($CD4^+CD8^-$; $CD4^-CD8^+$) T cells [8]. In the peripheral blood CXCR4 has been observed on the majority of both T helper cells and cytotoxic T cells with some preference for naive and resting cells carrying the CD45RA isoform [8, 9].

Recently, two murine homologs of human CXCR4 have been identified that are generated by alternate splicing of the region encoding the amino-terminal domain of the receptor. The short form lacks two amino acid residues (Val^6, Ser^7), resulting in an isoform more closely related to human CXCR4 [10–13]. Little is known about the expression and regulation of these receptor molecules during leukocyte maturation and activation. In this study, we characterized expression and regulation of the murine chemokine receptor CXCR4, a molecule that is evolutionarily highly conserved between various species including rat, cattle, sheep, monkey, and human [13]. We show that expression of murine CXCR4 on T cells is remarkably different from the situation described in human. It is interesting that the highest levels of CXCR4 on mature T cells were observed on $CD4^+$ and $CD8^+$ cells isolated from the bone marrow. In addition, we observe an organ-specific expression pattern of the receptor on T helper cells, whereas, on activation, CXCR4 is preferentially induced on $CD8^+$ T cells *in vivo*. On thymocytes, expression of CXCR4 is restricted to intermediate stages of T cell development as expression of this receptor was found on $CD4^+CD8^+$ thymocytes, but not on cells representing either earlier

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(Thy1.2$^+$CD3$^-$CD4$^-$CD8$^-$) or later (CD4$^+$CD8$^-$; CD4$^-$CD8$^+$) stages of T cell maturation. Together with the observation that stromal cell-derived factor-1α (SDF-1α) also reduces glucocorticoid-induced apoptosis in thymocytes to a certain extent, our data suggest that CXCR4 and its ligand are involved in T cell development and activation.

MATERIALS AND METHODS

Mice

NMRI and BALB/c mice were kept at the animal facility of the Max-Delbrück Center for Molecular Medicine, Berlin and were usually 6–10 weeks old when used.

Cloning of murine CXCR4

Reverse transcriptase polymerase chain reaction using total RNA preparation from murine thymus was carried out with degenerated primers. Five micrograms of total RNA served as template for the Superscript reverse transcriptase (GIBCO-BRL, Eggenstein, Germany) reaction in the presence of 50 fmol backward primers GSP-RT-CXCR4 (5'-ACG ATG CCG GGC AGG ATG AG-3'; 80°C, 5 min; ice, 37°C, 60 min). Tailing reaction was done for 5 min at 37°C, followed by 5 min at 65°C. In the first step of a nested polymerase chain reaction (PCR) the following primers and conditions were used: GSP-1-CXCR4 (5'-CAC AGA TGT ACC TGT CAT CC-3'); QT (5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCA AGCTTTTTT TTTTTTTTTT-3'); Q0 (5'-CCAGTGAGCAGAGTGACG-3'); conditions were 1 cycle at 98°C, 300 s; 75°C, 120 s; 50°C, 120 s; 72°C, 40 min; 30 cycles at 94°C, 60 s; 56°C, 60 s; 72°C, 180 s; 1 cycle at 72°C, 600 s. A second amplification step was done using GSP-2-CXCR4 (5'-CCT TGG CCT TTG ACT GTT GG-3') and Q1 (5'-GAGGACTCG AGCTCAAGC-3') primers at the following conditions: 30 cycles at 98°C, 120 s; 94°C, 60 s; 60°C, 60 s; 72°C, 180 s. After size fractionation fragments were isolated and cloned into the KpnI-HindIII site of a p-Bluescript KS(-) vector and then in a Rc-CMV expression plasmid as described earlier [8].

Antibodies

The following fluorescein isothiocyanate- (FITC), phycoerythrin-(PE), or biotin-labeled antibodies and conjugates were used: anti-CD4 (L3/T4, clone YTS 191.1), anti-CD8 (CT-CD8a), and anti-Thy 1.2 (clone 5a-8) purchased from Caltag (Medac, Hamburg, Germany); anti-CD62L and anti-CD44 from PharMingen (Hamburg, Germany); anti-CD3 from Sigma (Deisenhofen, Germany); and anti CXCR4 (clone 2B11) was generated in our laboratory and was described earlier [8].

Western blotting

Using thymocyte membranes solubilized with 0.025% Digitonin (Sigma) polyacrylamide gel electrophoresis (PAGE) and Western blotting were done as described previously [14].

Immunohistochemistry

Thymi were isolated from 6- to 10-week-old animals and snap frozen. Ten-micrometer cyrosections were incubated with biotinylated anti-CXCR4 mAb and revealed using FITC-labeled tyramide as described by the manufacturer (TSA-Direct, DuPont-NEN; Bad Homburg, Germany). Slides were analyzed by confocal microscopy (Leica, Bensheim, Germany).

Flow cytometry

Flow cytometry was done as described elsewhere [15]. In brief, using NH_4Cl lysis red blood cells were depleted from a single-cell suspension of spleen and peripheral blood. These cells and cells derived from further lymphoid organs were washed twice in FACS buffer [phosphate-buffered saline (PBS), 2% fetal calf serum (FCS), 0.1% NaN_3, 10 mM HEPES, pH 7.3] and were resuspended in FACS buffer containing 5% aggregated rat serum. Cells were subsequently stained with various combinations of FITC- and PE- or biotin-conjugated antibody for 30 min at 4°C. After two washes, biotinylated antibodies were revealed with streptavidin-PE conjugate or streptavidin-CyChrom (Jackson, 1:100). Flow cytometric analysis was done using a FACScan flow cytometer (Becton Dickinson, Heidelberg; Germany).

Cell culture

Lymphocytes were isolated from human blood by Ficoll density centrifugation. Murine spleens were disrupted by straining them through a 70-μm nylon mesh. Erythrocytes were lysed by NH_4Cl and macrophages were removed by plastic adherence. Lymphocytes were cultured in RPMI-1640 supplemented with 10% FCS at 37°C, 5% CO_2 for up to 6 days. To some samples phytohemagglutinin (PHA) was added at 2.5 μg/mL. After 3 days, medium was replaced.

Endocytosis assay

To test whether SDF-1α induces down-regulation of surface CXCR4, thymocytes and cells isolated from mesenteric lymph nodes were incubated with recombinant SDF-1α (Peprotech, London, UK or R & D Systems, Wiesbaden, Germany) at 37°C. After 45 min cells were washed extensively and stained with anti-CXCR4 mAb (30 min on ice) to determine the amount of surface-expressed CXCR4 by flow cytometry.

Apoptosis assay

Thymocytes (5×10^6/mL in RPMI, 10% FBS) were incubated with 2 μM dexamethasone in the presence of various concentrations of SDF-1α at 37°C, 5% CO_2. After 5 h cells were harvested and the percentage of apoptotic nuclei was determined by flow cytometry using recombinant annexin V-FITC (Boehringer Mannheim, Germany) and propidium iodide (Sigma) as described by others [16].

Assay for chemotaxis

The *in vitro* migration of cells in response to SDF-1α was assessed in a modified 96-well Boyden chamber (Neuro Probe Inc). Thirty microliters of SDF-1α dilutions in RPMI/0.5% BSA were loaded in the lower compartment in triplicate and 75,000 thymocytes in 50 μL RPMI were placed in the upper compartment. The two compartments were separated with a polyvinylpyrrolidone-free polycarbonate filter with 5-μm pores precoated with 20 μg/mL mouse type IV collagen. The chamber was incubated for 2 h at 37°C, 5% CO_2. The filter was fixed and stained in methanol supplemented with 5% glutardialdehyde and 1% crystal violet. Migrated cells were counted in 5–15 randomly selected high-power fields (×400) per sample.

RESULTS

Monoclonal antibody 2B11 binds to murine CXCR4

When testing mAb 2B11, an antibody raised against human CXCR4, we noticed that this mAb also stained some cells obtained from murine lymphoid organs. Therefore, we cloned a cDNA encoding the short isoform of the murine homolog of the receptor. The cDNA sequence obtained (EMBL accession number: Z80111) is identical to those published by others [12, 13] and demonstrates an overall identity of 91% to human CXCR4 on the amino acid level (data not shown). Subsequently, rat basophilic leukemia (RBL) cells, as well as NIH 3T3 cells, were transfected with an expression plasmid encoding a fusion protein consisting of the entire muCXCR4 (short isoform) and a myc epitope at the carboxy-terminal end. These transfected cells were used to determine whether 2B11 also recognizes the murine homolog. mAb 2B11 bound to transfected NIH 3T3 cells expressing muCXCR4 (**Fig. 1A**), but not to those

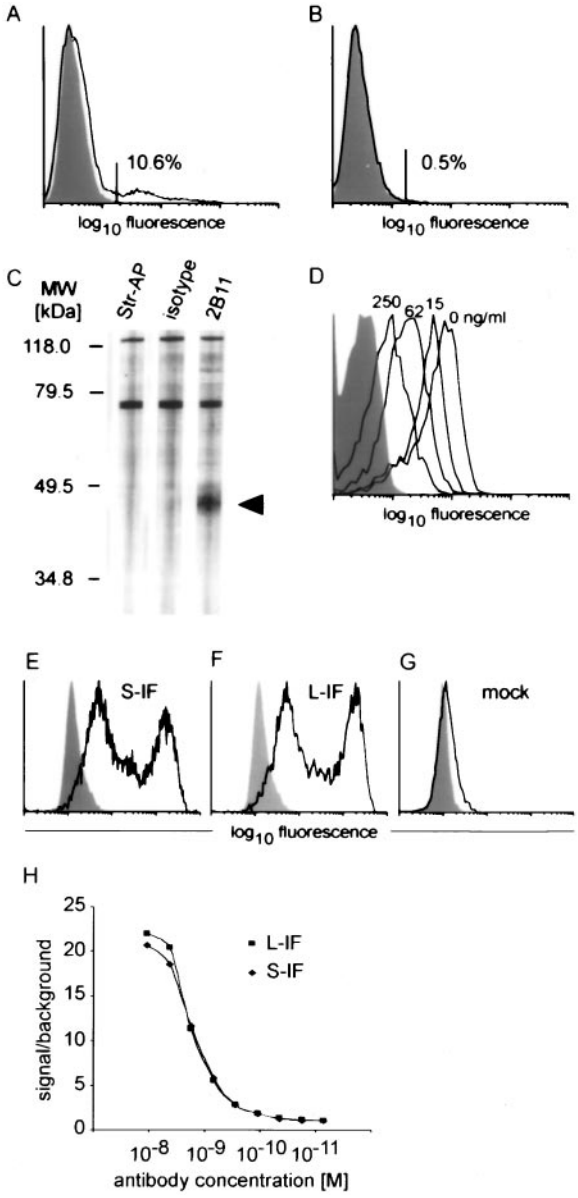

Fig. 1. mAb specific for muCXCR4, (A, B) Mouse NIH 3T3 cells were transiently transfected with expression clones encoding a myc-tagged muCXCR4 (A), or huCXCR5 (B). Samples were incubated with anti CXCR4 mAb 2B11 (solid line) or isotype control antibody (shaded area) and after counter-staining with secondary antibody analyzed by flow cytometry. (C) Immunoblotting of solubilized crude membranes of murine thymocytes with anti-CXCR4 mAb and isotype control antibodies as indicated. (D) Thymocytes were incubated for 45 min at 37°C with various concentrations of SDF-1α as indicated. Cells were washed, stained on ice with anti-CXCR4 mAb and subjected to flow cytometry. For details see Materials and Methods. (E–H) Sf9 cells were infected with bacculoviruses encoding either the short isoform (S-IF; E), the long isoform (L-IF; F), or huCXCR5 (mock; G). After 3 days cells were stained and analyzed as described for A and B. (H) Sf9 cells expressing either the S-IF or the L-IF of muCXCR4 were incubated with various concentrations of biotinylated mAb 2B11 or isotype control antibody. After counter-staining with streptavidin-PE, samples were subjected to flow cytometry and the mean fluorescence intensity (MFI) was determined. Shown are ratios of MFI (2B11) to MFI (isotype antibody) for various antibody concentrations.

expressing huCXCR5 (Fig. 1B) or various other chemokine receptors (data not shown). After SDS-PAGE of solubilized membranes derived from murine thymocytes and Western blotting, mAb 2B11 identifies a major band with an apparent molecular mass of about 44.6 kDa, which comes close to the predicted molecular mass derived from the amino acid sequence (41 kDa, see Fig. 1C). We further confirmed the specificity and selectivity of this mAb by incubating murine thymocytes for 45 min at 37°C with various concentrations of SDF-1α, a natural ligand of CXCR4. Because binding of SDF-1 to CXCR4 does not interfere with mAb 2B11 binding (not shown), this antibody was used to determine the amount of surface-expressed CXCR4. As previously demonstrated for human lymphocytes [8, 17], SDF-1α down-regulated the expression of muCXCR4 in a dose-dependent way, resulting in a loss of more than 85% of initial cell-surface levels at the highest concentration chosen (Fig. 1D). To test whether mAb 2B11 recognizes both isoforms of muCXCR4, Sf9 cells were infected with baculoviruses recombinant for either the short or the long isoform of muCXCR4. As shown in Figure 1 (E–G) mAb 2B11 binds to both isoforms of muCXCR4 but not to mock-infected Sf9 cells. Furthermore, we used baculovirus-infected cells to determine the avidity with which mAb 2B11 binds to the short and the long isoform of the receptor. To that end biotinylated mAb 2B11 and isotype control antibody were titrated on infected Sf9 cells and the mean fluorescence intensity of the cells (MFI) has been determined by FACS analysis. Calculating the ratio of MFI (mAb 2B11) to MFI (isotype control) for various antibody dilutions, similar binding curves were obtained for the short and the long isotype (Fig. 1H). Subjecting these values to non-linear regression analysis (Prism 2.0; GraphPad Software Inc.; San Diego, CA) K_d values of 1.33×10^{-9} M and 1.50×10^{-9} M were calculated for the long and the short isoform, respectively. Taken together, these data demonstrate that mAb 2B11 binds equally well to both isoforms of murine CXCR4.

Organ-specific expression of CXCR4 on T helper cells

We used mAb 2B11 to study expression and regulation of the receptor on mouse T cells derived from either NMRI or BALB/c mice by means of flow cytometry. Within the range of experiments performed, the expression pattern of CXCR4 was found to be identical in both strains. In the peripheral blood 5–10% of both $CD4^+$ or $CD8^+$ T cells were found to express CXCR4 (**Fig. 2**). This finding is contrasted by the observation made in human peripheral blood where 40% of the cytotoxic T cells and 40–70% of T helper cells express CXCR4 [8, 18]. Furthermore, CXCR4 expression was studied on T cells derived from several secondary lymphoid organs. The receptor was found on 6 ± 3, 11 ± 3, 21 ± 10, and 42 ± 17% (mean $\pm$ SD, $n = 6$) of $CD4^+$ cells isolated from inguinal lymph nodes (ILN), spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PP), respectively (Fig. 2A). On average, 15–30% of $CD8^+$ cells isolated from secondary lymphoid organs expressed this chemokine receptor (Fig. 2A). On human T cells CXCR4 is preferentially expressed on antigen-inexperienced, naive subpopulations [8, 18]. We therefore used three-color flow cytometry applying anti-CD3, anti-CXCR4, and mAb discriminating naive and memory T cells such as anti-CD44 or anti-CD62L. As shown in Figure 2, B and C, we could not observe any preferential expression of CXCR4 on naive T cells in the mouse.

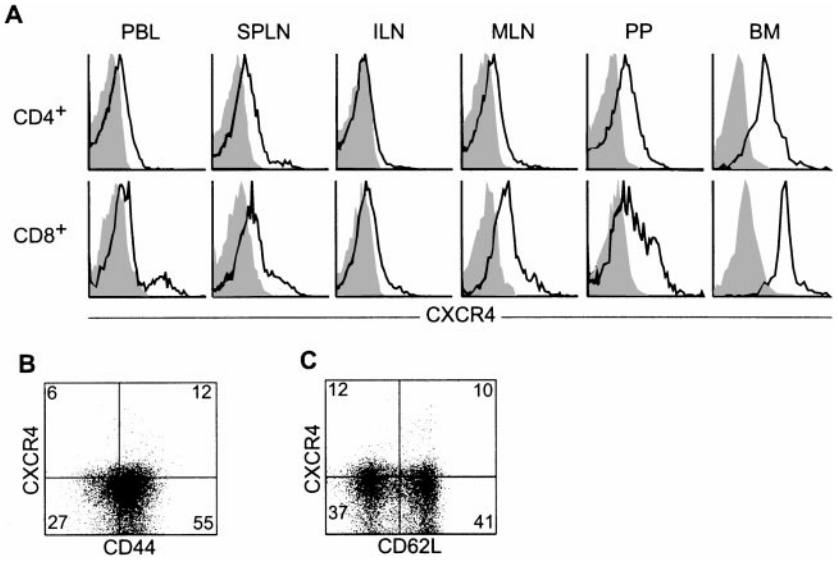

Fig. 2. Expression of CXCR4 on T cells isolated from various organs. Lymphocytes were isolated from various organs of BALB/c mice as indicated and double-stained with antibodies specific for CD4 or CD8 and anti-CXCR4 (solid lines) or isotype control antibody (shaded area, A). Lymphocytes isolated from mesenteric lymph nodes were triple-stained with anti-CD3, anti-CXCR4, and anti-CD44 (B) or anti-CD62L (C) and subjected to flow cytometry. The staining pattern of $CD3^+$ cells is shown. Numbers indicate the percentage of positive cells within each quadrant. One representative experiment of four to eight independent experiments is shown. (SPLN, spleen; ILN, inguinal lymph node; MLN, mesenteric lymph node; PP, Peyer's patches; BM, bone marrow).

Murine and human CXCR4 are differently regulated after T cell activation *in vitro*

Because induction of huCXCR4 has been observed after *in vitro* stimulation of T cells [9], we compared receptor expression during T cell activation *in vitro*. Freshly isolated human peripheral blood mononuclear cells (PBMC) or murine spleen cells were cultured in medium without or with PHA (2.5 μg/mL) for up to 6 days. Expression of CXCR4 was determined on days 0, 1, 2, 3, 4, and 6. Cells were double stained with anti-CD4-FITC or anti-CD8-FITC and biotinylated anti-CXCR4 or isotype control mAb followed by incubating cells with SA-PE. Gates were set to $CD4^+$ or $CD8^+$ cells and the mean fluorescence intensity of each cell population was determined for CXCR4 and isotype control mAb. The ratio of MFI (CXCR4)/MFI (control) has been determined and set to 100 for each cell population at $t = 0$. Changes of CXCR4 during culture were related to this setting. When culturing human or murine lymphocytes without PHA for 24 h we observed an increase in CXCR4 expression on T cells derived from both species. Relative expression levels of CXCR4 increased to 151 (mean, $n = 5$) and 137 on murine $CD4^+$ and $CD8^+$ cells, respectively (**Fig. 3A**). However, when culturing human lymphocytes under the same conditions, a much stronger increase in the expression of CXCR4 was observed, as relative expression values increased to 265 and 188 on $CD4^+$ and $CD8^+$ cells, respectively (Fig. 3A). Major differences in the regulation of CXCR4 between human and mouse became obvious once cells had been activated with PHA. On both murine $CD4^+$ and $CD8^+$ cells increased levels of CXCR4 could be observed during the first 2 days after stimulation, followed by a marked drop on day 3 and increasing expression from day 4 onward (Fig. 3, B and C). In contrast, after activating human T cells, expression of CXCR4 was highly reduced during the first 2 days of culture followed by a strong induction of receptor expression from day 4 onward (Fig. 3, B and C). These data demonstrate that CXCR4 is differently regulated on human and murine T cells during the initial phase of T cell activation.

Preferential induction of CXCR4 on $CD8^+$ cells after *in vivo* activation

Because we usually observed some higher expression of CXCR4 on $CD8^+$ cells than on $CD4^+$ cells (Fig. 2), we wondered whether CXCR4 is differently regulated on T cells after activation *in vivo*. We therefore injected mice intraperitoneally with 100 μg of the T cell-dependent antigen TNP-KLH in incomplete Freund's adjuvant. Twelve days later, at the peak of the germinal center reaction, T cells were isolated from spleen and mesenteric lymph node because these are the lymphoid organs that are primarily involved in the immune response elicited by intraperitoneal antigen application. Compared with untreated controls, no obvious changes could be observed on T helper cells regarding CXCR4 expression in spleen and mesenteric lymph nodes after immunization (**Fig. 4**, **A** and **B**). However, in all animals immunized ($n = 6$), we observed a strong increase in CXCR4 expression on all $CD8^+$ T cells isolated from these two organs (Fig. 4, C and D). These data indicate that on activation *in vivo* expression of CXCR4 in the T cell compartment is primarily induced on cytotoxic T cells. Alternatively, immunization selectively induces the migration of $CXCR4^+CD8^+$ cells from the periphery into these organs. Independent of the underlying mechanism it thus seems unlikely that increased expression of CXCR4 on T cells is due to antigen-specific activation as the frequency of antigen-specific T cells is usually well below 1 in 1000. It therefore seems more likely that increased expression levels of CXCR4 reflects an inflammatory response.

Differential expression of CXCR4 during T cell development

Analyzing CXCR4 on human thymocytes, we found expression of the chemokine receptor at all stages of T cell maturation including $CD4^+CD8^+$ double-positive and single-positive cells [8]. In contrast, in the mouse thymus, we identified a remarkably restricted expression pattern of the receptor during T cell maturation. Expression of the CXCR4 was limited to all cells

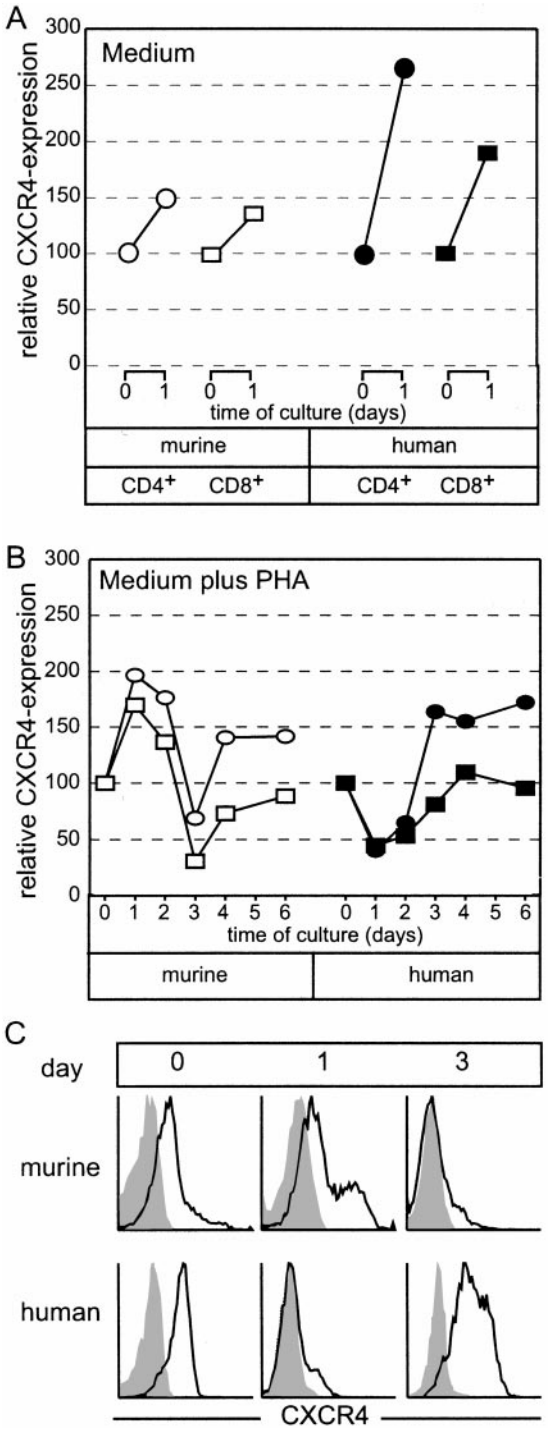

Fig. 3. Differential regulation of CXCR4 on murine and human T cells Murine lymphocytes (open symbols) or human lymphocytes (closed symbols) were cultured in medium (A) or in medium supplemented with 2.5 μg/mL PHA (B). At the time points indicated aliquots were withdrawn double-stained with anti-CD4 (circles) or anti-CD8 (quadrants) and anti-CXCR4 or isotype control antibodies. For each cell population ($CD4^+$ or $CD8^+$), the mean fluorescence intensity for CXCR4 or isotype staining has been determined and relative CXCR4 expression has been determined as described in Results. Shown are mean values of two to five independent experiments. The standard deviation averaged 19% of the mean values. (C) The staining pattern of murine and human $CD4^+$ cells are shown after different incubation periods with PHA and staining with anti-CXCR4 (solid lines) or isotype antibodies (shaded area); one representative experiment of data shown in panel B.

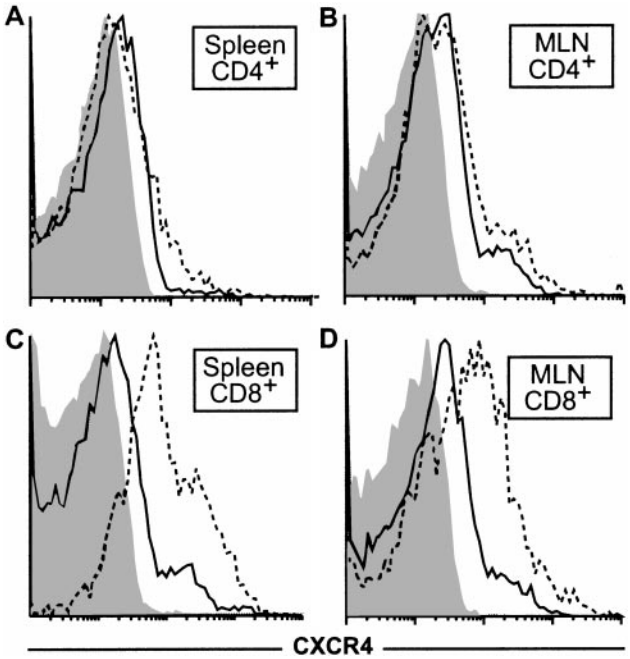

Fig. 4. Induction of CXCR4 on cytotoxic T cells after immunization. BALB/c mice had been untreated (solid lines) or intraperitoneally injected with DNP-KLH in incomplete Freund's adjuvant 12 days earlier (dashed lines). Lymphocytes were isolated from spleen and mesenteric lymph node (MLN) as indicated and stained with anti-CXCR4 (solid or dashed lines) or isotype control antibodies (shaded area). Shown is one representative result of 6 experiments.

representing an intermediate stage of T cell maturation ($CD4^+CD8^+$; **Fig. 5A**), but was virtually absent at earlier ($Thy1.2^+CD3^-CD4^-CD8^-$; Fig. 5B) or later stages ($CD4^+CD8^-$ and $CD4^-CD8^+$; Fig. 5, C and D) of T cell development. CXCR4 is not only expressed on the cell surface but is also stored in large intracellular granules in some human and murine lymphocytes ([8] and data not shown). We therefore subjected cryostat sections of the thymus to immunohistology to explore the possibility that mature thymocytes express the receptor intracellularly. However, we could not identify any medullary cells to express CXCR4 but found, as expected, high-level expression of the receptor on virtually all cells situated in the cortex (see Fig. 5E). These data demonstrate that the chemokine receptor CXCR4 is tightly regulated during thymocyte maturation in the mouse.

SDF-1α is chemotactic for thymocytes and reduces apoptosis

Chemotaxis toward SDF-1α has been reported for various leukocyte populations, including hematopoietic progenitor cells, T cells, B cells, monocytes, and neutrophils [7, 18–21]. Among various organs, SDF-1 was found to be transcribed in the thymus. Because CXCR4, a natural receptor for this peptide, was identified on a subpopulation of thymocytes, we tested the potency of SDF-1α to induce a chemotactic response of these cells *in vitro*. Performing a microchemotaxis assay using a modified 96-well Boyden chamber we detected specific migration of thymocytes toward SDF-1α, which peaked at 30–100 ng/mL of the chemoattractant (**Fig. 6A**). The same concentration was also found to be optimal for both lymphocytes isolated

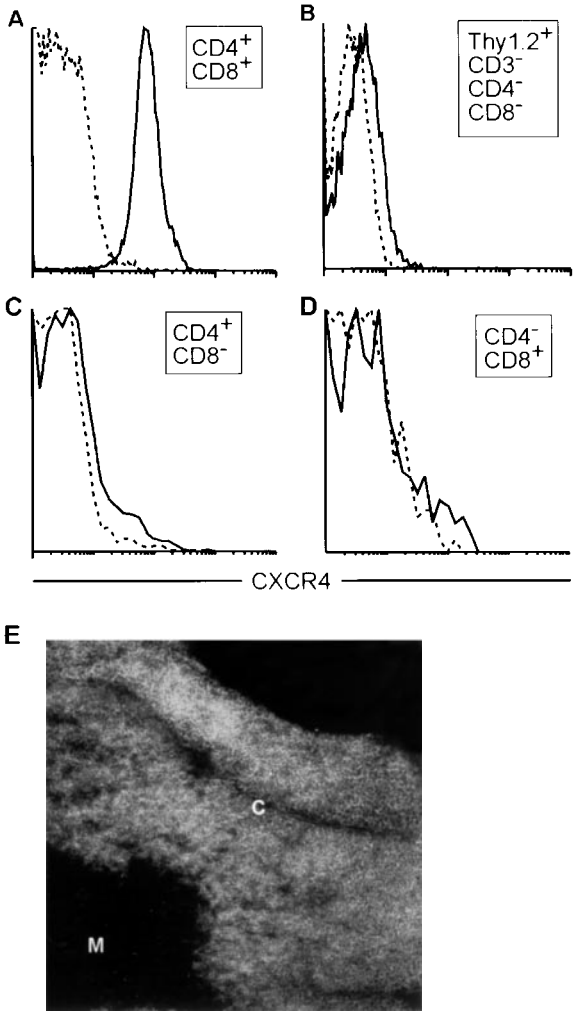

Fig. 5. Restricted expression of CXCR4 on thymocytes. (A–D) Thymocytes were isolated from BALB/c mice as described in Materials and Methods and incubated with biotinylated anti-CXCR4 mAb. After counter-staining with streptavidin-Cychrome (A–D) and various FITC- or PE-labeled mAb as indicated, cells were analyzed by flow cytometry (similar results were obtained from two further BALB/c and three NMRI mice). (E) Cryostat sections derived from the thymus were incubated with biotinylated anti-CXCR4 mAb. Binding was revealed after incubation with streptavidin-peroxidase applying FITC-labeled tyramide as described in Materials and Methods (C, cortex; M, medulla).

from mesenteric lymph nodes (Fig. 6B) and for MACS-purified splenic B cells (data not shown). The potency of SDF-1α to induce chemotaxis of murine lymphocytes seems to be much higher than previously reported for human cells where up to 1.5 μg/mL were needed to maximally induce chemotaxis [22].

CD4CD8 double-positive T cells are the dominating cell population within the thymus. Most of these immature cells undergo apoptosis unless they are rescued by defined signals generated during processes known as positive and negative selection [for reviews see refs. 23 and 24]. Thymocytes, in particular those expressing CD4 and CD8, are highly sensitive to glucocorticoid-induced apoptosis [25], but some cytokines such as interleukin-1 (IL-1), IL-2, and IL-4 have been described to counteract steroid-induced programmed cell death [26–28]. Because we found that expression of CXCR4 was restricted to a thymocyte population highly sensitive toward apoptosis, we were interested to test whether SDF-1α interferes with this process. To assess programmed cell death among thymocytes, we tested these cells for binding of annexin V, a marker of early stages of apoptosis [16]. Thymocytes were incubated with various concentrations of the chemokine with or without 2 μM dexamethasone, a concentration known to induce massive programmed cell death in thymocytes. After 5 h samples were stained with annexin V-FITC and PI and the percentage of apoptotic cells was calculated by determining the amount of annexin V^+ PI^- cells. Without the addition of dexamethasone we could not observe any significant interference of SDF-1α with apoptosis, although we regularly noted a slight reduction of spontaneous programmed cell death in the presence of the chemokine (Fig. 6C). However, when stimulating thymocytes with 2 μM dexamethasone the presence of SDF-1α prevented programmed cell death in a dose-responsive manner. Using SDF-1α at 500 ng/mL, the highest concentration tested, dexamethasone-induced cell death was reduced by 13% (mean value, range 10–22%; $n = 7$; Fig. 6D).

DISCUSSION

We studied the expression and regulation of the murine chemokine receptor CXCR4 and compared it to the situation found in humans. The observed 91% homology between mouse and human CXCR4 is exceptionally high for members of the

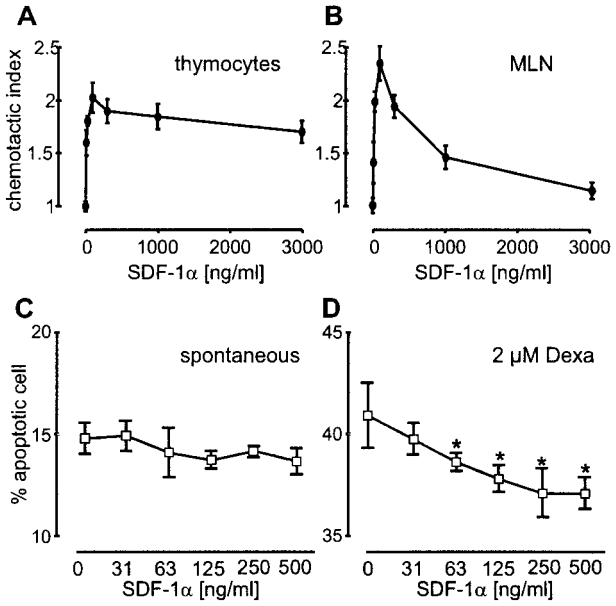

Fig. 6. SDF-1α induces thymocyte migration and reduces glucocorticoid-induced apoptosis. Murine thymocytes (A) and lymphocytes isolated from mesenteric lymph nodes (B) were tested in the modified 96-well Boyden chamber with polycarbonate filters and 5-μm pores for migration of various concentrations of SDF-1α. Data are expressed as the mean ± SEM of cell counts obtained by triplicate determinations. Similar results were obtained from three different experiments. (C, D) Thymocytes were incubated for 5 h with different concentrations of SDF-1α without (C) or with 2 μM dexamethasone (D). Cells were washed, stained with annexin V-FITC and propidium iodide (PI), and the percentage of apoptotic cells (annexin V^+ PI^-) has been determined by flow cytometry. Data shown are mean ± SD of triplicates from one experiment. Similar results were obtained from at least six different experiments (*$P < 0.05$, Wilcoxon test).

chemokine receptor family as only 83% for homology has been reported for human and mouse BLR1/CXCR5 [29, 30], 78% homology for CCR1 [31, 32], and 63% for CCR3 [32, 33]. Together with the fact that SDF-1 shows 99% identity between both species, one is tempted to speculate that both receptor and ligand might serve identical functions in both species. However, when analyzing CXCR4 on murine T cells, we found prominent difference from the situation observed in humans. In general, expression of CXCR4 is more restricted in murine than in human T cells. Both in peripheral blood and in secondary lymphoid organs fewer $CXCR4^+$ T cells are observed and expression levels of this receptor are usually lower in mice [8] (Fig. 2). Furthermore, on activation of human peripheral T cells with PHA, a marked drop of CXCR4 could be observed on both $CD4^+$ and $CD8^+$ cells during the first 2 days followed by an increase of cell surface levels on day 3. These observations are contrasted by the situation made with murine splenic T cells, which showed increased levels of CXCR4 for the first 2 days followed by a drop on day 3 (Fig. 3). Different sources of human (blood) and murine (spleen) T cells were used in this study. However, it seems most likely that the origin (human vs. mouse) rather than the localization (blood vs. spleen) of the T cells analyzed is reflected by our results because a high proportion of lymphocytes present in the blood had left secondary lymphoid organs, such as the spleen, during the last few hours. In addition, on human thymocytes, expression of CXCR4 was found on all stages of T cell development, whereas, on murine thymocytes, expression of CXCR4 was restricted to intermediate stages characterized by the co-expression of CD4 and CD8 (Fig. 5). Taken together, these data indicate that CXCR4 might serve different functions in humans and mice during T cell development and activation.

When analyzing murine T cells derived from different lymphoid tissues we observed an organ-characteristic expression pattern of CXCR4. Levels of this receptor were low or absent on T cells isolated from peripheral lymph node or spleen, intermediate on PP-derived T cells, and high on T cells isolated from the bone marrow. This finding is of particular interest as tissue specific homing has been observed for different T cell subpopulations such as naive or memory T cells. Some difference in tissue-specific migration has been attributed to different levels of surface-expressed adhesion molecules. L-selectin and CD44 are primarily expressed on naive cells and high levels of β7 integrin have been described for B and T cells, which preferentially home to gut-associated lymphoid tissue. However, the majority of adhesion molecules are expressed uniformly on leukocytes [reviewed in ref. 3]. In contrast, several chemokine receptors such as BLR1/CXCR5, CXCR4, and CCR7 [4, 8, 9, 15, 34], which are thought to activate adhesion molecules on binding to their ligands, are only expressed on lymphocyte subpopulations. This combination of differentially expressed chemokines and chemokine receptors would allow selective activation of defined adhesion molecules. Furthermore, it would also provide a model to explain differential homing of lymphocytes to various lymphoid organs. High levels of CXCR4 on bone marrow T cells as described in this study would support this hypothesis. They are also in line with a recent study by Sawada et al. who generated mice double transgenic for human CXCR4 and human CD4, both under the control of regulatory elements that restricted expression of the transgenes to $CD4^+$ cells. When analyzing the animals, they observed highly reduced numbers of $CD4^+$ T cells in the peripheral blood but, it is interesting to note, encountered a nearly 10-fold increase of $CD4^+$ cells in the bone marrow [35]. Furthermore, SDF-1 has been initially identified in bone marrow stoma cells. Taken together, all these data strongly suggest that CXCR4 might be a receptor used by T cells to home to the bone marrow.

Taking into account that chemokine-receptor interaction triggers chemotaxis, it seems plausible to assume that these classes of molecules also participate in those processes of T cell maturation in which migration is a prerequisite for organ differentiation. This view would be supported by several findings: (1) some chemokines such as thymus-expressed chemokine (TECK), macrophage-derived chemokine (MDC), and thymus- and activation-regulated chemokine (HTARC) are constitutively and preferentially expressed in the thymus; (2) TECK, a novel CC chemokine is expressed on thymic dendritic cells and is chemotactic for thymocytes [36]; (3) early studies on transgenic mice expressing the S1 unit of pertussis toxin have suggested a role of G-protein-coupled receptor for both entry of early progenitor cells into the thymus and emigration of mature T cells from the thymus [37]; (4) $G\alpha_{i2}$-deficient mice generated by gene targeting have shown profound alterations in thymocyte maturation and function [38]. The different stages of intrathymic maturation are confined to specific microenvironments, most probably reflecting directional migration of the maturating T cells from the cortex toward the medulla. The subcapsular region contains the earliest progenitors characterized by the absence of CD3, CD4, and CD8. These cells are believed to migrate through the cortex toward the medulla. Murine T cells of the thymic cortex are characterized by the co-expression of CD4 and CD8 molecules and, as demonstrated here, by high levels of CXCR4. The molecular mechanisms underlying the directional migration of maturating T cells are poorly understood. In addition to the above views, our findings show that a chemokine receptor and its natural ligand are probably involved in this process. In this study we found high-level expression of CXCR4 in the thymus limited to double-positive cells. This would argue against observations of others who found CXCR4-specific transcripts at all stages of T cell development of the murine thymus by means of RT-PCR analysis [11]. However, we consider the flow cytometry data to be more reliable, apart from the possible involvement of posttranscriptional mechanisms in the regulation of CXCR4 protein expression, which are beyond the scope of RT-PCR analysis. Moreover, Suzuki et al. recently demonstrated that within the thymus SDF-1 preferentially binds to double-positive cells [39]. This correlates to the restricted localization of CXCR4-positive cells to the thymic cortex (Fig. 5E) and demonstrates, for the first time, compartmentalization of thymocytes with regard to the expression of a chemokine receptor.

The fact that several chemokines are present in this organ indicates either synergistic or redundant function. Recent studies analyzing gene-targeted mice would support redundant function of the chemokine system during thymic T cell

development. Mice deficient for SDF-1 [40] or CXCR4 [41–43] show several abnormalities, including defective cardiac ventricular septum formation, deficient formation of blood vessels, derailed migration of cerebellar neurons, and impaired B lymphopoiesis and myelopoiesis. However, T lymphopoiesis seems unaffected in these mice. As outlined above, expression of numerous chemokines has been identified in the thymus. Therefore it seems most likely that other chemokines and chemokine receptors can compensate for both SDF-1 and CXCR4 deficiency during T cell development. The complex expression pattern of chemokines in the thymus also suggests that some members of this protein family might serve additional function apart from inducing cell migration. Indeed, SDF-1 has been originally identified as a factor that stimulates the growth of immature B cells [44] and CC chemokine I-309 has been described to counteract dexamethasone-induced apoptosis in T cell lymphomas [45]. In this study, we identified SDF-1 as a chemokine that interacts with anti-apoptotic processes in thymocytes. In the thymus, apoptosis is known to be a crucial step in T cell development. Among other cells, it affects double-positive thymocytes carrying T cell receptors that are potentially harmful and reactive to self antigens, a process known as negative selection. Thymic negative selection has been intensively studied during the last decade and many of the mechanisms involved in this process have been elucidated [reviewed in ref. 24]. However, little is known about factors that prevent apoptosis during thymocyte development. As outlined above, some growth factors such as IL-2 and IL-4 have been shown to exert such an effect in thymocytes. Studies on gene-targeted mice revealed that the stress-signaling kinase Sek-1 delivers cell survival signals during T cell development [46]. Because SDF-1 shows only moderate anti-apoptotic properties, it remains to be determined whether further thymus-expressed chemokines could also participate in thymocyte selection.

In summary, our data demonstrate that CXCR4, an important HIV receptor, is differently expressed and regulated in humans and mice. This might not only indicate different function of this receptor during T cell development and activation in both species but might also be relevant when testing CXCR4-antagonists in animal models as potential anti-HIV agents.

NOTE ADDED IN PROOF

A study by Suzuki et al. [47], published during the review process of this article, describes expression of CXCR4 on murine thymocyte subpopulations similar to results reported here.

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