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# CCR7 Directs the Migration of Thymocytes into the Thymic Medulla<sup>1</sup>

Joanne Kwan and Nigel Killeen<sup>2</sup>

**Developing thymocytes migrate from the cortex to the medulla of the thymus as a consequence of positive selection. This migration is likely to be essential for tolerance because it allows the developing cells to move into an environment that is optimal for negative selection. Guidance mechanisms that draw positively selected thymocytes into the medulla have not been clarified, but several studies have implicated chemokines in the process. CCR7, the receptor for the medullary chemokines CCL19 and CCL21, is induced on thymocytes during their positive selection. In this study we show that premature expression of CCR7 repositions CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells into the medulla of transgenic mice. This repositioning of the thymocytes is accompanied by impairment of their development. The data show the involvement of CCR7 in medullary migration and emphasize the importance of proper thymocyte positioning for efficient T cell development. *The Journal of Immunology*, 2004, 172: 3999–4007.**

**S**mall CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) cells are the most numerous hemopoietic cell type in the thymus (1). These short-lived cells are packed densely into the cortex of the thymus, where they will either die by apoptosis or receive signals that induce them to develop further. The few cells that differentiate into CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> (single-positive) cells subsequently complete their development in the thymic medulla. Both the cortex and the medulla are comprised of complex networks of epithelial, mesenchymal, and hemopoietic cells (2, 3). The division of the thymus into cortical and medullary regions is perhaps its most striking histological characteristic, yet comparatively little is known about how these regions are formed or how T cell precursors are induced to move between them during their development.

Blood-borne hemopoietic precursor cells enter the thymus from vessels that are located deep in its cortex near the boundary with the medulla (4, 5). The cells proliferate on entry and gradually migrate toward the outer regions of the cortex. As they move, they eventually cease dividing, express the recombinase-activating gene 1 (RAG-1)<sup>3</sup> and RAG-2 proteins and undergo VDJ rearrangements at the *Tcrb*, *Tcrg*, and/or *Tcrd* loci. Cells that complete productive *Tcrb* rearrangements can express TCR $\beta$  proteins and will display these on their surfaces in precursor-TCR (pre-TCR) complexes (1). Signals from the pre-TCRs then induce the cells to re-enter the cell cycle and ultimately to reverse their migration back toward the medulla while they differentiate into double-positive cells (4–6). After a rapid series of five or more divisions, the cells again cease proliferating and re-express RAG-1 and RAG-2 before rearranging the *Tcr* locus. Productive rearrangements this time permit the

expression of TCR $\alpha\beta$  complexes, which are capable of delivering signals to the cells when they engage peptide/MHC complexes (1).

Although the majority of double-positive cells die because they fail to receive the correct form of signal from the TCR $\alpha\beta$  they express, a minor fraction of them receive signals that allow for differentiation into single-positive cells. These cells migrate into the medulla as they down-regulate RAG-1 and RAG-2 and complete the changes in gene expression that define the CD4 or CD8 lineages (1). Interestingly, single-positive cells can reside in the medulla for 2–3 wk, a time period that may be essential for the completion of negative selection (7). At the end of this time, medullary single-positive cells leave the thymus, at least in part via the blood (8).

Chemokines are small diffusible or matrix-associated proteins that transmit chemotactic signals into cells through pertussis toxin-sensitive, G protein-coupled receptors (9, 10). Just as they have proved essential for lymphocyte migration in secondary lymphoid tissue, there are several indications that chemokines are important for the migration of cells within and from the thymus. Most notably, a number of studies have shown that pertussis toxin can impede the migration of single-positive cells into the medulla and prevent the exit of mature cells from the thymus to the periphery (11, 12). There are also thymic abnormalities in mice lacking G $\alpha_i$  (13, 14). In addition to these functional observations, the expression patterns of chemokines and their receptors are highly suggestive of potential roles in guiding cells in the thymus. Thus, passage through the TCR $\beta$  and TCR $\alpha$  developmental checkpoints is associated with the up-regulation of distinct chemokine receptors, the ligands for which are regionally localized in the thymus. For example, pre-TCR signaling leads to increased CCR9 expression, which would allow the cells to migrate toward CCL25 (thymus-expressed chemokine) (15–18). Similarly, CCR4, CCR7, and CCR8 are all up-regulated during positive selection and would permit migration toward medullary CCL22 (macrophage-derived chemokine) or CCL17 (thymus and activation-regulated chemokine), CCL19 (EB11-ligand chemokine, macrophage inflammatory protein-3 $\beta$ , and Exodus-3)/21 (secondary lymphoid tissue chemokine, 6-C-kine, Exodus-2, and thymus-derived chemotactic agent 4), and CCL1 (T cell activation gene-3), respectively (8, 19–22).

Notwithstanding their suggestive expression patterns, however, evidence for the involvement of specific chemokines in thymocyte migration *in vivo* has been limited to date. Mutant mice deficient

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<sup>3</sup> Abbreviations used in this paper: RAG, recombinase-activating gene; BrdU, 5-bromo-2'-deoxyuridine; PNA, peanut agglutinin; pre-TCR, precursor-TCR.

in the expression of individual chemokines or their receptors generally show well-defined cortical and medullary regions with little evidence for defects in the positioning of thymocytes in them (9). An exception to this is the recent demonstration that thymus-specific deletion of CXCR4 can impair the migration of double-negative thymocytes into the cortex (23). Interestingly, the defect in cortical migration is associated with impaired thymocyte development, suggesting that proper positioning may be essential for cells to receive the developmental cues they would normally require. Nonetheless, thymocytes can still complete their development without CXCR4, albeit at reduced efficiency compared with wild-type cells (23–25). Similarly, mice lacking CCR7 support largely normal thymocyte development, yet these mice show a defect in the emigration of mature cells from the neonatal thymus (8). Although it remains to be tested, an attractive hypothesis to reconcile the pertussis toxin, expression pattern, and genetic data is that multiple chemokines and their receptors are involved in positioning thymocytes, and that there is at least some redundancy in the positioning process at different thymocyte developmental stages.

As one means to examine the specific roles of CCR7 in thymocyte migration, we generated transgenic mice that express CCR7 prematurely. CCR7 is normally induced during positive selection, so it seemed reasonable that this receptor might be important for directing the migration of cells from the cortex to the medulla in response to the medullary chemokines CCL19 and CCL21 (20, 26). Our expectation was that premature expression of CCR7 might be sufficient to cause a redistribution of double-positive thymocytes and perhaps result in their inappropriate entrance into the medulla before positive selection had occurred. In this study we report the results of these experiments and identify CCR7 as a participant in the directed migration of thymocytes that accompanies positive selection.

## Materials and Methods

### *Mice, Abs, and flow cytometry*

A cDNA encoding a FLAG-tagged form of mouse CCR7, provided by Drs. V. Ngo and J. Cyster (University of California, San Francisco, CA); the FLAG epitope is Asp-Tyr-Lys-Asp-Asp-Asp-Lys (26), was cloned into untranslated sequence in the second exon of a mouse CD4 minigene that lacks the intronic transcriptional silencer (27). The resulting construct was linearized and injected into C57BL/6 $\times$ DBA/2 F<sub>2</sub> embryos. Founder transgenic CCR7 transgenic mice were identified by PCR, Southern blot, and flow cytometry, and these were then crossed to C57BL/6 (B6) mice for five or six generations. HY-specific B6.2.16 TCR $\alpha\beta$  transgenic mice and *plt/plt* were provided by Dr. E. Robey (University of California, Berkeley, CA) and Dr. J. Cyster (University of California, San Francisco, CA), respectively. CD45 congenic (B6.Ly5.2) mice were obtained from the National Cancer Institute Developmental Therapeutics Program. All experiments were performed according to protocols approved by the University of California-San Francisco, Institutional Animal Care and Use Committee.

Abs were purchased from BD Biosciences (Mountain View, CA; CD4, CD8, CD3 $\epsilon$ , CD69, Ly5.1), Caltag Laboratories (San Francisco, CA; CD4, CD8), and Sigma-Aldrich (St. Louis, MO; M2-FLAG). FITC-conjugated peanut agglutinin was purchased from Vector Laboratories (Burlingame, CA). The CCL19-Fc protein and human LFA3-Fc have been described previously (28). The T3.70 Ab specific for the B6.2.16 TCR (29) was purified from hybridoma supernatant fluid.

For flow cytometry, single-cell suspensions were prepared from thymuses, spleens, and lymph nodes using cell strainers (Falcon; BD Biosciences) and PBS containing BSA (0.3%, w/v) and 1.54 mM NaN<sub>3</sub>. The cells were washed two or more times and then incubated with Abs for 30 min on ice. The cells were washed an additional two times before analysis or incubation with secondary reagents.

### *Chemotaxis assays*

Chemotaxis assays were performed in RPMI 1640 containing 10% FBS as previously described (20). Thymocytes ( $10 \times 10^6$ ) in 0.1 ml of medium were placed in each of the upper compartments of Transwell chambers containing polycarbonate membrane (5- $\mu$ m pore size; Corning Costar,

Cambridge, MA). Variable concentrations of chemokines (CCL19 or CCL21; R&D Systems, Minneapolis, MN) in 0.5 ml of medium were placed in the bottom wells of the chambers. After 3-h incubation at 37°C, cells were recovered from the bottom chambers, counted, and analyzed by flow cytometry.

### *Immunohistochemistry and immunofluorescence*

Paraffin sections (3–5  $\mu$ m) were stained with H&E and prepared by Biopathological Sciences Medical (South San Francisco, CA). Frozen sections (6  $\mu$ m) were fixed with acetone, blocked with 0.1% BSA in TBS, and stained with biotin-labeled peanut agglutinin (PNA; Vector Laboratories), MTS10 (BD PharMingen, San Diego, CA), or CDR1 (American Type Culture Collection, Manassas, VA). Peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), HRP-conjugated  $\alpha$ -rat IgM (Southern Biotechnology Associates, Birmingham, AL), or HRP- and alkaline phosphatase-conjugated  $\alpha$ -rat IgG Abs (Jackson ImmunoResearch Laboratories) and 3,3'-diaminobenzidine (Sigma-Aldrich) or Fast Red (Sigma-Aldrich) were subsequently added. After washing with TBS and water, sections were counterstained with hematoxylin Gill's Formulation 1 (Fisher Scientific, Pittsburgh, PA).

For immunofluorescence, frozen sections (6  $\mu$ m) were blocked with 5% dry milk in PBS and stained with FITC-labeled PNA (Vector Laboratories), biotin-labeled anti-Ly5.1 Ab (BD Biosciences), and Cy3-labeled streptavidin (Jackson ImmunoResearch Laboratories) in 0.1% BSA/1% normal mouse serum/PBS. Sections were examined with a DML fluorescence microscope (Leica, Deerfield, IL) or a LSM 510 confocal microscope (Zeiss, Thornwood, NY).

### *Cell transfers*

Bone marrow cells were collected from wild-type B6.Ly5.2/Cr and Ly5.1-expressing CCR7 transgenic or nontransgenic littermates using IMDM containing 15% FCS. The CCR7 transgenic or nontransgenic cells were mixed with B6.Ly5.2 cells in various ratios ranging from 1:19 to 1:1, and a total of  $5 \times 10^6$  cells were injected into each lateral tail vein of lethally irradiated (two doses of 550 rad, 3 h apart), 6- to 7-wk-old, B6.Ly5.2 mice. The recipients received water containing neomycin (Sigma-Aldrich) and polymyxin B sulfate (Paddock Laboratories, Minneapolis, MN) for 6 wk.

### *5-Bromo-2'-deoxyuridine (BrdU) labeling of thymocytes*

Eight-week-old mice received one i.p. injection of 1.8 mg of BrdU (Sigma-Aldrich) dissolved in 200  $\mu$ l of water and were thereafter provided with BrdU (0.8 mg/ml) and 1% glucose drinking water for up to 8 days. Thymuses were harvested at various time points and stained for BrdU incorporation. BrdU detection was performed as previously described with some modifications (30). Briefly,  $2 \times 10^6$  thymocytes were stained with Abs specific for cell surface Ags. After washing twice with PBS, the cells were fixed for 30 min in 2% paraformaldehyde and subsequently permeabilized with 0.1% Nonidet P-40. After this step, the cells were pelleted, incubated with 50 Kunitz U of DNase I (Sigma-Aldrich), washed with PBS, and then stained with FITC-conjugated anti-BrdU Ab (BD Biosciences).

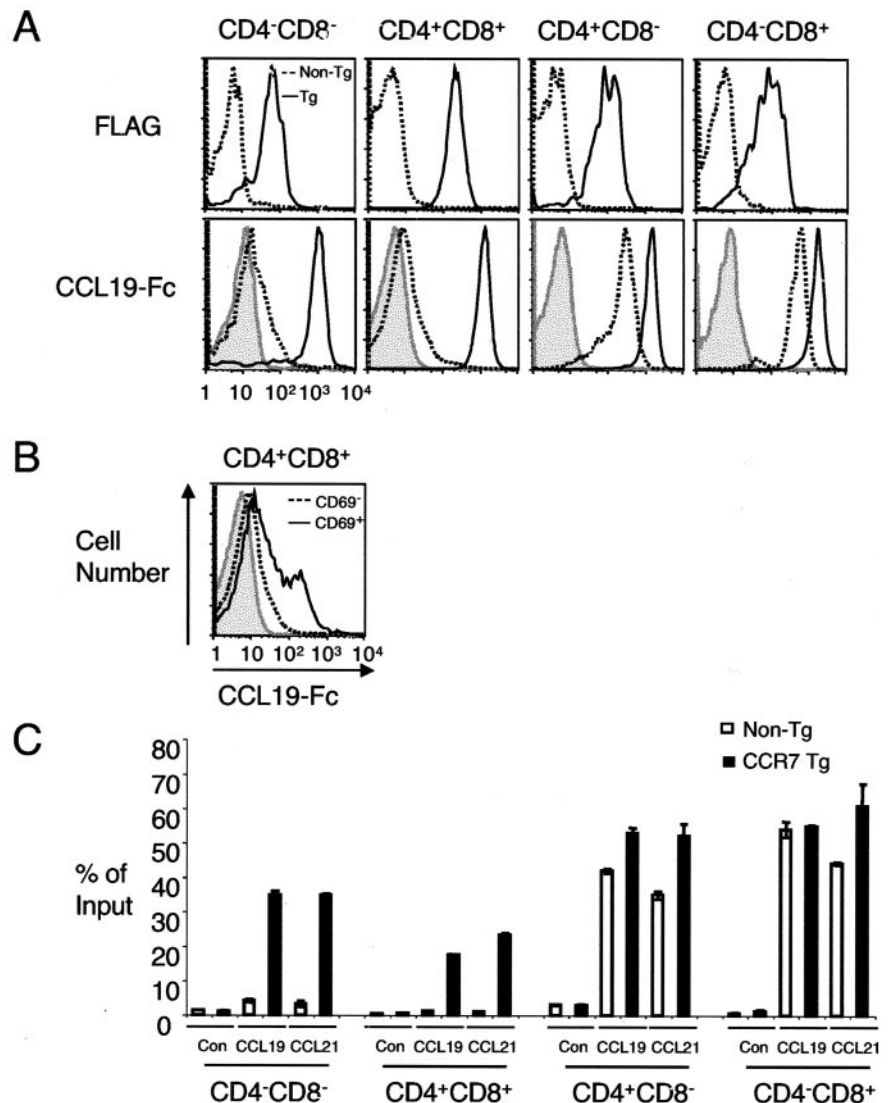
### *Intrathymic injections*

Five- to 6-wk-old mice were anesthetized with avertin (tribromoethanol; Sigma-Aldrich). An incision was made in the sternum to reveal the thymus, and 20  $\mu$ l of an FITC (Molecular Probes, Eugene, OR) solution (1 mg/ml) in PBS was injected into each thymic lobe. Spleen and mesenteric lymph node cells from mice with equivalent levels of FITC labeling in the thymus were analyzed by flow cytometry 16 h after injection for the presence of FITC-labeled recent thymic emigrants.

## Results

### *Expression and function of CCR7 on thymocytes from transgenic mice*

To express CCR7 on immature thymocytes, we generated transgenic mice in which expression of the chemokine receptor was controlled by regulatory elements from the mouse *Cd4* gene (27). A cDNA encoding a FLAG-tagged form of CCR7 (26) was embedded in a *Cd4* minigene construct that lacked the transcriptional silencer normally present in the first intron of the *Cd4* gene. CCR7 expression on cells in these mice could be detected either with an Ab specific for the FLAG epitope or with a CCL19-Fc fusion protein (28). As shown in Fig. 1, A and B, all transgenic thymocyte



**FIGURE 1.** Expression and function of CCR7 on thymocytes from transgenic mice. *A*, Thymocytes from CCR7 transgenic (solid line) and nontransgenic littermate mice (dashed line) were stained with an Ab specific for the FLAG epitope (*upper panel*) that detects the transgene-encoded protein or a CCL19-Fc fusion protein (*lower panel*) that detects both transgene-encoded and endogenous CCR7. The shaded curve shows control staining with human LFA3-Fc. *B*, The histogram shows binding of CCL19 to CD69<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> (solid line) or CD69<sup>-</sup>CD4<sup>+</sup>CD8<sup>+</sup> (dashed line) cells. The shaded curve shows control staining with human LFA3-Fc. *C*, CCR7 transgenic and nontransgenic thymocytes were placed in the upper compartments of Transwell chambers containing 500 ng/ml CCL19, 500 ng/ml CCL21, or no chemokine in the lower compartment. Cells that had migrated into the lower chamber were detected by flow cytometry after 3 h. The data shown are representative of three independent experiments.

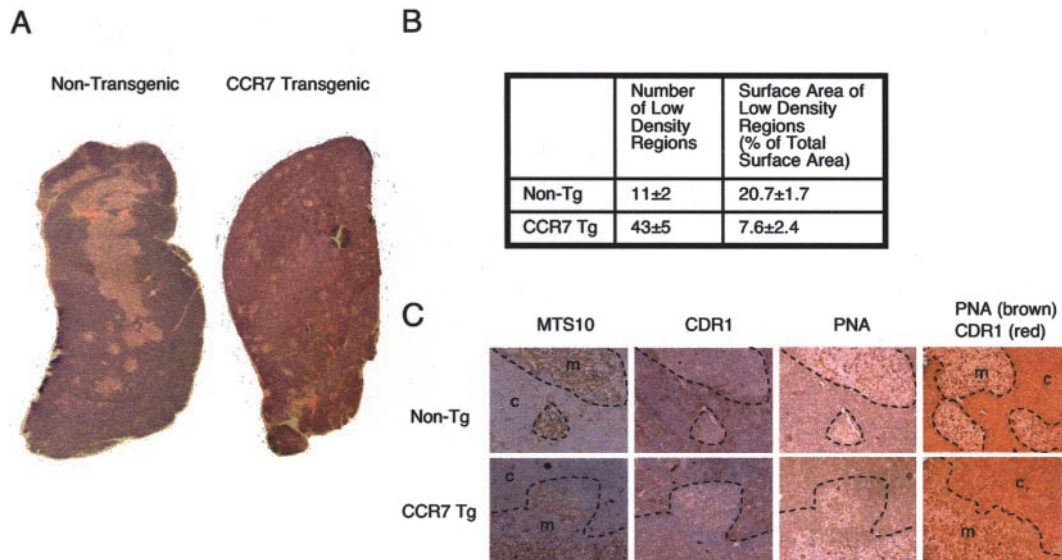
subsets expressed CCR7, whereas the endogenous protein was expressed on post-positive selection, double-positive thymocytes (i.e., CD69<sup>high</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells) and CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> single-positive thymocytes. Consistent with the expression pattern, both immature and mature thymocytes from the transgenic mice migrated toward a source of CCL19 or CCL21 in Transwell chemotaxis assays (Fig. 1C), with double-negative cells demonstrating slightly enhanced migration relative to double-positive cells. In contrast, only the mature cells from nontransgenic mice demonstrated CCL19- or CCL21-dependent chemotaxis. These data show that expression of the transgene-derived CCR7 was sufficient to render immature thymocytes responsive to chemokines that would normally only induce the migration of CD69<sup>high</sup>CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells and mature single-positive cells (20, 26).

#### *CCR7 expression induces the movement of double-positive cells into the thymic medulla*

The cortical and medullary regions of wild-type mice can be readily distinguished from one another in fixed sections of thymus tissue stained with H&E. In the representative image shown in Fig. 2A, the medulla can be seen as a large central and mostly contiguous lightly stained area. Such medullary areas were more difficult to identify in sections of CCR7 transgenic thymuses, which typi-

cally showed much smaller islands of weak staining amid an expanded area of dense staining. Specifically, compared with wild-type thymuses, the CCR7 transgenic mice had a 4-fold increase in areas of weak staining, which together accounted for 2- to 3-fold less surface area (Fig. 2B).

The reduction in the weakly stained areas visible in H&E-stained sections could represent either a compression of the medullary space or an invasion of the medulla by double-positive thymocytes brought on by CCR7-dependent chemotaxis. To determine which of these possibilities was correct, we stained serial sections of thymuses from the two types of mice with two mAbs that distinguish cortical from medullary epithelium. We also stained the sections with PNA, a lectin that binds to desialylated O-linked glycans, which are much more abundant on double-positive than on single-positive thymocytes. As shown in Fig. 2C, CCR7 transgenic and wild-type thymuses contained similar masses of cells that stained with MTS10, which is specific for medullary epithelium. These same medullary masses could be identified in the serial sections by their absence of staining with CDR1, which binds to cortical epithelium. Strikingly, whereas PNA<sup>+</sup> cells were largely excluded from the wild-type medullary areas identified by the absence of CDR1, they were clearly present in the transgenic equivalents. This presence of CCR7 transgenic (but not nontransgenic) PNA<sup>+</sup> cells in the medullae was apparent



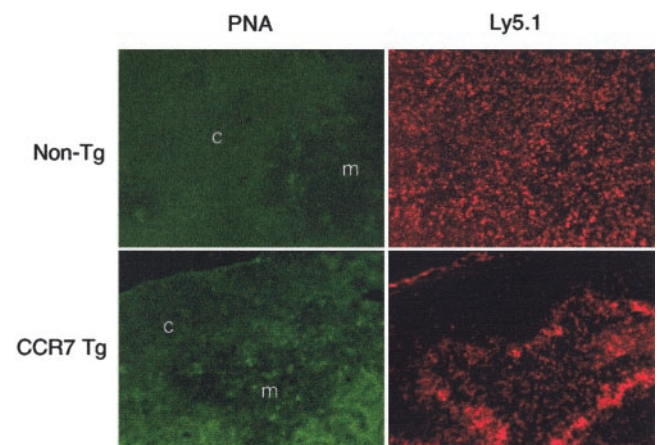
**FIGURE 2.** Abnormal architecture of CCR7 transgenic thymuses. *A*, Paraffin sections of CCR7 transgenic and nontransgenic thymuses were stained with H&E. Images were taken at  $\times 4$  magnification. The data shown are representative of sections from three or more tissue blocks. *B*, The table shows the mean number of low density regions (i.e., weakly stained regions) and the total surface area they occupied in thymic sections. Means were calculated from the analysis of eight sections in each case. The difference in surface area of low density regions was determined to be significant using Student's *t* test ( $p < 0.0001$ ). *C*, Adjacent sections of CCR7 transgenic and nontransgenic thymuses were stained with MTS10, CDR1, or PNA (brown) and counterstained with hematoxylin. Alternatively, different sections (*far right*) were double-stained with PNA (brown) and CDR1 (red). Note that in addition to staining cortical double-positive cells, PNA normally stains elements of the medullary stroma, but these stromal elements can be readily discriminated because of their large size and the fact that they stain more intensely than the thymocytes. The dashed lines correspond to the medullary and cortical regions defined by CDR1 staining. m, medulla; c, cortex. Images were taken at  $\times 5$  magnification.

both from the analysis of serial sections and from images of double-stained sections (Fig. 2*C*, *far right*), in which the brown wash of PNA-stained cells characteristically extended well beyond the red CDR1<sup>+</sup> cortical areas. These data therefore indicate that the transgenic thymuses have similar areas of medullary epithelium to those of wild-type mice, with the striking difference being that PNA<sup>+</sup> double-positive cells are present in the former, but not the latter. Thus, the premature expression of CCR7 leads to invasion of the medulla by double-positive cells.

As an additional means to visualize whether premature CCR7 expression could induce double-positive thymocytes to migrate into the medulla, we performed adoptive transfer experiments that would allow for the relative locations of transgenic and nontransgenic cells to be compared within individual thymuses. Lethally irradiated B6.Ly5.2 mice were reconstituted with B6.Ly5.2 bone marrow cells mixed with variable percentages of transgenic or nontransgenic Ly5.1<sup>+</sup> bone marrow cells. The recipients were then allowed to recover for 6 wk to allow for repopulation of the thymus with cells from the injected bone marrow. Sections of thymuses from the aged chimeras were stained with PNA to detect double-positive cells together with an Ab specific for Ly5.1 to distinguish between the transgenic and nontransgenic cells. Representative images from chimeras containing 5% Ly5.1 marked transgenic or nontransgenic cells are shown in Fig. 3. In marked contrast to their nontransgenic counterparts, the transgenic PNA<sup>+</sup> immature cells were preferentially localized to the medulla and the cortico-medullary junction of the thymus. These experiments, therefore, provide additional evidence that CCR7 expression is sufficient to relocate immature cells from the cortex to the medulla and suggest that the normal induction of CCR7 during positive selection is important for the migration of positively selected cells into the medulla.

#### Premature expression of CCR7 impairs the development of single-positive thymocytes

The presence of double-positive cells in the thymic medulla raised the possibility that early expression of CCR7 might affect the development of thymocytes. This seemed reasonable given the demonstrated importance of cortical epithelium in positive selection and of the medulla in negative selection (1). Thus, inappropriate



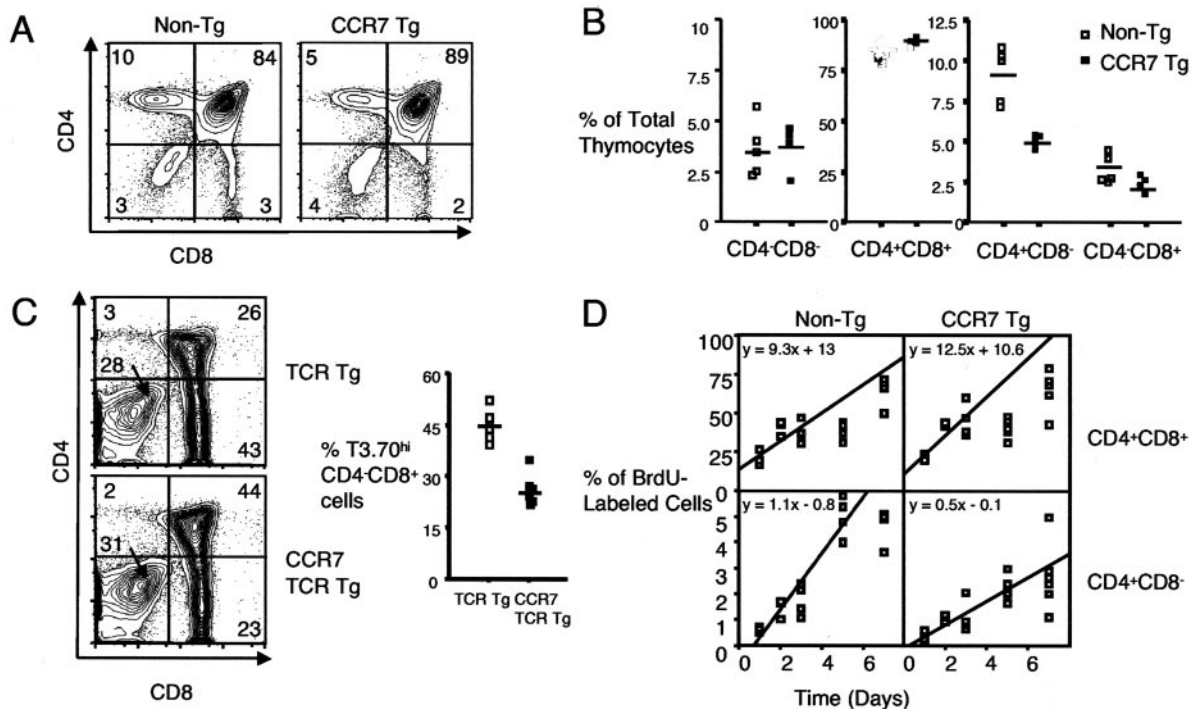
**FIGURE 3.** CCR7 expression induces movement of double-positive cells into the thymic medulla. Lethally irradiated mice were reconstituted 6 wk before analysis with mixtures of wild-type and allotype-marked (Ly5.1<sup>+</sup>) transgenic or nontransgenic cells. Thymic sections were stained with PNA and an Ab specific for Ly5.1. Representative sections from chimeras reconstituted with 95% Ly5.1<sup>-</sup> and 5% Ly5.1<sup>+</sup> cells are shown at  $\times 5$  magnification.

apposition of double-positive thymocytes with medullary epithelium might be expected to impair positive selection or perhaps to accentuate negative selection.

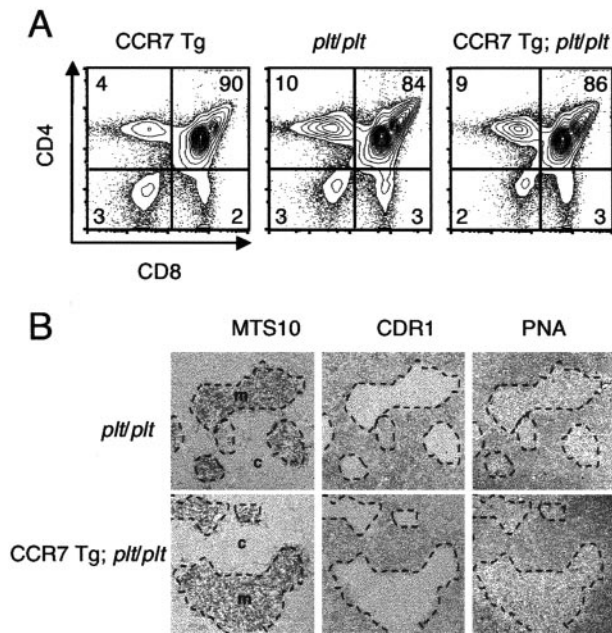
Using flow cytometry, we saw no effect of premature CCR7 expression on early thymocyte development (as assessed by CD44 and CD25 expression on double-negative cells), and there was no obvious decrease in the average number of double-positive cells present in the transgenic thymuses. We also found that double-positive cells were not enriched for annexin V<sup>+</sup> cells relative to wild-type controls. Thus, the mislocalization of cells had little detectable impact on development before positive selection, and it likewise did not appear to induce an increase in the apoptosis of double-positive cells. There was, however, an obvious effect of the transgene on the representation of CD4<sup>+</sup>CD8<sup>-</sup> single-positive cells in the thymus. Most notably, we observed a 2-fold reduction in the percentage of CD4<sup>+</sup>CD8<sup>-</sup> cells relative to other cells in the thymus (Fig. 4, A and B). As the total cellularity of the thymus was unchanged by the presence of the transgene, there was a commensurate decrease in the absolute number of CD4<sup>+</sup>CD8<sup>-</sup> cells per thymus. Reduced numbers of single-positive cells were apparent in the fetal and neonatal transgenic thymuses, and this developmental effect was also reflected in the reduced numbers of CD4<sup>+</sup> T cells present in the lymph nodes and spleens of young adult transgenic mice (data not shown). Despite the developmental abnormalities, however, the CD4<sup>+</sup> T cells that emerged from the transgenic thy-

muses retained the capacity to mediate immune responses, as shown by a test of delayed-type hypersensitivity (data not shown).

The frequency of CD4<sup>-</sup>CD8<sup>+</sup> cells was not obviously different in transgenic compared with nontransgenic mice. We therefore crossed the CCR7 transgenic mouse to a mouse expressing an MHC class I-restricted TCR transgene, so that selection of the CD4<sup>-</sup>CD8<sup>+</sup> cells could be examined with greater sensitivity. The TCR transgenic mouse we chose for this purpose encoded the well-characterized B6.2.16 TCR $\alpha\beta$  specific for the H-2D<sup>b</sup>-restricted H-Y Ag from the *Smcy* gene (31). Most thymocytes expressing this TCR are deleted at the double-negative stage of development or just after it in male H-2<sup>b</sup> thymuses (32), but they are efficiently selected into the CD8 lineage in female thymuses (29). We observed no effect of premature CCR7 expression on the deletion of H-Y-specific thymocytes (data not shown), but we consistently observed a reduced frequency of CD4<sup>-</sup>CD8<sup>+</sup> single-positive cells in the double-transgenic female thymuses. More strikingly, we found that compared with TCR transgenic mice lacking the CCR7 transgene, the double-transgenic thymuses contained fewer CD4<sup>-</sup>CD8<sup>+</sup> cells that stained brightly with the T3.70 anti-clonotype mAb (Fig. 4C). This phenotype suggests that premature expression of CCR7 compromised the selection of clonotype-bearing cells, such that cells using endogenously encoded TCR  $\alpha$ -chains were at a selective advantage. Cumulatively, the data indicate that, as seen for CD4<sup>+</sup>CD8<sup>-</sup> cells, the presence



**FIGURE 4.** Premature expression of CCR7 impairs the development of single-positive thymocytes. *A*, Representative flow cytometric data showing decreased numbers of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes in a CCR7 transgenic mouse compared with a nontransgenic control mouse. *B*, Scatter plots showing the relative percentages of thymocyte subsets identified by CD4 and CD8 expression from 7-wk-old mice. The absolute numbers of thymocytes for transgenic and nontransgenic mice were  $89 \pm 11 \times 10^6$  and  $82 \pm 17 \times 10^6$ , respectively. The difference in percentages of CD4<sup>+</sup>CD8<sup>-</sup> cells was determined to be significant using Student's *t* test ( $p < 0.002$ ). *C*, Impaired selection of clonotype-expressing CD4<sup>-</sup>CD8<sup>+</sup> thymocytes in mice transgenic for both CCR7 and the H-Y-specific B6.2.16  $\alpha\beta$  TCR. The contour plots show the expression of CD4 and CD8 on thymocytes expressing high levels of the  $\alpha\beta$  TCR clonotype identified with the T3.70 Ab. The scatter plot shows the frequency of T3.70<sup>high</sup>CD4<sup>-</sup>CD8<sup>+</sup> cells in the two types of TCR transgenic thymuses. The percentages of CD4<sup>-</sup>CD8<sup>+</sup> T cells were significantly different by Student's *t* test ( $p < 0.001$ ). *D*, The kinetics of formation of CD4<sup>+</sup>CD8<sup>-</sup> cells in CCR7 transgenic mice were examined by continuous *in vivo* BrdU labeling, followed by flow cytometry. The relative frequencies of BrdU-labeled cells are plotted for CD4<sup>+</sup>CD8<sup>+</sup> (upper panel) and CD4<sup>+</sup>CD8<sup>-</sup> (lower panel) cells. In each case, the curves were generated using three data points, and the slopes were estimated using the ordinary least square method. The slopes for formation of CD4<sup>+</sup>CD8<sup>-</sup> cells (lower panel) were significantly different when compared using an F test ( $p < 0.05$ ).



**FIGURE 5.** Chemokine ligands of CCR7 direct impaired selection of CD4<sup>+</sup>CD8<sup>-</sup> cells in CCR7 transgenic mice. CCR7 transgenic mice were crossed with *plt/plt* mice that show reduced or absent expression of CCL21 and CCL19, respectively. **A**, Thymocytes from the mice were analyzed by flow cytometry using Abs specific for CD4 and CD8. **B**, Adjacent sections of *plt/plt* and CCR7 transgenic; *plt/plt* thymuses were stained with MTS10, CDR1, or PNA (brown) and counterstained with hematoxylin. Images were taken at  $\times 5$  magnification.

of the CCR7 transgene and the mislocalization it induced, interfered with the positive selection of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

In an attempt to clarify whether the impaired development of CD4<sup>+</sup>CD8<sup>-</sup> single-positive thymocytes in CCR7 transgenic mice was due to a decrease in their rate of formation, the kinetics of thymocyte development were examined by tracing BrdU incorporation after continuous BrdU oral administration in their drinking water. In the thymus, BrdU is incorporated into rapidly proliferating, double-negative cells, with little incorporation after the cells reach the double-positive stage of development, where they largely, but not completely, cease dividing (33, 34). Thus, the rate of appearance of the BrdU label in single-positive cells is a useful index of the rate of formation of these cells from double-positive precursors. As shown in Fig. 4D, the kinetics of double-positive thymocyte labeling were similar in CCR7 transgenic and nontransgenic mice. In contrast, CD4<sup>+</sup>CD8<sup>-</sup> single-positive cells in CCR7 transgenic mice showed a reduced rate of BrdU labeling compared with CD4<sup>+</sup>CD8<sup>-</sup> single-positive cells from nontransgenic mice. Consistent with the analysis of steady-state thymocyte frequencies, this result suggests that premature expression of CCR7 causes a decrease in the fraction of cells that commits to the CD4 lineage during positive selection.

#### Chemokine ligands of CCR7 direct impaired selection of CD4<sup>+</sup>CD8<sup>-</sup> cells in CCR7 transgenic mice

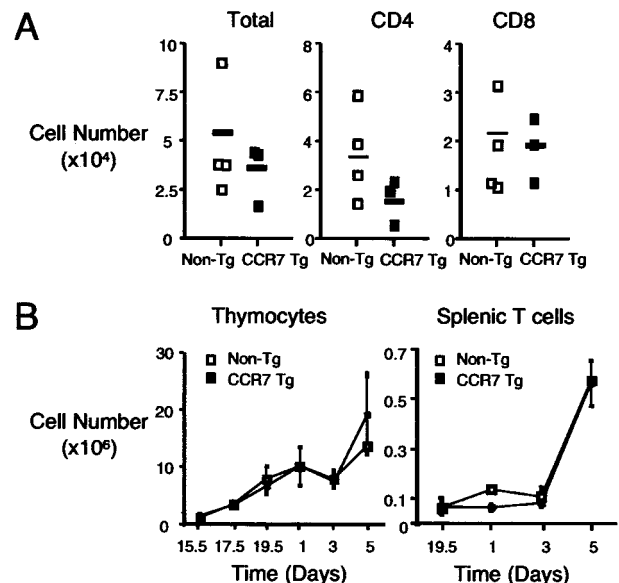
The data shown in Figs. 2 and 3 indicate that premature CCR7 expression causes the redistribution of immature thymocytes toward the thymic medulla. To show that the developmental effects associated with this redistribution were dependent on interactions between CCR7 and its chemokine ligands, CCL19 and CCL21, we crossed CCR7 transgenic mice to *plt/plt* mice that show impaired expression of these chemokines (35, 36). The *plt* mutation is a deletion of a large genomic region that contains the gene encoding CCL19 and one of two linked genes encoding CCL21. Mice ho-

mozygous for the *plt* mutation lack expression of CCL19 and show much reduced expression of CCL21 (36–38). As shown in Fig. 5A, the effect of premature CCR7 expression on T cell development was effectively suppressed in *plt/plt* mice. In addition, PNA-positive thymocytes were no longer present in the *plt/plt* CCR7 transgenic medullae, and the thymuses had normal cortical and medullary areas (Fig. 5B). These data indicate that, as expected, the positioning and developmental effects of the transgene were dependent on CCL19/21.

#### Effect of transgenic CCR7 on the exit of T cells from the thymus and their accumulation in the spleens of newborn mice

The most abundant source of CCL19 and CCL21 in the thymus is medullary epithelial cells, but there is also some expression of CCL19 on thymic endothelial cells. This latter site of CCL19 expression has recently been implicated as being important for the directed emigration of thymocytes from the medulla at the completion of their development (8). Support for this idea came principally from the finding that neonatal mice lacking CCR7 expression showed delayed accumulation of recent thymic emigrants in their spleens compared with wild-type littermates. Furthermore, CCL19, but not CCL21, could induce the migration of cells when it was applied to thymic organ cultures. Because of these findings, it was important to test whether premature exit of cells from the thymus might be associated with the developmental effects caused by transgenic CCR7 expression.

Two assays were used to quantify recent thymic emigrants in the CCR7 transgenic mice. In one, a solution of FITC was injected directly into the thymuses of transgenic and nontransgenic mice, and the numbers of FITC-labeled emigrant cells in secondary lymphoid organs were then determined by flow cytometry the following day. In the second, thymocyte and splenic T cell numbers were counted in neonatal mice. As shown in Fig. 6, neither assay showed any evidence of an increase in thymic emigration as a consequence of expression of the transgene. In fact, in both cases,



**FIGURE 6.** Effect of transgenic CCR7 on the exit of T cells from the thymus and their accumulation in the spleens of newborn mice. **A**, A solution containing FITC was injected into the thymuses of CCR7 transgenic and nontransgenic mice. One day later, FITC<sup>+</sup> recent thymic emigrants (all FITC<sup>+</sup> cells or the CD4<sup>+</sup> or CD8<sup>+</sup> fractions thereof) were enumerated in the spleens of the injected mice. **B**, The graphs show the numbers of thymocytes or splenic T cells in embryonic and newborn transgenic or nontransgenic mice.

there was reduced accumulation of CD4<sup>+</sup> cells in the transgenic mice, reflective of the developmental effect described above.

Finally, we also examined neonatal and adult blood and lymphoid tissues for the presence of elevated numbers of double-positive cells, but again could find no evidence that the transgene was inducing early exit of these cells from the thymus (data not shown).

## Discussion

In this study we have shown that CCR7 can promote the migration of double-positive cells from the cortex to the medulla of the thymus. This role was apparent both from the steady state analysis of CCR7-transgenic thymuses and from the positioning of the transgenic thymocytes in radiation chimeras. Inappropriate localization of double-positive thymocytes reduced the efficiency with which single-positive cells were formed, indicating that the correct positioning of thymocytes is essential for their development. The data suggest that regulating responsiveness to medullary chemokines is a crucial component of the normal development of thymocytes and highlight CCR7 as a participant in cortical to medullary thymocyte migration.

CCR7 binds to CCL19 and CCL21, both of which are encoded by a small cluster of genes on mouse chromosome 4. In the thymus, CCL19 is expressed by medullary epithelial cells, CD11c<sup>+</sup> dendritic cells, and CD31<sup>+</sup> endothelial cells. The expression of CCL21 overlaps with that of CCL19, but its range of action may be somewhat more constrained, because, unlike CCL19, it has a charged C-terminal extension that allows for binding to the extracellular matrix (39–41). We have shown that the effect of the CCR7 transgene on thymocyte development is lost in mice that are homozygous for the *plt* mutation, in which both CCL19 and CCL21 expression is impaired. Although this observation makes clear that the transgenic phenotype is ligand dependent, it leaves open the question of whether CCR7-directed migration into the medulla might normally be dependent on both or just one of the two chemokines.

The *plt/plt* mice have clearly defined cortical and medullary regions in the thymus, and they support overtly normal development of thymocytes. Similar statements apply to mice that lack CCR7 (8, 42). Such observations could be used to infer the absence of a role for CCR7 in medullary translocation and to call into question the conclusions of our study. It remains true, however, that positive selection in normal mice results in up-regulation of CCR7, and as we show in this study, the acquisition of CCR7 is sufficient to move double-positive cells into the medulla. Other chemokine receptors are also up-regulated during positive selection, raising the possibility that the migration of cells into the medulla could be brought about by the combined influence of multiple redundant mechanisms. Possibilities in this respect would include CCR4 and CCR9. CCR4 may be an especially attractive candidate, because, like CCR7, its expression is markedly induced as a consequence of positive selection. Moreover, CCL22, one of the ligands for CCR4, is also expressed in the medulla, and this ligand is an efficacious attractant for cells undergoing positive selection (i.e., CD69<sup>+</sup> cells) (16, 20, 43, 44). CCR9 is expressed by double-positive cells, but its greatest increase in expression may occur after the pre-TCR signal (15–17). The ligand for CCR9, CCL25, is found in both the cortex and the medulla, and thymocytes show apparently equivalent migration toward it both before and immediately after positive selection (15, 17, 20). Mice lacking CCR4 (45) or CCR9 (18, 46) have been generated, but neither mouse has to date been described as showing a defect in thymocyte positioning or in the formation of cortical or medullary areas. To date, mice showing defective responses to combinations of chemokines (e.g., mice lacking both CCR4 and CCR7) have not been generated, but such mice are of

obvious importance for discerning the nature of the redundancy in the migration mechanism. If CCR4 and CCR7 are the major contributors to medullary migration, then the loss of both receptors would presumably have a marked effect on migration and development.

In addition to its capacity to guide cells toward the medulla, CCR7 and its ligands are apparently also involved in directing cells out of the thymus. Neonatal mice lacking CCR7 or mice treated with a CCL19 antagonist show decreased emigration of cells from the thymus to the spleen, although in neither case is emigration completely impaired (8). Thus, as just argued for migration to the medulla, the dependency of thymocytes on CCR7-directed chemotaxis for emigration is not complete, and other factors are also likely to be involved in this process. Consistent with this, we found that transgenic expression of CCR7 on double-positive cells and mature single-positive cells was insufficient to direct them out of the thymus, and we saw no detectable increase in thymocyte emigration in the transgenic mice using two separate assays. Emigration from the thymus could involve both lymphatic and blood vessels, and the mechanisms that regulate it are largely unclear. As CCR7 is expressed from an early point after positive selection, yet single-positive cells can reside in the medulla for 2 wk or more (7), additional signals may be required to prime cells for exit, and these signals may only be received after the cells have resided in the medulla for a substantial length of time.

The migration path of thymocytes is circuitous, involving movement from the medulla to the outer cortex, followed by re-entry into the medulla after TCR $\alpha\beta$  selection (4–6). As mentioned in the introduction, recent data implicate CXCR4 in the first step of this path, i.e., the guidance of progenitor cells into the cortex (23). When in competition with wild-type thymocytes, CXCR4-deficient cells failed to enter the cortex, and their development was arrested at the double-negative stage. In a similarly competitive situation, CCR7 transgenic cells did not show the same type of developmental block despite the fact that they were found concentrated in the medulla and showed little, if any, penetration into the cortex. Although the intrathymic migration phenotypes of CXCR4-deficient and CCR7 transgenic cells appear to be superficially similar, additional experiments will be required to determine whether this is, in fact, the case. It remains possible, for example, that CXCR4 expression on progenitor thymocytes allows them to move toward CXCL12-secreting cells close to the medullary boundary, and that limited penetration into the cortex of this sort is sufficient for development to proceed.

The thymic medulla is rich in cell types that are effective in inducing negative selection. Double-positive cells are especially sensitive to apoptosis, in part because of their reduced expression of proteins such as Bcl-2 that would protect them against programmed cell death (1). It seemed possible, therefore, that by causing double-positive cells to move into the medulla prematurely, there would be an increase in apoptosis because of the induced proximity of sensitive cells to APCs replete with costimulatory ligands. Although we did observe a defect in thymocyte development in the transgenic mice, we found no evidence that this was due to increased cell death. Thus, the overall cellularity of the thymus was not decreased, and the only observable defects were those in the rate of formation of CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> single-positive cells. Cortical epithelial cells are distinctive in their capacity to promote the positive selection of cells (2), and it could be that less of the transgenic cells are able to interact efficiently with these cells because of premature migration toward medullary stromal elements. Alternatively, the apposition of the cells next to medullary components may, in fact, suppress signals that are necessary for positive selection. Whatever the basis of the effect, the data make clear that normal thymocyte development hinges on the



proper regulation of cortico-medullary migration and retention of cells in the cortex until positive selection signals have been received.

Patterning of the thymus during and after embryogenesis is a dynamic process that can be reinitiated in response to thymocyte depletion and that can also be modeled in vitro in reaggregate organ culture systems. The formation of the cortex and medulla in both contexts is crucially dependent on the presence of thymocytes (2). There is, for instance, a lack of recognizable medullary or cortical areas in mice that lack intrathymic T cell precursors (e.g., mice deficient in both c-Kit and common  $\gamma$ -chain expression or mice that overexpress human CD3 $\epsilon$ ) (47, 48). Such thymuses are extremely small and contain seemingly disorganized aggregations of epithelial cells. RAG-1- or RAG-2-deficient mice that lack double-positive thymocytes have larger thymuses, but these are still much smaller than wild-type thymuses. Although cortical epithelium forms in these thymuses, they contain hypoplastic and disorganized medullae (49, 50). TCR $\alpha$ -deficient thymuses are also devoid of properly organized medullae despite the presence of abundant cortical epithelium (51). Cumulatively, observations of this sort show that the formation of the medulla is dependent on the presence of mature, single-positive cells, whereas the cortex can be organized in response to the presence of double-negative thymocytes.

The data presented in this paper show that the medulla could remain organized despite the presence of invading double-positive cells. Thus, double-positive cells do not appear to recruit cortical epithelial cells to their vicinity, nor do they appear to suppress the formation of the medulla. Recent data indicate that lymphotoxin  $\beta$  receptor on single-positive thymocytes and the NF- $\kappa$ B-inducing kinase in medullary epithelial cells are essential for proper organization of the medulla (52). These data support a model for the development of the medulla that depends on inducing signals from single-positive cells, but is not dependent on the exclusion of double-positive cells.

Through their capacity to bring stromal and thymocyte components together, it is sensible to consider a role for chemokines in the organizing process that forms cortical and medullary regions. We show in this study that chemokine receptors can drive associations between thymocytes and medullary cells consistent with the involvement of chemokines in selection and perhaps cortico-medullary patterning. Transgenic gain-of-function systems such as the one used in this study may provide further insight into the signals that are important for formation of the thymic medulla.

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