

# A CXCR4-Dependent Chemorepellent Signal Contributes to the Emigration of Mature Single-Positive CD4 Cells from the Fetal Thymus<sup>1</sup>

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Developing thymocytes undergo maturation while migrating through the thymus and ultimately emigrate from the organ to populate peripheral lymphoid tissues. The process of thymic emigration is controlled in part via receptor-ligand interactions between the chemokine stromal-derived factor (SDF)-1, and its cognate receptor CXCR4, and sphingosine 1-phosphate (S1P) and its receptor S1PR. The precise mechanism by which S1P/S1PR and CXCR4/SDF-1 contribute to thymic emigration remains unclear. We proposed that S1P-dependent and -independent mechanisms might coexist and involve both S1P-induced chemoattraction and SDF-1-mediated chemorepulsion or fugetaxis of mature thymocytes. We examined thymocyte emigration in thymi from CXCR4-deficient C57BL/6 embryos in a modified assay, which allows the collection of CD62L<sup>high</sup> and CD69<sup>low</sup> recent thymic emigrants. We demonstrated that single-positive (SP) CD4 thymocytes, with the characteristics of recent thymic emigrants, failed to move away from CXCR4-deficient fetal thymus in vitro. We found that the defect in SP CD4 cell emigration that occurred in the absence of CXCR4 signaling was only partially overcome by the addition of the extrathymic chemoattractant S1P and was not associated with abnormalities in thymocyte maturation and proliferative capacity or integrin expression. Blockade of the CXCR4 receptor in normal thymocytes by AMD3100 led to the retention of mature T cells in the thymus in vitro and in vivo. The addition of extrathymic SDF-1 inhibited emigration of wild-type SP cells out of the thymus by nullifying the chemokine gradient. SDF-1 was also shown to elicit a CXCR4-dependent chemorepellent response from fetal SP thymocytes. These novel findings support the thesis that the CXCR4-mediated chemorepellent activity of intrathymic SDF-1 contributes to SP thymocyte egress from the fetal thymus. *The Journal of Immunology*, 2005, 175: 5115–5125.

The completion of T cell-lineage differentiation depends on the active movement of hemopoietic progenitors from the blood to the thymus, and then on the intricate migratory pathway that developing thymocytes follow from the corticomedullary junction, to the outer cortex, and finally to the thymic medulla (1). Systemic immune surveillance requires the emigration of newly derived T cells from the thymus into the peripheral circulation. Although many aspects of intrathymic thymocyte migration have been described, the mechanism by which mature thymocytes exit the thymus is less well understood. There is evidence to support the thesis that chemokinetic agents, including chemokines that signal via G protein-coupled receptors, are involved in thymic emigration by virtue of the fact that this process is pertussis toxin (PTX)<sup>3</sup> sensitive (2, 3). The chemokine CCL19, which is highly expressed by endothelial venules in the thymic medulla, is be-

lieved to serve as a chemoattractant for mature T cells generated within the thymus, guiding thymic emigration into venules (4). Recent studies also point to a pivotal role for sphingosine 1-phosphate (S1P) and its receptor S1PR in thymic emigration (5–7). However, there is also evidence that the action of chemoattractants alone does not fully explain the process of thymic emigration.

It was recently shown that chemorepellent signals elaborated by thymic stroma, including the chemokine stromal-derived factor (SDF)-1 (or CXCL12), might also contribute to thymic emigration. SDF-1 at concentrations >100 nM repels T cells via a CXCR4 receptor-mediated, PTX- and wortmannin-sensitive manner in vitro and in vivo, termed chemorepulsion or fugetaxis (8, 9). We have previously shown that CD4 and CD8 SP T cells migrate away from both human and murine thymic fragments and a thymic organoid in a system in which the presence and action of an extrinsic chemoattractant was excluded (10). This process was dependent on a G<sub>ai</sub> protein-coupled receptor and PI3K signal transduction pathway (9). More recently, it has been proposed that a chemorepellent chemokine signaling pathway exists via CCR7, which directs immature thymocytes away from the medulla to the cortex of the thymus (11, 12).

Studies using CXCR4 knockout mice initially failed to demonstrate a significant role of CXCR4 on T cell development. In fact, whereas the absence of CXCR4 seemed to be critical for myeloid and B lymphopoiesis, no significant defect in T cell development was observed nor were defects in lymphoid organ repopulation found when CXCR4<sup>-/-</sup> thymi were engrafted into congenic mice (13–15). However, subsequent studies demonstrated that expansion of T cell precursors in embryonic thymi was impaired in the absence of CXCR4. Moreover, chimeric mice competitively

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<sup>3</sup> Abbreviations used in this paper: PTX, pertussis toxin; FTOC, fetal thymus organ culture; RTE, recent thymic emigrant; SDF-1, stromal-derived factor 1; SP, single positive; S1P, sphingosine 1-phosphate; WT, wild type; MFI, mean fluorescence intensity; DP, double positive; CD62L, CD62 ligand; DN, double negative.

repopulated with CXCR4<sup>-/-</sup> fetal liver cells displayed markedly reduced donor-derived thymocytes, supporting the idea that CXCR4 plays a role in expansion of T cell precursors in thymus. Our previous studies explored the role of CXCR4 and SDF-1 in thymic emigration from normal thymi (8–10).

By using a modified fetal thymus organ culture (FTOC) system in which intrathymic signaling can direct the movement of cells away from the thymus in the absence of extrathymic chemoattractants, we demonstrated that in the presence of CXCR4/SDF-1 signaling, thymocytes with the phenotypic pattern of recent thymic emigrants (RTE) were able to migrate out of the thymus. However, despite normal thymic development, mature SP thymocyte emigration was shown to be severely impaired in fetal thymi from CXCR4<sup>-/-</sup> mice. This defect led to a significant reduction in SP CD4 thymocyte emigration and the retention of this subpopulation in the thymus. Addition of SDF-1 to the lower chamber of the FTOC system significantly reduced the emigration of SP CD4 thymocytes, due to ablation of the chemokine gradient. Treatment of FTOC from CXCR4<sup>-/-</sup> mice with exogenous S1P, serving as an extrathymic chemoattractant in the experimental system, partially restored SP CD4 emigration but failed to significantly affect SP CD4 emigration from normal thymi. We found that treatment of CXCR4<sup>+/-</sup> mice or thymi with the CXCR4 antagonist AMD3100 recapitulated the defect in SPCD4 emigration. We therefore demonstrated that the chemorepellent action of the SDF-1/CXCR4 axis contributes to the egress of mature SP CD4 cells from the fetal thymus via an S1P-independent mechanism.

## Materials and Methods

### Mice

E15.5 CXCR4<sup>-/-</sup> embryos were generated by breeding CXCR4-deficient heterozygous mice on a C57BL/6 (B6) background. Presence of the vaginal plug was considered to represent gestational day 0.5. Mice and embryos were genotyped as previously described (15, 16). Mice used in the current study were bred in a pathogen-free facility, in accordance with National Institutes of Health animal research guidelines.

### FTOC

Pregnant females at day 15.5 of gestation were sacrificed by CO<sub>2</sub> asphyxiation, and embryos were chilled on ice. Fetal thymi were carefully removed under a dissecting microscope taking care to keep the anatomically identifiable lobes joined together. Thymi were placed in HBSS and kept on ice. Individual thymi were gently transferred onto the surface of a polycarbonate membrane of a 6.5-mm Transwell insert with a 3.0- $\mu$ m pore size (Corning Life Sciences). DMEM (360  $\mu$ l) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin,  $2 \times 10^{-5}$  M 2-ME, 0.1 mM nonessential amino acids, 10% HEPES, and 10% FBS (Sigma-Aldrich) was added to the lower chamber of the Transwell. This amount of medium allowed the thymus to be at the interface of medium and air. The cultures were incubated at 37°C in 5% CO<sub>2</sub> and medium was replaced every 3 days. Thymic emigrants were collected from each thymus by transferring each Transwell insert containing one thymus onto a new well 24 h before harvesting emigrants present in the lower chamber for analysis. On the day of analysis, two thymi were harvested and emigrants were pooled from at least four thymic lobes from CXCR4<sup>+/-</sup> and CXCR4<sup>-/-</sup> mice. For each time point, CXCR4<sup>+/-</sup> and CXCR4<sup>-/-</sup> thymi from one or two pregnant females bred at the same time were analyzed, aiming to minimize differences in the stage of maturation and total cell recovery. Wild-type (WT) E15.5 (CXCR4<sup>+/+</sup>) thymi were obtained from fetuses from B6 mice breedings. For inhibition studies on thymic emigration, PTX (100 ng/ml; Sigma-Aldrich) or murine recombinant SDF-1 (50, 500, and 1000 nM; PeproTech) was added to WT FTOC at day 14 and thymus and emigrants were harvested at day 15 of culture (9). FTOC from 15.5-day postcoitum (CXCR4<sup>+/-</sup> or CXCR4<sup>-/-</sup>) embryos were also prepared as discussed and S1P (10 or 100 nM) or AMD3100 (0.5 or 1  $\mu$ g/ml; Sigma-Aldrich) was added to the lower chamber of day 14 FTOC. Thymic emigrants were then collected after 24 h (7, 17).

### Immunofluorescence staining and flow cytometric analysis

Thymic lobes were harvested and transferred to a 4-ml round-bottom polystyrene tube containing 0.4 mg/ml collagenase (from *Clostridium histolyticum*, type V, clostridiopeptidase A; Sigma-Aldrich), 10% FBS, EDTA 0.2 mg/ml, and 0.2 M phosphate. After 30 min incubation at 37°C, thymi were disaggregated by gentle pipetting until no visible fragments remained. Cell suspensions were washed once in HBSS and 10% FBS to prevent further enzyme action. Cells were resuspended in PBS containing 0.1% BSA and counted using a hemocytometer. After incubation for 30 min on ice with 0.5  $\mu$ g of 2.4G2 Ab to block Fc binding, direct staining for three- or four-color analysis was performed. Cell suspensions were incubated for 15 min at 4°C and then washed twice with staining buffer and analyzed using a FACSCalibur (BD Biosciences). Data analysis was performed with FlowJo software (Tree Star). The following mAbs were used: anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-TCR- $\beta$  (clone H57-597), anti-TCR- $\gamma\delta$  (clone GL3), anti-CD69 (clone HI-2F3), anti-CD62 ligand (CD62L, clone MEL-14), anti-CD3 $\epsilon$  (clone 145-2C11), anti-CXCR4 (clone 2B11/CXCR4), anti- $\alpha_4$  integrin (clone 9C10), anti- $\alpha_5$  integrin (clone 5HIO-27), anti-LFA-1 (clone 2D7), and anti-CD44 (clone IM7) (BD Pharmingen). For annexin V and propidium iodide staining, an annexin V-FITC apoptosis detection kit I was used (BD Pharmingen). For each marker, the threshold of positivity was found beyond the nonspecific binding observed in the presence of irrelevant control Ab. Mean log fluorescence intensity (MFI) values were obtained by subtracting the MFI of the isotype control from the MFI of the positively stained sample. The Kolmogorov-Smirnov test was used for statistical analysis of histograms.

### Measurement of proliferative capacity of thymocytes

Embryonic thymocytes from E15.5 were processed as described. A total of  $1 \times 10^5$  thymocytes was cultured in duplicate in complete DMEM. Cells were allowed to proliferate for 2 days in round-bottom 96-well plates precoated with 10, 50, and 100 ng/ml anti-CD3 mAb (145.2C11; BD Pharmingen) in the presence of 1  $\mu$ g/ml soluble anti-CD28 mAb and 25 U/ml murine rIL-2. On day 3, cells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine/well and harvested after 18 h (18).

Single-cell suspensions of thymocytes from E15.5 CXCR4<sup>+/-</sup> and CXCR4<sup>-/-</sup> thymi at day 14 of culture were prepared as described. Thymocytes were resuspended at a density of  $1 \times 10^6$  cells/100  $\mu$ l PBS. An equal volume of 2  $\mu$ M CFSE (Molecular Probes) in PBS was added, and the cell suspension was gently mixed for 10 min at room temperature. Labeled thymocytes were plated at  $2 \times 10^5$  cells/well in round-bottom 96-well plates. Cells were allowed to proliferate for 2 days in round-bottom 96-well plates precoated with 100 ng/ml anti-CD3 mAb in the presence of 1  $\mu$ g/ml soluble anti-CD28 mAb and 25 U/ml murine rIL-2. At the time of harvest, CFSE-labeled thymocytes were washed twice in cold PBS buffer and stained with anti-CD4 and anti-CD8 mAb and flow cytometry was performed.

### Transmigration assays

Quantitative transmigration assays were performed using a Transwell system (Costar; Corning) with 6.5 mm diameter, 5- $\mu$ m pore size polycarbonate membrane as previously described (8). Purified fetal (E16) thymocyte subpopulations ( $5 \times 10^4$  cells) were prepared by FACS as previously described (9) and were added to the upper chamber of each well in a total volume of 150  $\mu$ l of DMEM containing 0.5% FCS. Cell populations were shown to be >99.9% pure by flow cytometric analysis (data not shown). Human SDF-1 (PeproTech) was used at concentrations of 100 ng/ml and 1 or 10  $\mu$ g/ml in the lower, upper, or both lower and upper chambers of the Transwell to generate a checkerboard analysis matrix of positive, negative, and absent gradients of SDF-1, respectively. Thymocyte subpopulations were also pretreated with PTX (100 ng/ml) or anti-CXCR4 mAb (10  $\mu$ g/ml) as previously described and before addition into the transmigration assay (9).

### Thymic emigration in newborns treated with AMD3100

Normal newborn mice were injected i.p. twice a day with AMD3100 at a concentration of 1  $\mu$ g/kg for 5 days from birth (days 21–23 of gestation). Thymus spleen and lymph nodes were then harvested by a dissecting microscope and single-cell suspensions prepared. Lymph node cell preparations consisted of all visualized nodes including axillary, inguinal, and popliteal nodes.

Total cell number was determined using a hemocytometer, and subset analysis for CD4, CD8, and CD3 was performed on a FACSCalibur.

## Results

### *Emigrant thymocytes from FTOC are clearly distinct from intrathymic cells and have the immunophenotype of RTE*

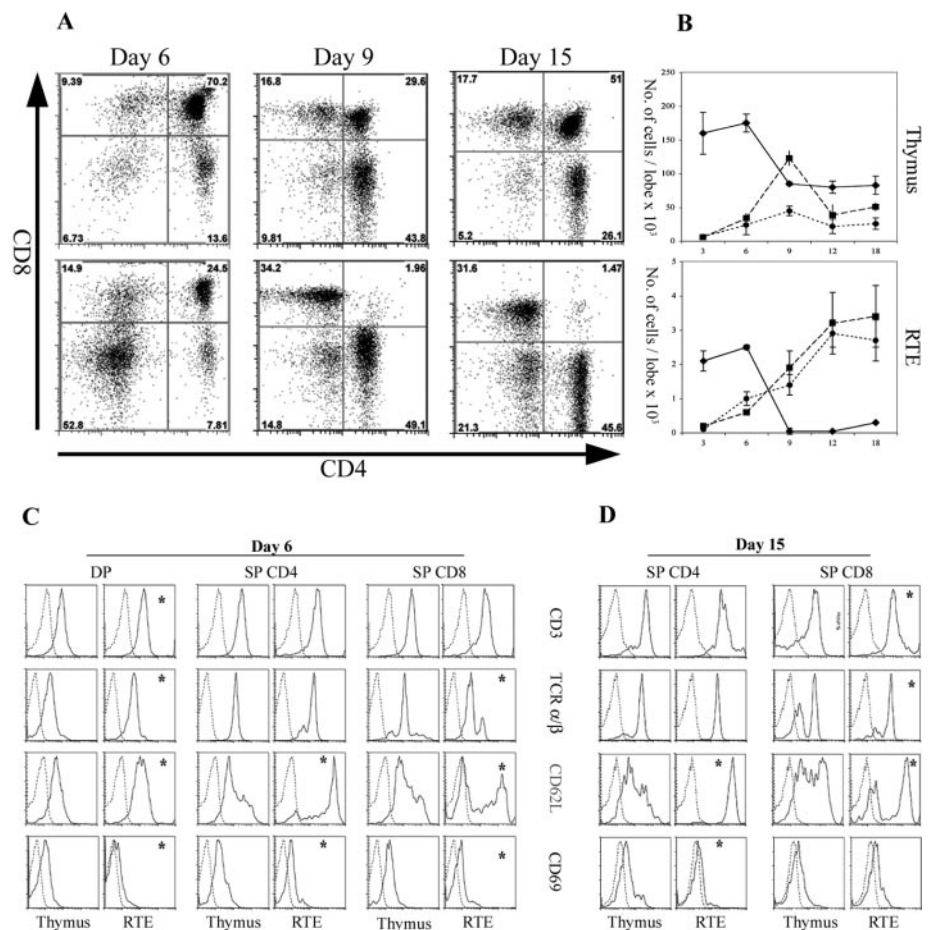
The expression of CD3, CD69, CD62L, TCR- $\alpha\beta$ , and TCR- $\gamma\delta$  was determined for intrathymic thymocytes and for thymic emigrants following 24-h time periods during FTOC. Total cellularity of E15.5 FTOC was maximal at day 6 of culture ( $250 \pm 45.9 \times 10^3$ , mean  $\pm$  SD) (Fig. 1, A and B). This finding was consistent with previous studies (19). Day 6 of culture was characterized by the predominance of intrathymic double-positive (DP) cells and was followed by a subsequent peak of SP CD4 cells at day 9. In late-stage FTOC, DP cells were still predominant but the total cellularity of the thymus was reduced.

Analysis of emigrants from CXCR4-WT B6 FTOC revealed an immunophenotypic pattern that was distinct from that of intrathymic thymocytes (Fig. 1A). DP cells, which were present among emigrants until day 6 of culture, showed significantly higher expression of CD3 and TCR- $\alpha\beta$  (MFI  $547 \pm 147$  and  $54 \pm 9.2$ , respectively) compared with intrathymic DP cells (MFI  $209 \pm 139$  and  $40 \pm 15.5$ , respectively;  $p < 0.01$ ) (Fig. 1C). From day 9 onward, only SP cells emigrated from the thymus into the lower chamber of the Transwell, and they were characterized by the simultaneous loss of CD69 and increased expression of CD62L, a pattern that has been proposed to specifically distinguish RTE from medullary SP (20) (Fig. 1D). SP CD4 cells expressing CD62L<sup>high</sup> (MFI  $1951 \pm 236$ ) were found among emigrants, whereas a CD62L<sup>low</sup> (MFI  $265 \pm 44.3$ ) pattern of expression characterized the corresponding intrathymic SP CD4 population (Fig. 1D). Compared with SP CD4 thymocytes, CD62L<sup>high</sup> and CD62L<sup>low</sup> SP CD8 thymocytes were present in the thymus, sug-

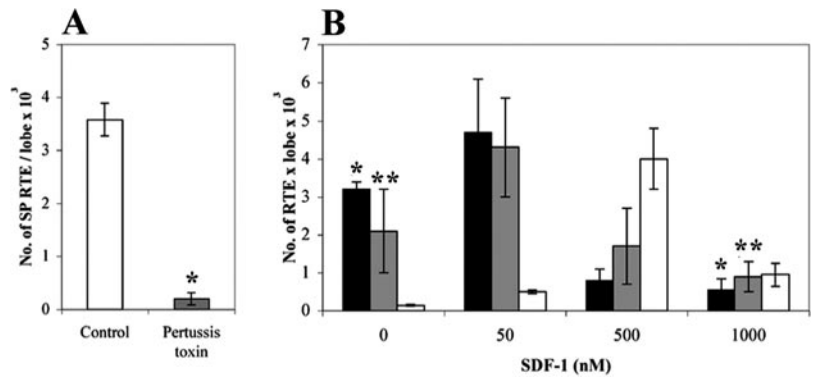
gesting that SP CD8 cells do not migrate immediately after acquisition of high levels of L-selectin (Fig. 1D). As seen in emigrants from early-stage FTOC, two distinct SP CD8 populations expressing CD62L<sup>high</sup> and CD62L<sup>low</sup> were present in the emigrant fraction (Fig. 1D). Double negative (DN) cells were stably present among emigrants. However, DN thymocytes emigrating from FTOC showed a more mature phenotype compared with intrathymic DN cells as  $71 \pm 24\%$  (mean  $\pm$  SD) of DN emigrants (day 15 of FTOC) showed expression of CD3 (MFI  $256 \pm 96$ ) compared with only  $47 \pm 23.8\%$  DN intrathymic cells (MFI  $99 \pm 77$ ) ( $p = 0.012$ , fraction of intrathymic DN CD3<sup>+</sup> vs DN emigrants CD3<sup>+</sup>) (data not shown).

### *Inhibition of thymic emigration by PTX and the presence of extrathymic SDF-1*

Thymic emigration has been previously shown to be a PTX-sensitive process involving the action of G<sub>o</sub>i protein-coupled receptors, including CXCR4 and S1PR. To evaluate our system for quantitating thymic emigration we incubated day 15 FTOC with PTX. Emigration of SP CD4 and CD8 thymocytes was significantly inhibited compared with untreated FTOC ( $p = 0.002$ ) (Fig. 2A). The finding of the immunophenotypic resemblance of emigrants with RTE and the PTX sensitivity of thymic emigration in our system confirmed the physiological relevance of this in vitro system to thymic emigration in vivo. This finding incidentally underscored the fact that passive leakage of mature thymocytes did not contribute significantly to emigration in our system. Furthermore, passive leakage of thymocytes from the thymus was clearly effectively minimized in this study by using a membrane with a fixed 3- $\mu$ m pore size study as compared with previous study in



**FIGURE 2.** PTX and SDF-1 block emigration of SP thymocytes from normal FTOC. The absolute number of RTE SP cells (A) is shown for control thymi and thymi treated with PTX (\*,  $p = 0.002$ ). B, The absolute number of RTE SP CD4 (■), CD8 (▨), and DP (□) migrated in the presence of different concentrations of recombinant SDF-1 is shown. \*,  $p = 0.016$ ; \*\*,  $p = 0.20$ .



which a nylon screen with a pore size of 100  $\mu\text{m}$  was used to examine thymic emigration (21).

Because, we postulated that intrathymic SDF-1 acts as a chemorepellent for mature SP thymocytes in a concentration-dependent manner in our system we proposed that incubation of FTOC with extrathymic SDF-1 at high concentrations would impair thymocyte emigration by nullifying the chemokine gradient. Chemotactic concentrations of SDF-1 (50 nM), added to the lower chamber of the FTOC system were effective in attracting more SP CD4 and CD8 thymocytes into the lower chamber of the Transwell (Fig. 2B). When SDF-1 concentrations were increased up to 500 nM and 1  $\mu\text{M}$ , the number of SP RTE was significantly reduced ( $p = 0.016$ ) (Fig. 2B). SP CD8 emigration was not significantly affected by high levels of extrathymic SDF-1 ( $p = 0.2$ ) (Fig. 2B). These data support the view that emigration of mature SP thymocytes in our system is a  $G_{\alpha i}$  protein-coupled seven-transmembrane receptor-dependent process in which intrathymic SDF-1 serves as a chemorepellent agent for mature thymocytes.

#### Thymocyte emigration from CXCR4<sup>-/-</sup> embryonic thymi

The total cellularity of CXCR4<sup>-/-</sup> thymi was initially less than that of control CXCR4<sup>+/-</sup> thymi but this increased progressively and eventually exceeded the cellularity of control FTOC in late stages of culture. These differences did not reach statistical significance (total cellularity: days 3–6,  $p = 0.073$ ; days 12–15,  $p = 0.45$ ). The appearance of DP thymocytes in CXCR4<sup>-/-</sup> thymi was delayed until day 6 of culture as compared with CXCR4<sup>+/-</sup> thymi but DP cells reached a normal proportion of intrathymic thymocytes beyond this time (Fig. 3). SP CD4 thymocytes were delayed in development in CXCR4<sup>-/-</sup> FTOC. The fraction and the absolute number of SP CD4 cells were significantly reduced until day 9 of culture in CXCR4<sup>-/-</sup> thymi ( $p = 0.026$ ) (Fig. 3C). However, from day 9 onward, SP CD4 cells in CXCR4<sup>-/-</sup> thymi progressively increased in absolute number and eventually these thymi contained more of this cell type compared with control FTOC, although this was not statistically significant ( $p = 0.071$ ) (Fig. 3C).

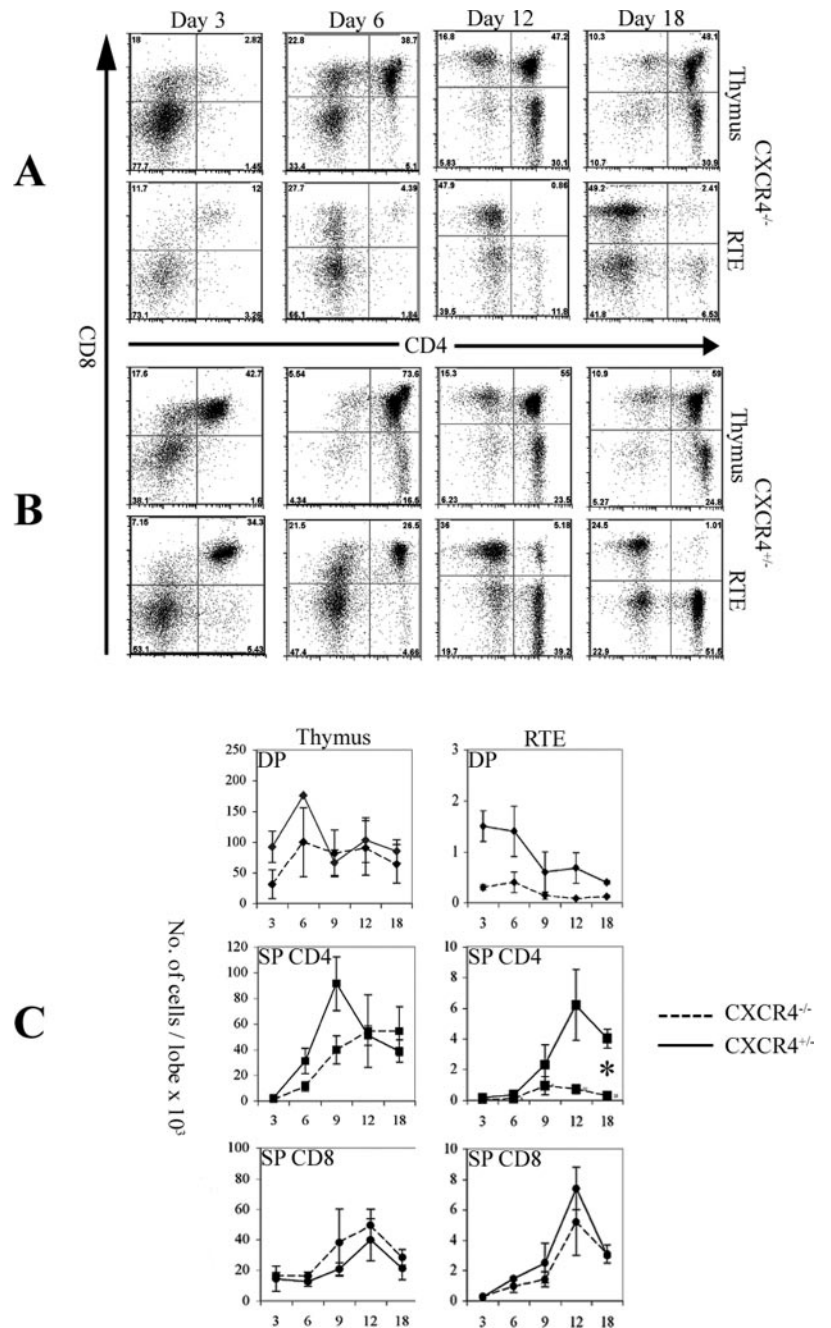
Analysis of changes in the immunophenotype of CXCR4<sup>-/-</sup> emigrants over time showed a marked derangement in the composition of different thymocyte subpopulations (Fig. 3). The total number of CXCR4<sup>-/-</sup> emigrants was always significantly reduced compared with control CXCR4<sup>+/-</sup> thymic emigrants ( $p = 0.001$ ) (data not shown). At early stages in FTOC, DP emigrants were significantly reduced in comparison to control thymi ( $p = 0.031$ ). SP CD4 cells showed the most striking difference in their ability to emigrate away from CXCR4<sup>-/-</sup> (Fig. 3, A and C). SP CD4 emigrants from CXCR4<sup>-/-</sup> thymi were significantly reduced in number in late-stage FTOC as compared with SP CD4 cells from CXCR4<sup>+/-</sup> thymi ( $p = 0.008$ ) (Fig. 3C). In marked contrast, the

fraction of SP CD8 cells among CXCR4<sup>-/-</sup> emigrants was similar to that seen in control thymi ( $p = 0.35$ ).

Analysis of TCR- $\alpha\beta$  expression in intrathymic CXCR4<sup>-/-</sup> SP CD4 cells from day 15 FTOC showed no significant differences in the pattern of maturation compared with control FTOC (MFI  $115 \pm 1.6$  and  $107 \pm 5.4$ , respectively;  $p = 0.35$ ) (data not shown). CD3 expression was significantly higher in intrathymic CXCR4<sup>-/-</sup> SP CD4 cells compared with control cells (MFI  $560 \pm 129.6$  and  $333 \pm 69.1$ , respectively;  $p = 0.045$ ) (data not shown). Analysis of CD62L expression in CXCR4<sup>-/-</sup> FTOC was consistent with an accumulation of mature SP CD4 cells in the thymus. The MFI and the percentage of cells expressing CD62L<sup>high</sup> in intrathymic CXCR4<sup>+/-</sup> CD4 SP cells was significantly lower compared with CXCR4<sup>-/-</sup> SP CD4 intrathymic cells (MFI  $214 \pm 65.4$  vs  $355 \pm 97.5$ ,  $p < 0.01$ ; CD62L<sup>high</sup>  $30.4 \pm 11.4$  vs  $57.5 \pm 18.7\%$ ,  $p = 0.008$ , respectively) (Fig. 4, A–C) suggesting that, in the absence of CXCR4, SP CD4 thymocytes with the characteristics of RTE were retained in the thymic medulla. The expression of CD69 was slightly but still significantly higher in the SP CD4 thymocytes from CXCR4<sup>-/-</sup> cultures compared with SP CD4 thymocytes from control FTOC (MFI  $63 \pm 18.5$  vs  $47 \pm 5.7$ ;  $p = 0.043$ ) (Fig. 4, A and B). The fraction of apoptotic thymocytes from day 15 CXCR4<sup>-/-</sup> FTOC was not significantly different compared with CXCR4<sup>+/-</sup> thymocytes ( $23.8 \pm 4.1\%$  vs  $22.9$  vs  $4.3\%$ , respectively;  $p = 0.089$ ) (data not shown). These data ruled out the possibility that diminished survival of CXCR4<sup>-/-</sup> thymocytes accounted for the impaired emigration of SP CD4 cells and further support the view that this thymocyte subpopulation, with the immunophenotype of RTE, is severely impaired in its ability to emigrate from the thymus in the absence of CXCR4. In contrast, the emigration of SP CD8 thymocytes appears to be CXCR4-independent.

#### Ab-induced proliferation of CXCR4<sup>-/-</sup> thymocytes

Having found that the absence of CXCR4 interferes with the emigration of phenotypically mature SP CD4 but not SP CD8 cells, we studied whether there were functional differences between SP CD4 thymocytes from CXCR4<sup>-/-</sup> or CXCR4<sup>+/-</sup> thymi. We addressed this question by analyzing Ab-induced proliferation of control and CXCR4-deficient thymocytes. As shown in Fig. 4D stimulation of total thymocytes from day 14 FTOC with three different concentrations of anti-CD3 mAb resulted in a lower but not statistically significant level of [<sup>3</sup>H]thymidine incorporation in CXCR4<sup>-/-</sup> thymocytes compared with control thymocytes ( $p = 0.062$ ). Proliferation, as evaluated by [<sup>3</sup>H]thymidine incorporation, reflected the overall proliferation of all thymocyte subpopulations and could not completely rule out a proliferative defect of SP CD4 cells in CXCR4<sup>-/-</sup> FTOC. Consequently, we analyzed specific Ab-induced proliferation in SP CD4 thymocytes from CXCR4<sup>-/-</sup>



**FIGURE 3.** Thymic maturation and pattern of emigration in CXCR4<sup>-/-</sup> FTOC. E15.5 FTOC from CXCR4<sup>-/-</sup> (A) or CXCR4<sup>+/-</sup> (B) embryos were cultured and analyzed for CD4 and CD8 expression (A and B) and absolute number (C). Emigrants from at least four thymi of each genotype were analyzed. \*, *p* = 0.008.

FTOC using CFSE (Fig. 5). The fraction of SP CD4 and CD8 thymocytes that underwent cell division during a 48-h time period was not significantly different in CXCR4<sup>-/-</sup> thymocytes (Fig. 5, D–F) compared with control cells (Fig. 5, A–C) (*p* = 0.44 and 0.62, respectively; Fig. 5A). These results rule out a functional defect in the proliferative capacity of SP CD4 thymocytes as an explanation for the impairment in their emigration from CXCR4<sup>-/-</sup> FTOC.

*Integrin expression does not account for impaired emigration of SP CD4 thymocytes from CXCR4<sup>-/-</sup> thymi*

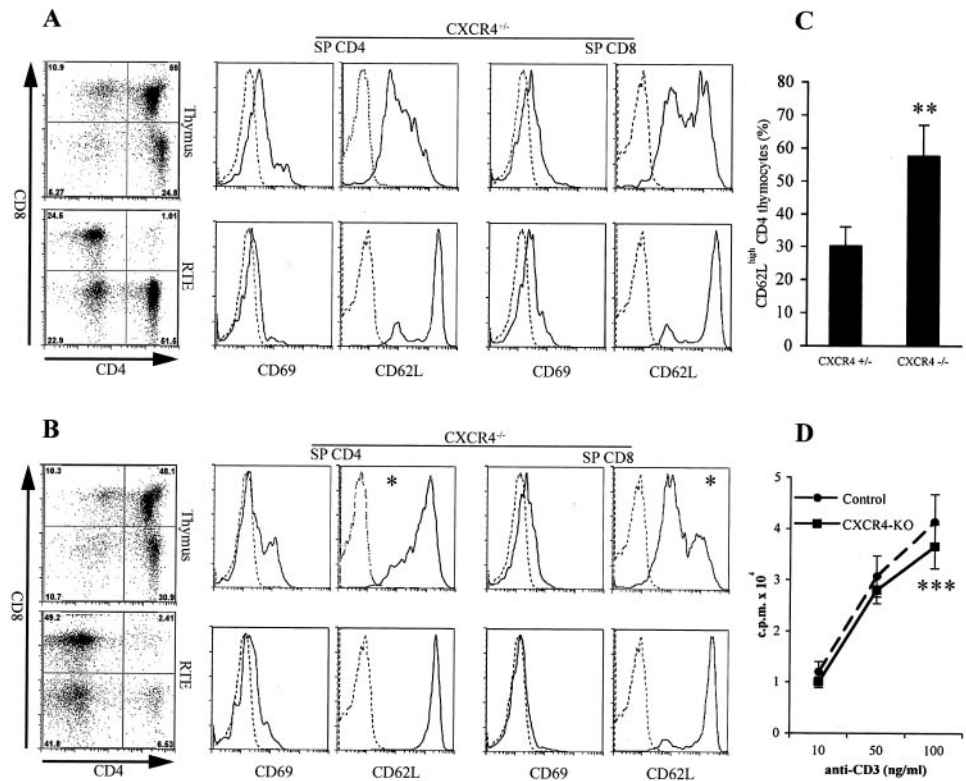
Although integrins have been shown contribute to thymic development, the precise role of integrins in thymic emigration is not fully understood. Similar levels of expression of CD44, LFA-1, and  $\alpha_4$  and  $\alpha_5$  integrins have been previously found on RTE and intrathymic cells (21). It has been previously demonstrated that

SDF-1 up-regulates lymphocyte adhesion via  $\alpha_4\beta_7$  integrin (22) and  $\alpha_4$  and  $\alpha_5$  integrins have been shown to play a role in the proliferation of fetal thymocytes (23, 24). We studied the expression of these integrins in FTOC. The level of expression of  $\alpha_4$  and  $\alpha_5$  integrins, LFA-1, and CD44 was not significantly different in CXCR4<sup>-/-</sup> and CXCR4<sup>+/-</sup> thymi (Fig. 6) supporting the view that a difference in integrin expression did not explain the failure of SP CD4 thymocytes to emigrate from CXCR<sup>-/-</sup> thymi.

*Extrathymic S1P elicits chemoattraction of SP CD4 thymocytes from CXCR4<sup>-/-</sup> FTOC*

S1P receptors have been shown to play a crucial role in the egress of mature T cells from the thymus into the circulation (5, 7). We studied the role of S1P as a chemoattractant in CXCR4<sup>-/-</sup> thymi. Day 14 control and CXCR4<sup>-/-</sup> FTOC were incubated in the upper chamber with 10 or 100 nM S1P in the lower chamber of the

**FIGURE 4.** Impaired emigration of mature SP CD4 thymocytes from late-stage CXCR4<sup>-/-</sup> FTOC. Expression profiles of CD4 and CD8 and levels of expression of CD69 and CD62L in SP cells were analyzed from control CXCR4<sup>+/-</sup> (A) and CXCR4<sup>-/-</sup> (B) FTOC after 15 days of culture. Cells were stained with control (dotted histograms) or experimental (solid histograms) mAbs. RTE were collected from at least four thymi. \*,  $p < 0.01$ ; Ag expression in CXCR4<sup>-/-</sup> vs CXCR4<sup>+/-</sup> intrathymic thymocytes (Kolmogorov-Smirnov test). The frequency (mean  $\pm$  SD;  $n = 3$ ) of SP CD4 thymocytes expressing high levels of CD62L in control and CXCR4<sup>-/-</sup> FTOC is shown. \*\*,  $p = 0.008$  (C). Thymocytes from day 14 CXCR4<sup>-/-</sup> or control FTOC, were activated with anti-CD3 and anti-CD28 mAb in the presence of IL-2 and proliferation was quantitated by [<sup>3</sup>H]thymidine (D). \*\*\*,  $p = 0.062$ .



FTOC. Intrathymic cells and emigrants were harvested 24 h later (Fig. 7). Treatment of CXCR4<sup>+/-</sup> FTOC with S1P did not significantly affect the total number of SP CD4 and CD8 emigrants although a trend toward an increased fraction of SP CD8 emigrants was observed (CD8 control  $1.9 \pm 1.4 \times 10^3$ ; CD8 S1P  $3 \pm 1.2 \times 10^3$ ,  $p = 0.08$ ; data not shown) (Fig. 7A). S1P was able to attract a significantly greater number of SP CD4 cells out of CXCR4<sup>-/-</sup> thymi compared with untreated CXCR4<sup>-/-</sup> FTOC ( $p = 0.033$ ). S1P-mediated chemoattraction of SP CD4 thymocytes from CXCR4<sup>-/-</sup> FTOC was therefore concentration dependent. Analysis of intrathymic CD62L showed that extrathymic S1P lead to the emigration of a fraction of CXCR4<sup>-/-</sup> SP CD4 thymocytes expressing CD62L<sup>high</sup>, which were otherwise retained in CXCR4<sup>-/-</sup> thymi that were not treated with S1P (Fig. 7B). This finding suggests that the failure of CD4 SP thymocytes to emigrate from CXCR4<sup>-/-</sup> thymi as a result of SDF-1 driven chemorepulsion was partially compensated for by S1P-mediated chemoattraction in this system.

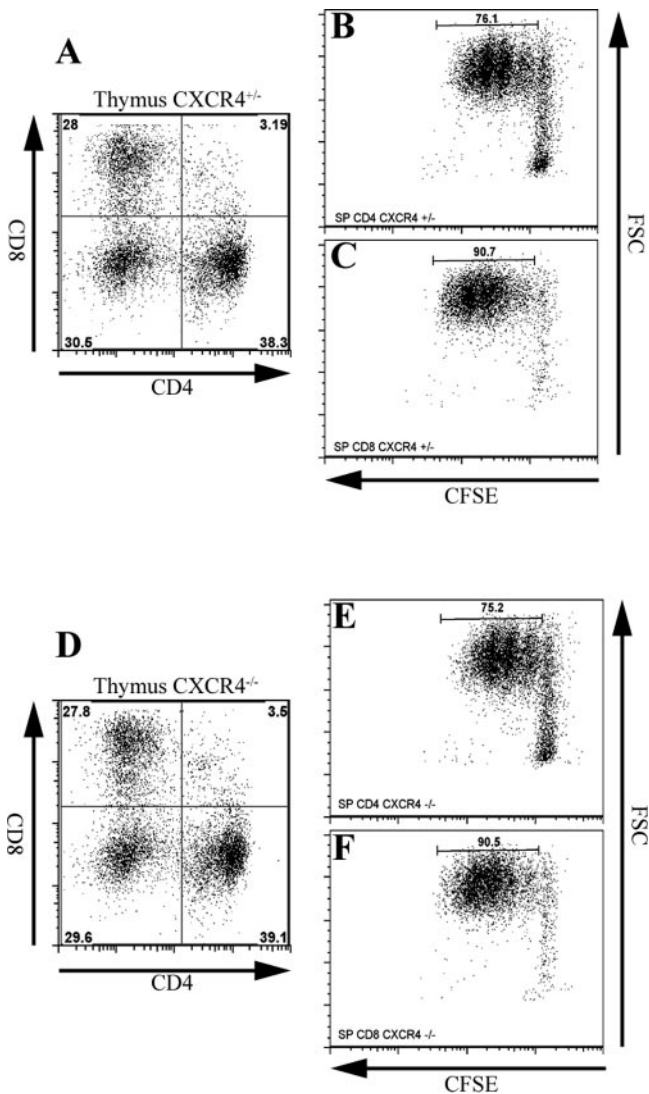
#### The CXCR4 antagonist AMD3100 impairs normal SP CD4 cell emigration from FTOC

Having found that emigration of SP CD4 thymocytes was severely impaired in CXCR4<sup>-/-</sup> FTOC, we aimed to study whether blocking CXCR4 would affect emigration of cells from CXCR4<sup>+/-</sup> thymi. We addressed this question by using the specific nonpeptide CXCR4 antagonist, AMD3100. AMD3100 is a bicyclam derivative first described for its potent activity against HIV infection (17, 25). AMD3100 has been shown to inhibit intracellular Ca<sup>+</sup> flux and chemotactic responses of murine splenocytes to SDF-1 (26). CXCR4 expression was measured in SP and DP thymocytes from day 14 FTOC (data not shown). As expected, CXCR4 was expressed at a higher level in DP thymocytes (MFI  $52.1 \pm 11.3$ ) compared with SP cells. SP CD4 thymocytes showed significantly higher expression of CXCR4 compared with SP CD8 cells (MFI  $29.1 \pm 6.5$  vs  $15.5 \pm 7.3$ , respectively;  $p = 0.01$ ). FTOC at day 14

of culture was incubated for 24 h with 1  $\mu$ g/ml AMD3100. Treatment of FTOC with 1  $\mu$ g/ml AMD3100 significantly increased the total number of cells in the thymus as compared with untreated FTOC ( $349 \pm 99.5 \times 10^3$ /lobe vs  $247 \pm 72.9 \times 10^3$ /lobe, respectively;  $p = 0.022$ ) (Fig. 8A). The total number of SP CD8 emigrants did not differ between AMD3100-treated compared with untreated FTOC ( $p = 0.93$ ). However, in contrast, SP CD4 thymocytes were severely impaired in their ability to emigrate from AMD3100-treated thymi as compared with untreated controls ( $1.1 \pm 0.15 \times 10^3$ /lobe vs  $4.07 \pm 0.12 \times 10^3$ /lobe, respectively;  $p = 0.005$ ) (Fig. 8, A and C). Analysis of CD62L expression revealed that retention of CD62L<sup>high</sup> CD4 SP cells in the thymus of AMD3100-treated FTOC had occurred in comparison to untreated CXCR4<sup>+/-</sup> thymi ( $p = 0.017$ ) (Fig. 8, B and D). CD62L expression by SP CD8 thymocytes was not affected in AMD3100-treated thymi ( $p = 0.56$ ) (Fig. 8, B and D).

#### Movement of fetal SP CD4 thymocytes away from recombinant SDF-1

In view of the fact that we proposed that mature SP CD4 thymocytes migrate out of FTOC as a result of SDF-1-induced chemorepulsion, we examined whether high concentrations of the chemokine could evoke a chemorepellent response from sorted SP thymocytes. SP CD4 and CD8 thymocytes demonstrated a maximal fugetactic response to SDF-1 at a concentration of 10  $\mu$ g/ml, under which conditions  $18.2 \pm 1.7$  and  $12 \pm 1.0\%$  of CD4 and CD8 cells transmigrated, respectively (SP CD4 vs SP CD8 thymocytes,  $p = 0.054$ ). The chemorepellent effect of SDF-1 for SP CD4 and SP CD8 thymocytes was inhibitable by PTX (SP CD4,  $p < 0.0001$ ; SP CD8,  $p < 0.001$ , respectively) (data not shown). SP CD4 thymocyte and SP CD8 fugetaxis was inhibitable by anti-CXCR4 (SP CD4,  $1.3 \pm 0.3\%$ ; SP CD8,  $4.8 \pm 0.8\%$ ) ( $p < 0.0001$  and  $p < 0.0188$ , respectively). DN and DP thymocytes showed minimal fugetactic responses to chemorepellent concentrations of SDF-1 ( $1.2 \pm 0.3$  and  $1.9 \pm 0.4\%$ , respectively) (data not shown).

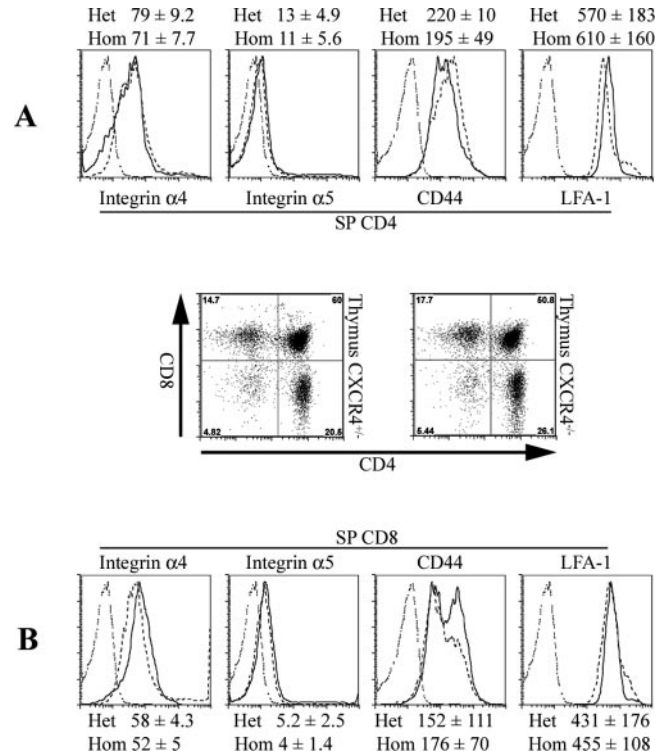


**FIGURE 5.** Kinetic analysis of T cell division in populations of CFSE-labeled thymocytes from E15.5 CXCR4<sup>+/+</sup> and CXCR4<sup>-/-</sup> FTOC. Expression profiles of CD4 and CD8 after proliferation (A and D) and CFSE profiles (B, C, E, and F) of CXCR4<sup>+/+</sup> (A–C) and CXCR4<sup>-/-</sup> (D–F) SP CD4 and CD8 are represented. The frequency of SP thymocytes that underwent at least one cell division is gated.

These data support the thesis that high levels of SDF-1 exert a chemorepulsive effect on SP CD4 and to a lesser extent on CD8 thymocytes.

#### *AMD3100 results in retention of SP CD4 thymocytes in the thymus in vivo*

To evaluate whether antagonism of the CXCR4/SDF-1 axis impairs thymocyte emigration in vivo, we administered AMD3100 to newborn mice. In contrast to a recent study in which data were presented that support the view that CXCR4 contributes to homeostatic egress of thymocytes (27), we found that a single i.p. dose of AMD3100 (1 μg/kg) in newborn mice did not have a significant effect on thymic cellularity or on the relative numbers or proportions of intrathymic thymocyte subpopulations (data not shown). When AMD3100 was administered twice a day for 5 days, the total number of intrathymic thymocytes in AMD3100-treated animals was not significantly different from untreated animals ( $91.5 \pm 5.5 \times 10^6$  vs  $90.7 \pm 20.6 \times 10^6$ , mean  $\pm$  SEM, respectively;  $p = 0.8$ ). However, the total number of SP CD4 thymocytes in thymi

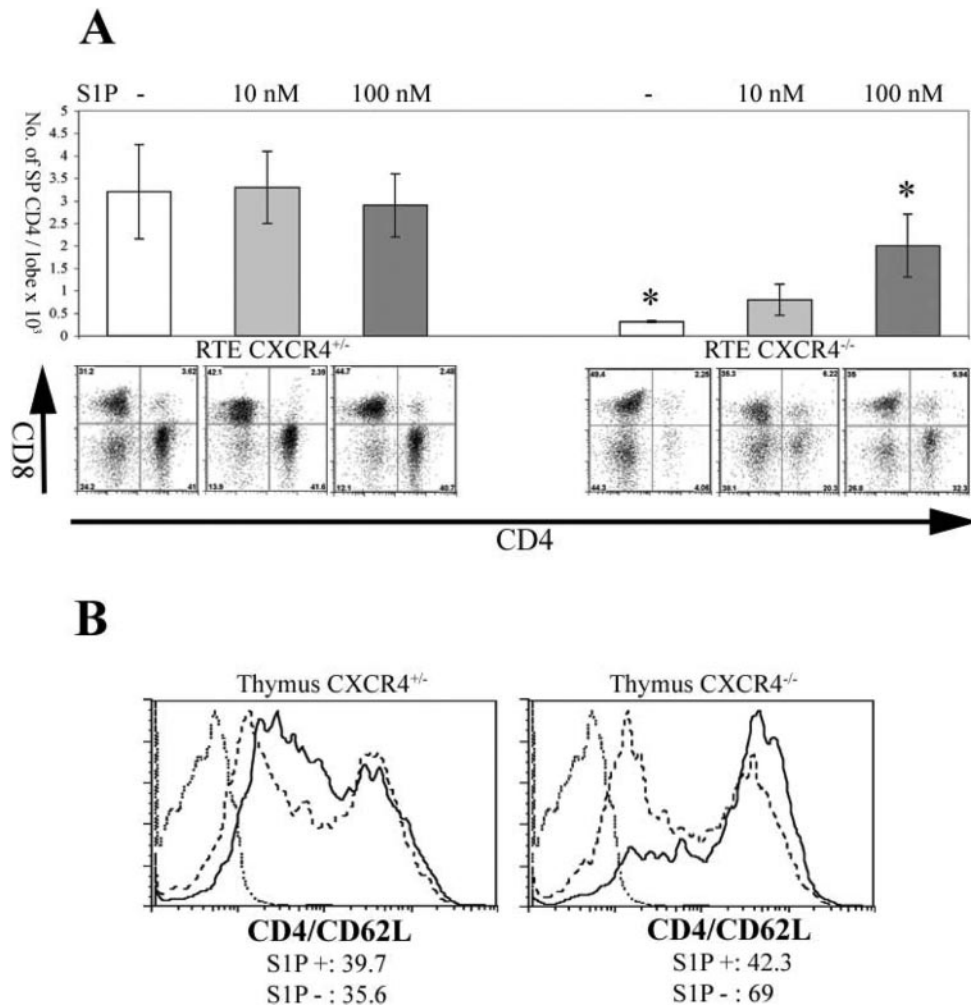


**FIGURE 6.** Absence of CXCR4 does not significantly affect the adhesion molecule expression profile of thymocytes. The levels of expression of  $\alpha_4$  and  $\alpha_5$  integrins, CD44 and LFA-1 are shown in SP CD4 (A) and SP CD8 (B) intrathymic cells from control and CXCR4<sup>-/-</sup> FTOC at day 14 of culture. For each histogram, the MFI value of heterozygote and homozygote thymocytes is shown. Isotype control (dotted histogram), CXCR4<sup>-/-</sup> (dashed histogram), and CXCR4<sup>+/+</sup> (solid histogram) are shown.

from mice treated with AMD3100 was significantly increased compared with control thymi ( $p = 0.044$ ) (Fig. 9). Although the total number of CD3<sup>+</sup> cells in spleens from AMD3100-treated newborns was significantly reduced compared with control spleens ( $p = 0.039$ , data not shown), SP CD4 and CD8 splenocytes from AMD3100-treated newborns were also reduced compared with control spleens from untreated mice, with a trend toward statistical significance only for SP CD4 cells (SP CD4,  $p = 0.063$ ; SP CD8,  $p = 0.36$ ) (Fig. 9B). No differences were observed in the SP CD4 and CD8 lymph nodes population in AMD3100-treated mice compared with control (Fig. 9B).

## Discussion

This study demonstrates that the emigration of SP CD4 thymocytes from the fetal thymus is dependent on the CXCR4/SDF-1 axis. Migration of mature CD4 and CD8 T cells away from WT FTOC followed programmed differentiation and occurred mainly as a result of intrathymic decisional processes. SP thymocytes that exited the thymus clearly resembled RTE and were phenotypically distinct from their intrathymic counterparts. Furthermore, when thymic maturation and emigration from CXCR4<sup>-/-</sup> FTOC was studied, a profound abnormality in thymic emigration was found despite normal thymic development. In this setting mature CXCR4<sup>-/-</sup> SP CD4 thymocytes were severely impaired in their ability to move out of the thymus, and this resulted in their retention and accumulation in the thymus. Blocking CXCR4 with the antagonist, AMD3100, as well as nullifying the gradient by adding SDF-1 to the lower chamber of the Transwell recapitulated the defect in emigration of SP CD4 cells from CXCR4<sup>-/-</sup> cultures.



**FIGURE 7.** S1P chemoattraction of SP thymocytes in control and CXCR4<sup>-/-</sup> FTOC. Thymi and RTE were collected after 14 days of culture following 24-h incubation with 10 or 100 nM S1P in the lower chamber or with control buffer used for dissolving S1P. *A*, The absolute number of SP CD4 RTE and the pattern of expression of CD4 and CD8 RTE from control and S1P-treated (10 or 100 nM) FTOC is represented. *B*, The fraction of SP CD4 thymocytes from CXCR4<sup>+/-</sup> and CXCR4<sup>-/-</sup> FTOC expressing CD62L<sup>high</sup> after incubation with S1P (100 nM) or buffer. \*,  $p = 0.033$ . Isotype control (dotted histogram), S1P (100 nM, dashed histogram), and no S1P (solid histogram) are shown.

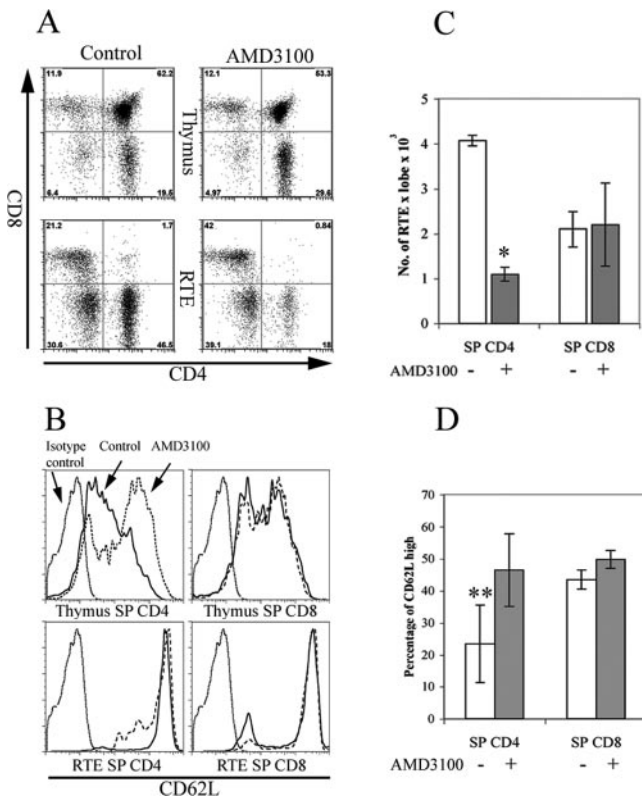
Retained CXCR4<sup>-/-</sup> SP CD4 thymocytes were also able to respond to the chemoattractant S1P. SP CD4 thymocytes in CXCR4<sup>-/-</sup> thymi display a normal pattern of maturation as well as normal Ab-induced proliferation, suggesting that the absence of CXCR4/SDF-1 signaling does not affect TCR activation (28). Furthermore, the finding that thymocytes accumulating in the CXCR4<sup>-/-</sup> thymus phenotypically resembled RTE is consistent with their normal anatomical localization within the CXCR4<sup>-/-</sup> thymic medulla.

Our system allows the study of thymic emigration from fetal thymi, which differs from emigration from adult thymus. The analysis of emigrants from day E15.5 fetal thymi reflects the export of the first generation of thymocytes generated from lymphoid colonization of the fetal thymus (10.5 and 12 days of fetal life). Thymocytes generated from this first wave of progenitors are endowed with the ability to become functional and to emigrate and this process plays a major role in the establishment of the peripheral T cell pool (29–31). This pattern of emigration clearly differs from adult thymic egress where the peripheral T cell compartment is already functional.

Our data, based on the analysis of each subpopulation of emigrants, demonstrated that CD62L expression alone delineated SP emigrants from medullary thymocytes. This phenotypic difference has been previously used to distinguish RTE from medullary SP thymocytes (20). In a previously published study, thymic emigrants isolated from FTOC were shown to have a high degree of heterogeneity in CD62L expression (21). However, emigrant iso-

lation was performed using a nylon screen with a fixed 100-micron pore size and furthermore emigrant subpopulation analysis was not performed. This may be of particular relevance as we found two different subpopulations of SP CD8 emigrants, one expressing CD62L<sup>low</sup> and the other CD62L<sup>high</sup>. It has been previously reported that two main subpopulations of SP CD8 thymic emigrants exist, each expressing different levels of L-selectin and characterized by a different homing commitment (32, 33). In addition, our data show that DP thymocytes are able to emigrate from fetal thymi at early but not at late-stage culture. Although this result clearly differs from thymic export from adult thymi, it is consistent with previous reports demonstrating the presence of a consistent fraction of DP CD4 and CD8 thymocytes (~30% of total cells) in lymph nodes of 3-day-old mice (34). This population of peripheral DP thymocytes has been shown to represent a lesser fraction of peripheral cells in 8-day-old mice, reiterating the rapid decrease in DP emigrants seen in day 6–12 FTOC. Therefore, our findings are concordant with the view that DP cells have migratory properties at an early stage of development. DN cells have been previously reported to be found among thymic emigrants (35). In our study we were also able to demonstrate that the emigration of DN cells was insensitive to PTX inhibition and associated with a higher level of CD3 expression. Although this serves as evidence that the emigration of DN cells in our system is not due to passive leakage the precise mechanism by which this process occurs is unclear.

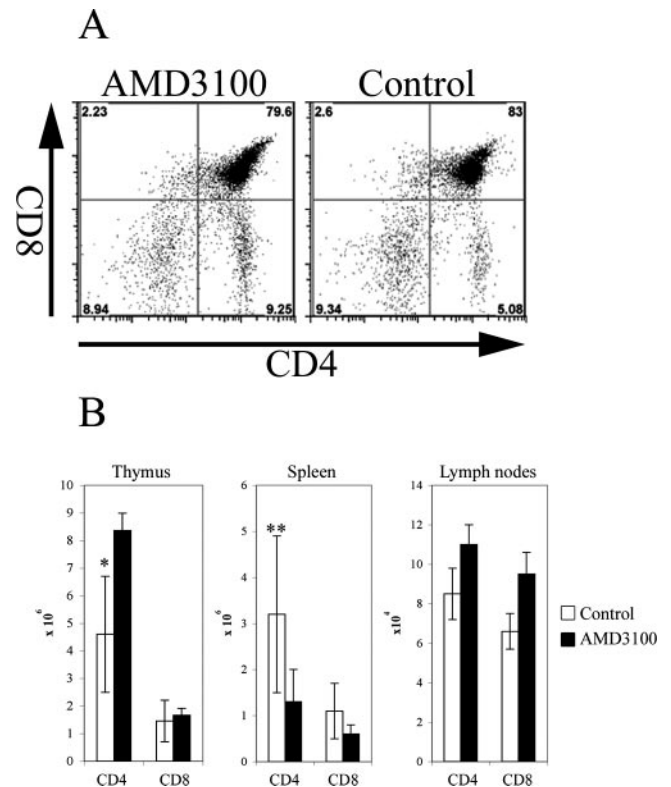
Chemokines, such as SDF-1 have been predominantly shown to serve as chemoattractants for leukocyte subpopulations as a result



**FIGURE 8.** The CXCR4 antagonist AMD3100 reduces emigration of SP CD4 thymocytes from normal FTOC. CXCR4<sup>+/-</sup> E15.5 FTOC ( $n = 4$ ) were cultured for 14 days and then treated for 24 h with 1  $\mu$ g/ml AMD3100. Thymic cells and RTE were collected and stained for CD4 and CD8 (A and C) and CD62L (B and D). The absolute number (mean  $\pm$  SEM) of SP CD4 and CD8 RTE (C, \*,  $p = 0.005$ ) and the fraction (mean  $\pm$  SEM) of CD62L<sup>high</sup> SP intrathymic thymocytes (D, \*\*,  $p = 0.017$ ) from AMD3100-treated and untreated FTOC are shown.

of their binding to G<sub>αi</sub> protein-coupled seven-transmembrane receptors including CXCR4 via a PTX-sensitive mechanism (36). However, it has been shown previously that mature SP thymocytes can also actively migrate away from thymic stroma as a result of a process mediated in part by high concentrations of the chemokine SDF-1, via a mechanism termed chemorepulsion or fugetaxis (8, 9). Previous and current data are also consistent with persistent thymocyte migration without a loss of responsiveness of murine thymocytes to SDF-1 during cortical to medullary transition (37). Recently, data supporting an additional role of chemorepulsion in intrathymic trafficking of early thymocytes have been reported. Misslitz et al. (11) found evidence to support the view that CCR7 and its chemokine ligands, CC19 and CCL21, may direct the movement of DN thymocytes from the corticomedullary region to the cortical region as a result of chemorepulsion (11, 12). We demonstrate that SDF-1-mediated repulsion of SP cells is gradient dependent. High concentrations of SDF-1 in the lower chamber of the Transwell significantly impaired the emigration of SP CD4 and, to a lesser extent, SP CD8 emigrants from FTOC.

Disruption of SDF-1 and CXCR4 genes in mice have been associated with impaired hemopoiesis. However, the role of CXCR4 in T cell development is controversial. Normal T cell development as well as normal migration to lymphoid organs was found when day 17.5 CXCR4<sup>-/-</sup> thymi were engrafted into mice lacking TCR- $\alpha$  (13–15). In contrast with this result, Ara et al. (38) demonstrated that CXCR4 deficiency affects the generation of mature thymocytes in adult chimeric mice. Our results are partially con-



**FIGURE 9.** Increased number of intrathymic SP cells and reduction of splenic T cells after AMD3100 treatment of newborn mice. SP CD4 and CD8 expression profiles (A) and the absolute number of SP CD4 and CD8 cells in the thymus, spleen, and lymph nodes (B) of AMD3100 treated CXCR4<sup>+/-</sup> mice compared with control are shown. \*,  $p = 0.039$ ; \*\*,  $p = 0.063$ .

sistent with these previous studies as we found a delay in the appearance of DP and SP thymocytes in CXCR4<sup>-/-</sup> thymi. However, a normal compartment of DP and SP thymocytes eventually developed in these thymi, suggesting that the initial defect in development does not impair the ability of CXCR4<sup>-/-</sup> thymocytes to expand and reach DP and SP maturation. We demonstrate for the first time that SP CD4 thymocytes expressing high levels of CD62L accumulate in the thymus in the absence of CXCR4. This pattern has been recently shown to characterize a correct medullary localization of SP thymocytes, which are unable to leave the thymus in the absence of S1P (5–7). It is important to note that the defect in thymic emigration from CXCR4 knockout thymi is precisely reiterated in vivo in normal mice treated with the CXCR4 antagonist AMD3100 and by treating WT FTOC with the same antagonist. These internally consistent data support the view that SP CD4 thymocytes simply fail to emigrate and then subsequently accumulate within the thymus.

There is evidence that thymic emigration occurs as a result of a multistep process starting with the correct localization of mature T cells to the thymic medulla and terminating with the movement of thymocytes from the medulla into the blood through the endothelial venules (1). The first step involving migration of mature T cells up to the basal surface of the medullary endothelial cell is thought to be controlled by CCR7 and its ligand, CCL19 (4, 39). The movement of T cells from the medulla to the periphery probably depends on other G protein signals, which could act through a repulsive, attractive, or a combination of these mechanism. S1P has been recently shown to attract T cells from the medulla to the periphery based probably on its high levels in the peripheral blood

(5, 7). Our data support the existence of an additional CXCR4-dependent mechanism that contributes to fetal thymic emigration, based on repulsion of mature T cells from the medulla to the periphery. In addition, we found that S1P was able to act as an extrathymic chemoattractant for CXCR4-deficient SP CD4 thymocytes expressing high levels of CD62L, thus resembling RTE, which accumulated in the thymic medulla in the absence of CXCR4.

Our results confirm that homeostatic control of SP CD4 thymocyte egress is a CXCR4-dependent mechanism and support the view that S1P-induced thymocyte egress is at least partially independent of CXCR4. Our data also demonstrate that absence of CXCR4/SDF-1 signaling does not prevent normal thymocyte migration and localization to the medulla as CXCR4-deficient thymocytes emigrated in response to S1P. This finding is also consistent, in part, with recent data from Yopp et al. (27) who showed that in vivo administration of AMD3100 prevented thymic T cell egress, which was partially restored by the administration of the S1P agonist FTY720. Furthermore, the minimal chemoattractant effect of S1P on SP intrathymic thymocytes expressing CD62L<sup>high</sup> was evident in the normal thymus only in the fraction of SP CD8 expressing CD62L<sup>high</sup> but not in SP CD4 thymocytes that expressed lower levels of CD62L. The fact that we did not observe a significant emigration of thymocytes from normal FTOC may suggest that S1P signaling acts by regulating responsiveness to attractive chemokines or other retention signals that are not active in our system (5). Of interest, our in vivo data are concordant with previous observations that CXCR4 blockade does not affect lymph node migration, suggesting a secondary role of CXCR4/SDF-1 in lymph node trafficking (27, 40).

In contrast to SP CD4 thymocytes, SP CD8 cells were not impaired in their ability to emigrate away from CXCR4<sup>-/-</sup> thymi. This supports the view that at least two discrete mechanisms govern thymic emigration. The migratory responses of SP CD4 and CD8 thymocytes do not appear to differ in vitro to SDF-1 (9, 35), although these data were generated from cells in suspension or from excised thymic fragments rather than intact organs. However, there is data to support the view that SP CD4 thymocytes are distinct from SP CD8 thymocytes with regards to specific aspects of their intrathymic migration. Histological data from Suzuki et al. (41) demonstrated that PTX had a greater effect on the distribution of SP CD4 thymocytes than SP CD8 thymocytes at the thymic corticomedullary junction. There are data that suggests that the medullary stage of thymocyte development, which precedes emigration, differs between SP CD4 and CD8 thymocytes (42, 43). Even in the presence of similar expression of adhesion molecules in normal and CXCR4-deficient thymocytes, selectins and integrins have been described to be colocalized in the microvilli of the T cell surface where CXCR4 and CD4 molecules form homogeneous microclusters (44). This does not appear to be the case for CD8 and CXCR4. Chemokine responsiveness has also been found to differ in thymocyte subpopulations as it has been recently shown that the chemokine CCL25 exerts chemotactic activity on SP CD8 but not SP CD4 RTE (45). Taken together these data support the view that thymic emigration is differentially regulated in SP CD4 and CD8 cells.

The involvement of the combination of chemorepellents and chemoattractants in guiding a cellular developmental processes is not unprecedented in biology. Agents acting as both chemoattractants and chemorepellents, including SDF-1, have been shown to guide the directional migration of axonal growth cones toward or away from the midline in the developing CNS (46–48). Our data support a new thesis that both intrathymic chemorepellent and extrathymic chemoattractant agents govern the egress of mature thy-

mocytes from the thymus (12). Furthermore, our results validate a role for CXCR4 in regulating the homeostatic emigration of SP CD4 thymocytes via a mechanism in which intrathymic SDF-1 acts as a chemorepellent and extrathymic S1P serves as a chemoattractant for this cell subpopulation.

This work significantly adds to our understanding of how mature leukocytes exit the organs in which they develop and may provide new insights into therapeutic agents, including AMD3100 and FTY720, which may antagonize this process. In conclusion, our work provides further evidence for the physiological relevance of leukocyte fugetaxis or chemorepulsion.

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## Disclosures

The authors have no financial conflict of interest.

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