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Characterization of CCR9 Expression and CCL25/Thymus-Expressed Chemokine Responsiveness During T Cell Development: $CD3^{high}CD69^+$ Thymocytes and $\gamma\delta TCR^+$ Thymocytes Preferentially Respond to CCL25[1]

Shoji Uehara,* Kaimei Song,† Joshua M. Farber,† and Paul E. Love[2]*

CCR9 mediates chemotaxis of thymocytes in response to CCL25/thymus-expressed chemokine, and its mRNA is selectively expressed in thymus and small intestine, the two known sites of T lymphopoiesis. To examine the expression of CCR9 during lymphocyte development, we generated polyclonal Ab that recognizes murine CCR9. CCR9 was expressed on the majority of immature $CD4^+CD8^+$ (double-positive) thymocytes, but not on immature $CD4^-CD8^-$ (double-negative) thymocytes. CCR9 was down-regulated during the transition of double-positive thymocytes to the $CD4^+$ or $CD8^+$ (single-positive) stage, and only a minor subset of $CD8^+$ lymph node T cells expressed CCR9. All $CCR9^+$ thymocyte subsets migrated in response to CCL25; however, $CD69^+$ thymocytes demonstrated enhanced CCL25-induced migration compared with $CD69^-$ thymocytes. Ab-mediated TCR stimulation also enhanced CCL25 responsiveness, indicating that CCL25-induced thymocyte migration is augmented by TCR signaling. Approximately one-half of all $\gamma\delta TCR^+$ thymocytes and peripheral $\gamma\delta TCR^+$ T cells expressed CCR9 on their surface, and these cells migrated in response to CCL25. These findings suggest that CCR9 may play an important role in the development and trafficking of both $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ T cells. *The Journal of Immunology,* **2002, 168: 134–142.**

Chemokines are a group of small proteins with molecular mass between 8 and 14 kDa. Chemokines play key roles in the development and trafficking of hematopoietic cells through interactions with a subset of seven-transmembrane, G protein-coupled receptors (1, 2). Thymocyte subsets, distinguished by their expression of CD4 and CD8, localize to distinct regions of the thymus and display differential chemotactic behavior in response to thymus-expressed chemokines (3, 4). Consequently, it has been suggested that chemokines are likely to play an important role in regulating the trafficking of developing T cells within the thymus. Various chemokines such as CCL17, CCL19, CCL21, CCL22, CCL25/thymus-expressed chemokine, and CXCL12 are expressed in the thymus. One possible chemokine regulating the trafficking of thymocytes is CCL25. CCL25 mRNA is specifically expressed in the thymus and small intestine (5, 6). In the thymus, CCL25 is produced by medullary dendritic cells and cortical epithelial cells, and induces the migration of thymocytes but not mature peripheral T cells (5, 6). Recently, GPR9-6/CCR9 was found to be the receptor for CCL25. CCR9 mRNA is detected in immature and mature thymocytes but not mature T cells (6–11). CCL25 mRNA is also expressed in the epithelium of the small intestine and, in humans, CCR9 is selectively expressed on intestinal homing T lymphocytes and mucosal lymphocytes in the small intestine (6, 11–13). Based on these data, it has been suggested that CCR9 might play a role in the attraction and/or retention of T cell progenitors to these sites.

To characterize more precisely the expression of CCR9 during T cell ontogeny, we generated polyclonal Ab that recognizes murine CCR9, and we examined the relationship between CCR9 surface expression and the specific chemotactic activity to CCL25 during T cell development. Our results demonstrate that CCR9 is expressed on the cell surface of both $\alpha\beta$ and $\gamma\delta TCR^+$ thymocytes and that these cells are specifically responsive to CCL25. Moreover, in $\alpha\beta$ T lineage cells, CCR9 expression is developmentally regulated, and CCL25 responsiveness is influenced by activation through the TCR. Collectively, these data support the idea that CCR9 plays an important role in T cell development in both the $\alpha\beta$ and $\gamma\delta$ T lineages.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were bred within our facility. Embryos at various stages of gestation were obtained from time-mated pregnant mice. The date on which a vaginal plug was observed was designated gestation day 0.5. Mutant strains of mice used in this study included Rag-$1^{-/-}$ (14), TCR-α chain$^{-/-}$ (15), and MHC class I × $II^{-/-}$ (β_2-microglobulin$^{-/-}$ × $A\beta^{-/-}$) (16).

Generation of anti-murine CCR9 Ab

Rabbit polyclonal Ab against murine CCR9 was prepared according to standard methods. A 16-aa NH_2-terminal peptide of murine CCR9 (CM FDDFSYDSTASTDD) was coupled to keyhole limpet hemocyanin. Immune serum was produced in New Zealand White rabbits, and Ab was purified using the immunizing peptide coupled to normal human serum-activated Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). Control rabbit IgG was purified from preimmune serum by affinity to protein A (Pierce, Rockford, IL).

*Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, and †Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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[2] Address correspondence and reprint requests to Dr. Paul E. Love, Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Building 6B, Room 2B210, MSC 2780, Bethesda, MD 20892. E-mail address: pel@helix.nih.gov

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Validation of anti-murine CCR9 Ab

Mouse CCR9 cDNA was amplified from mouse thymocyte cDNA by PCR using the following *Eco*RI site containing primers: forward primer, 5′-CCGGAATTCTGAATAGCCCTCCTGAAGCTGATTGGC-3′; reverse primer, 5′-CCGGAATTCCCAAAAAGGACCATATGCCCT-3′. The PCR fragment was inserted into the *Eco*RI site of the retroviral vector hCD4.RV (17), which was kindly provided by T. Murphy, Washington University School of Medicine (St. Louis, MO). hCD4.RV contains a multiple cloning site, followed by an internal ribosome entry site, followed by sequences encoding a truncated human CD4. The plasmids containing CCR9 in sense and antisense orientations and control plasmids without insert were used to transfect Phoenix-*Eco* cells, obtained from G. Nolan, Stanford University (Stanford, CA), through the American Type Culture Collection (Manassas, VA), using the protocol on G. Nolan's web site at http://www.stanford.edu. Two days after transfection, the cells were stained for CD4 and with the affinity-purified biotinylated anti-murine CCR9 and control Abs, followed by PE-conjugated streptavidin (av-PE).[3]

Abs and reagents

Abs used for flow cytometric analysis were purchased from BD PharMingen (San Diego, CA) and included FITC, PE, or CyChrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD44, anti-B220, anti-$\alpha\beta$TCR, anti-$\gamma\delta$TCR, anti-Vγ2TCR, and anti-Vγ3TCR mAbs. Unconjugated anti-FcγRII (2.4G2) was used to block nonspecific binding of the labeled Ab. av-PE and CyChrome-conjugated streptavidin (av-CyChrome) were also purchased from BD PharMingen. Murine CXCL12 and CCL25 were obtained from PeproTech (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN), respectively.

Chemotaxis assays

Chemotaxis assays were performed as described (10), with modifications, using 6.5-mm Transwell tissue culture inserts with a 5-μm pore size (Costar, Cambridge, MA). Thymocytes were suspended at 1×10^7 cells/ml in RPMI 1640 plus 0.5% BSA, and 100 μl of cell suspension was added to an insert in a well with 600 μl of medium. After equilibration at 37°C for 1 h, chemokines were added to the wells and the plates were incubated for an additional 2 h before cells were harvested, collected by centrifugation, and counted. Duplicate wells were used for each condition.

Cell preparation

Thymi, lymph nodes, and spleens were excised from mice, and single cell suspensions were prepared. Intestinal intraepithelial lymphocytes (iIEL) were isolated as previously described (18). Briefly, small intestines were cut longitudinally and then into 5-mm pieces, and washed three times with CMF (Ca^{2+}-, Mg^{2+}-free HBSS with 1 mM HEPES, 2.5 mM $NaHCO_3$, pH 7.3) containing 2% calf serum. Washed intestinal pieces were stirred at 37°C for 20 min in CMF containing 10% calf serum and 1 mM dithioerythritol. This step was repeated, the resultant supernatants were rapidly filtered through nylon wool, and the filtrate was centrifuged through a 44/67% Percoll gradient (Amersham Pharmacia Biotech). The cells at the interface of the gradient were collected. For purification of $\alpha\beta$TCR$^+$ and $\gamma\delta$TCR$^+$ iIEL, cells were stained with biotinylated anti-TCR$\alpha\beta$ or anti-TCR$\gamma\delta$, and with anti-FcγR to prevent nonspecific binding of the labeled mAb, followed by incubation with streptavidin-coupled microbeads. Stained cells were applied to a MACS column (Miltenyi Biotec, Auburn, CA), and adherent cells were recovered.

Immunofluorescence analysis

Standard flow cytometry was performed, as described previously (19), using a FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

Enrichment of DN cells from thymus

Double-negative (DN) thymocytes were enriched by MACS. Briefly, thymocytes were stained with a mixture of biotinylated anti-CD4, anti-CD8, anti-B220, anti-TCR$\alpha\beta$, and anti-FcγR to prevent nonspecific binding of the labeled mAb, followed by incubation with streptavidin-coupled microbeads. Stained cells were applied to a MACS column, and nonadherent cells were recovered.

Thymocyte stimulations

In vivo CD3 cross-linking. Rag1$^{-/-}$ mice were injected i.p. with 100 μg of anti-CD3 mAb (2C11). Mice were sacrificed 1, 3, or 5 days after injection, and thymocytes were harvested and stained as described above.

In vitro CD3 cross-linking. Twenty-four-well plates were coated with 10 μg/ml anti-CD3 (2C11) in PBS overnight at 4°C and subsequently washed with hybridoma serum-free medium (Life Technologies, Rockville, MD). A total of 4×10^6 thymocytes was resuspended in 2 ml of hybridoma serum-free medium and plated in uncoated or anti-CD3-coated wells for 20 h at 37°C, 5% CO_2. After incubation, thymocytes were pelleted, washed, and resuspended at 1×10^7 cells/ml in RPMI 1640 with 0.5% BSA, and chemotaxis assays were performed as described above.

Northern blotting and semiquantitative RT-PCR

Poly(A)$^+$ RNA was isolated from small intestines of B6 and Rag1$^{-/-}$ mice using the MicroFast Track kit (Invitrogen, San Diego, CA), fractionated on a 1% agarose/formaldehyde gel, and transferred onto GeneScreen Plus nylon membrane (NEN, Boston, MA). The membrane was hybridized with ^{32}P-labeled cDNA fragments encoding mouse CCR9, mouse CCL25, or human elongation factor-1α. For quantitative RT-PCR analysis, total RNA was isolated from purified iIEL subsets using TRIzol reagent (Life Technologies), and first-strand cDNA template was synthesized using SuperScript II reverse transcriptase (Life Technologies) with random hexamers. Serial dilutions of these cDNA templates were subjected to PCR amplification using sets of primers of CCR9 (forward primer, 5′-ATTGCACAA GAGTGAAGACC-3′; reverse primer, 5′-GTCAACAGCCTGCACTAC AA-3′) or CD3ε (forward primer, 5′-GCCTCAGAAGCATGATAAGC-3′; reverse primer, 5′-AGACTGCTCTCTGATTCAGG-3′). Cycling parameters were 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C for 35 cycles to detect CCR9 mRNA, and 32 cycles for CD3ϵ. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide.

Results

Generation of rabbit anti-murine CCR9 Ab

We and others have found that CCR9 mRNA is selectively expressed in the thymus and small intestine, as assessed by Northern blot and RT-PCR analysis (Refs. 6, 7, and 9 and data not shown). However, the surface expression of CCR9 on murine T cells subsets has not been examined. To investigate the role of CCR9 and its ligand, CCL25, during T cell development, we generated polyclonal Ab against murine CCR9. Rabbits were immunized with a keyhole limpet hemocyanin-coupled peptide consisting of a 16-aa NH_2-terminal fragment of murine CCR9. Within this 16-aa peptide, only six amino acid residues are conserved between mouse and human CCR9 gene. Immune serum was affinity-purified on a CCR9 peptide column, biotinylated, and used for staining. Biotinylated IgG, purified from preimmune serum, was used as control Ab.

The specificity of the anti-peptide Ab was confirmed by staining a human embryonic kidney cell line transfected with murine CCR9 cDNA. Affinity-purified Ab against CCR9 peptide reacted with cells transfected with vector DNA containing mouse CCR9 sequences in the sense orientation, but not with cells transfected with vector without insert (Fig. 1*A*), or with vector containing CCR9 sequences in the antisense orientation (data not shown). Similar results were obtained with NIH3T3 cells transduced using murine CCR9-encoding retrovirus (data not shown). In addition, the anti-CCR9 Ab did not react with mouse CCR6-, CCR8-, or CXCR3-expressing cells (data not shown).

Since CCL25 is the only known ligand for CCR9, we evaluated whether anti-CCR9 Ab inhibits CCL25-induced migration. CCL25-mediated chemotaxis of thymocytes was not inhibited by pretreatment of cells with anti-CCR9, indicating that the Ab does not block CCL25 binding to CCR9 (data not shown). However, preincubation of thymocytes with CCL25 at 37°C for 30 min decreased the level of staining with anti-CCR9 in a dose-dependent manner (Fig. 1*B*). Incubation with CXCL12 at 37°C or with

[3] Abbreviations used in this paper: av-PE, streptavidin-PE; av-CyChrome, streptavidin-CyChrome; DN, double-negative; DP, double-positive; iIEL, intestinal intraepithelial lymphocyte; SP, single-positive.

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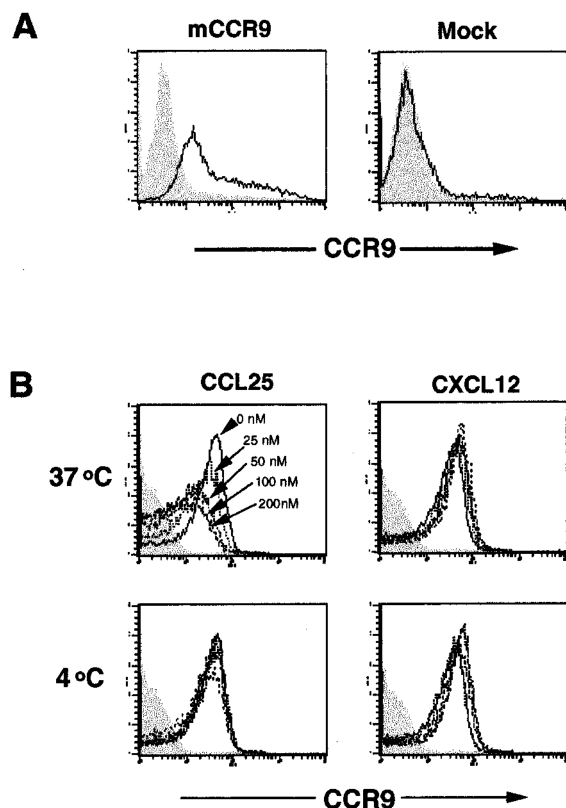

FIGURE 1. FACS analysis of CCR9 surface expression on transfected cells using polyclonal anti-CCR9. *A*, Phoenix *Eco*-CCR9 cells or mock-transfected Phoenix-*Eco* cells were incubated with biotinylated anti-CCR9 (open histogram) or biotinylated control rabbit Ig (shaded histogram), then labeled with av-PE and analyzed by FACS. *B*, Thymocytes were suspended in RPMI 1640 with 0.5% BSA containing 200, 100, 50, or 25 nM CCL25 or CXCL12 at 1×10^7 cells/ml. After incubation at 37 or 4°C for 30 min, thymocytes were spun, washed, and stained with biotinylated anti-CCR9, followed by av-PE. Background staining was obtained by staining with control rabbit Ig.

CCL25 at 4°C had no effect on CCR9 staining (Fig. 1*B*). These results suggest that CCL25 may induce down-modulation of CCR9 on thymocytes.

CCR9 expression on adult and fetal thymocytes and T cell subsets

Using affinity-purified anti-CCR9, we studied the expression of CCR9 on thymocytes and mature T cell subsets. Surface staining demonstrated that CCR9 is expressed on most double-positive (DP) thymocytes and is down-regulated on transitional single-positive (SP; $CD4^+CD8^{low}$ and $CD4^{low}CD8^+$) and mature SP ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes (Fig. 2*A*). Most DN cells did not express CCR9 (Fig. 2*A*), and analysis of immature DN thymocyte subsets (distinguished on the basis of CD25 and CD44 expression) failed to reveal detectable CCR9 surface expression (Fig. 3*A*). These data are consistent with previous results obtained using RT-PCR analysis (9, 10). In the periphery, CCR9 was expressed on a small subset of $CD8^+$ T cells but was not detectable on $CD4^+$ T cells (Fig. 2*B*). Chemotactic assay of lymph node cells showed preferential migration of $CD8^+$ T cells (~8-fold) relative to $CD4^+$ T cells in response to CCL25 (data not shown). Among $CD8^+$ T cells, naive ($CD44^{low}CD62L^{high}$) cells preferentially migrated to CCL25 (data not shown), supporting the idea that recent thymic $CD8^+$ emigrants still express CCR9 and can respond to CCL25. CCR9 expression was undetectable on CD3-activated mature T cells, NK cells, and $NK1.1^+$ T cells (data not shown).

To examine the expression of CCR9 during T cell ontogeny, we next analyzed total thymocytes from fetal and newborn mice. CCR9 expression was detected only on thymocytes from newborn mice (Fig. 3*B*). Interestingly, although thymocytes from gestation day 17.5 contain DP cells, these cells did not express CCR9 (Fig. 3*B*). Injection of anti-CD3 mAb into $Rag1^{-/-}$ mice mimics pre-TCR signals promoting cell proliferation and transition of DN thymocytes to the DP stage. It was previously reported that CCR9 mRNA expression is strongly induced following anti-CD3 treatment of $Rag2^{-/-}$ thymocytes (9). To examine the role of pre-TCR signaling in regulating surface expression of CCR9, $Rag1^{-/-}$ mice were injected with 100 μg of anti-CD3, and CCR9 expression was examined 3 and 5 days later. Untreated $Rag1^{-/-}$ thymocytes did not express surface CCR9 (Fig. 4) and could not respond to CCL25 (data not shown). Three days after stimulation with anti-CD3, $Rag1^{-/-}$ thymi contained DP cells; however, these cells remained $CCR9^-$ (Fig. 4*B*). Detectable CCR9 surface expression was only observed at day 5 when >90% of thymocytes were DP (Fig. 4*B*). Taken together, these data indicate that although CCR9 mRNA is rapidly induced by pre-TCR engagement (9), CCR9 surface expression begins only after thymocytes have developed to the DP stage.

TCR signaling enhances CCL25 responsiveness but down-regulates CCR9 surface expression

To determine whether $\alpha\beta$TCR engagement affects CCR9 expression, CCR9 levels were examined on $CD3^{low}CD69^-$, $CD3^{low}CD69^+$, and $CD3^{high}CD69^+$ thymocyte subsets, because CD69 and CD3 are up-regulated on DP thymocytes after engagement of the TCR by positively or negatively selecting ligands in the thymus (20, 21). Comparison of gated $CD3^{low}CD69^-$ and $CD3^{low}CD69^+$ cells did not reveal any difference in the level of CCR9 surface expression; however, CCR9 expression was slightly lower on $CD3^{high}CD69^+$ thymocytes (data not shown). When the chemotactic response of thymocytes was examined, $CD69^+$ thymocytes exhibited enhanced migration to CCL25 relative to $CD69^-$ thymocytes (Fig. 5). Analysis of the surface phenotype of migrating cells revealed that $CD3^{high}CD69^+$ cells were especially responsive to CCL25 (Fig. 5), and most of these cells were CD4/CD8 SP thymocytes (data not shown). CXCL12 also induced preferential migration of $CD69^+$ thymocytes, but these cells were predominantly $CD3^{low}$ DP (Fig. 5 and data not shown).

We next examined CCR9 expression and CCL25 responsiveness using thymocytes from MHC class $I/II^{-/-}$ and TCR-$\alpha^{-/-}$ mice. MHC class $I/II^{-/-}$ and TCR-$\alpha^{-/-}$ mice contain DP thymocytes, but these cells fail to undergo positive selection and lack $CD69^+$ cells due to the absence of TCR engagement or TCR expression, respectively (15, 16). Although CCR9 surface expression levels were similar on DP thymocytes from control (B6), MHC class $I/II^{-/-}$, and TCR-$\alpha^{-/-}$ mice, thymocytes from MHC class $I/II^{-/-}$ and TCR-$\alpha^{-/-}$ mice exhibited reduced CCL25-induced migration as compared with B6 mice (Fig. 6). To determine whether TCR-mediated signaling enhances CCL25-induced chemotaxis, MHC class I/II-deficient thymocytes were cultured with or without plate-bound anti-CD3 in serum-free media for 20 h and then assayed for chemotaxis. TCR stimulation significantly enhanced migration in response to CCL25 (Fig. 7*A*). Migration to CXCL12 was unchanged by TCR stimulation. Although migration to CCL25 was enhanced in cells stimulated by TCR cross-linking,

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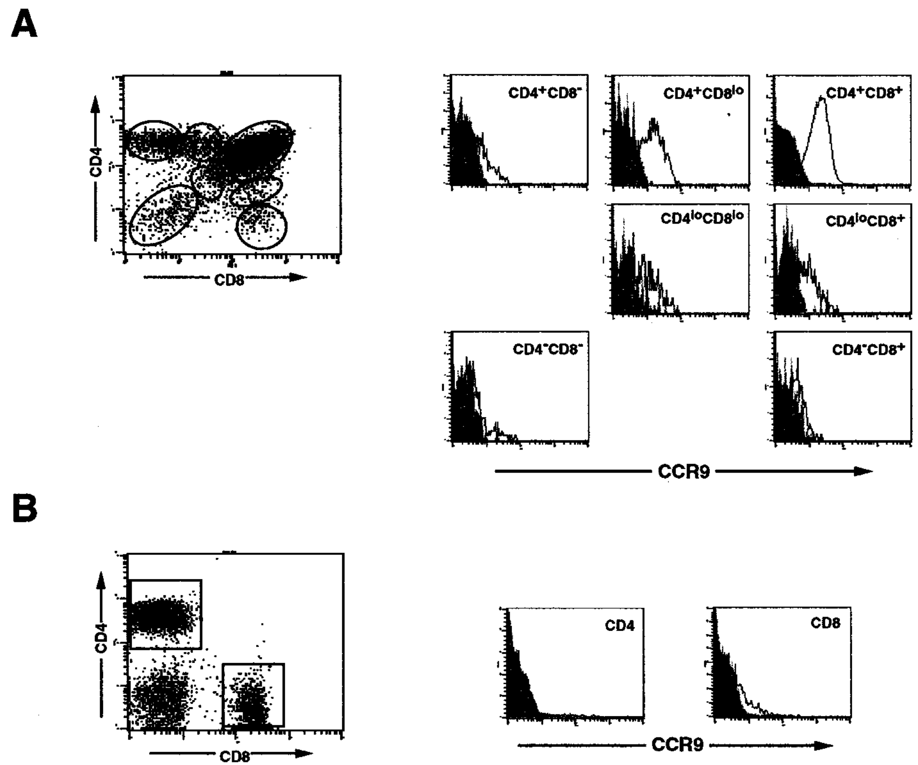

FIGURE 2. Expression of CCR9 on thymocyte and lymph node T cell subsets. Three-color staining was performed to analyze the level of CCR9 on $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ subsets. *A*, For thymocytes, seven gates were set on the basis of CD4 and CD8 expression, and the CCR9 profile of each subpopulation is depicted. Background staining was obtained by staining with control rabbit Ig. *B*, For lymph node cells, $CD4^+$ T cells and $CD8^+$ T cells were gated, and CCR9 expression was analyzed.

surface levels of CCR9 were down-regulated (Fig. 7*B*). Thus, although TCR cross-linking results in down-regulation of CCR9 surface expression, it augments chemotactic activity to CCL25.

Expression of CCR9 on γδ T lineage cells

Total populations of DN thymocytes exhibited a weak but significant chemoattractant activity to CCL25 (Ref. 10 and data not shown). DN thymocytes are heterogeneous and contain mature $\gamma\delta TCR^+$ cells in addition to immature $\alpha\beta$ lineage cells. To determine which subsets of DN thymocytes respond to CCL25, we performed chemotaxis assay on total DN thymocytes after depletion of $CD4^+$, $CD8^+$, $B220^+$, and $\alpha\beta TCR^+$ cells. Migrated cells were stained with anti-CD3 and anti-$\gamma\delta$TCR and examined by FACS (Fig. 8*B*). In the absence of chemokine, <1% of DN cells migrated in this assay (Fig. 8*A*). CCL25 induced the chemotaxis of 60% of $\gamma\delta TCR^+$ thymocytes but only 3% of $\gamma\delta TCR^-$ DN cells. $\gamma\delta TCR^+$ thymocytes were less responsive to CXCL12, as only 10% of $\gamma\delta TCR^+$ thymocytes migrated to this chemokine. Thus,

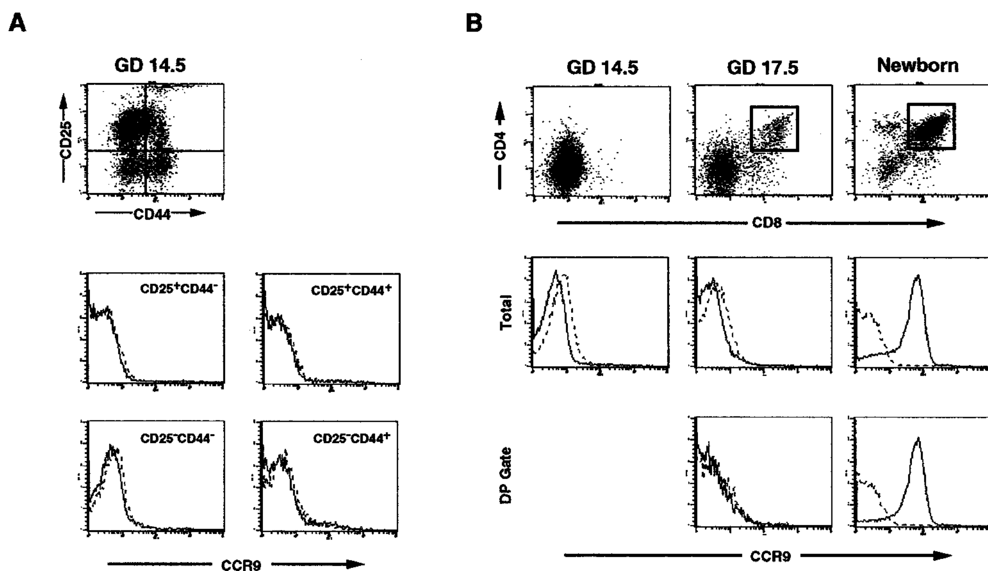

FIGURE 3. CCR9 expression on thymocytes from fetal and newborn mice. *A*, Thymocytes from gestation day 14.5 fetal mice were stained with FITC-labeled anti-CD44, PE-labeled anti-CD25, and biotinylated anti-CCR9 plus av-CyChrome. Gates were set on the basis of CD25 and CD44 expression, and the CCR9 profile of each subpopulation is depicted. *B*, Thymocytes from gestation day 14.5, gestation day 17.5, and newborn mice were stained with FITC-labeled anti-CD8, PE-labeled anti-CD4, and biotinylated anti-CCR9 plus av-CyChrome. For gestation day 17.5 and newborn mice, CCR9 expression on gated $CD4^+CD8^+$ cells was analyzed.

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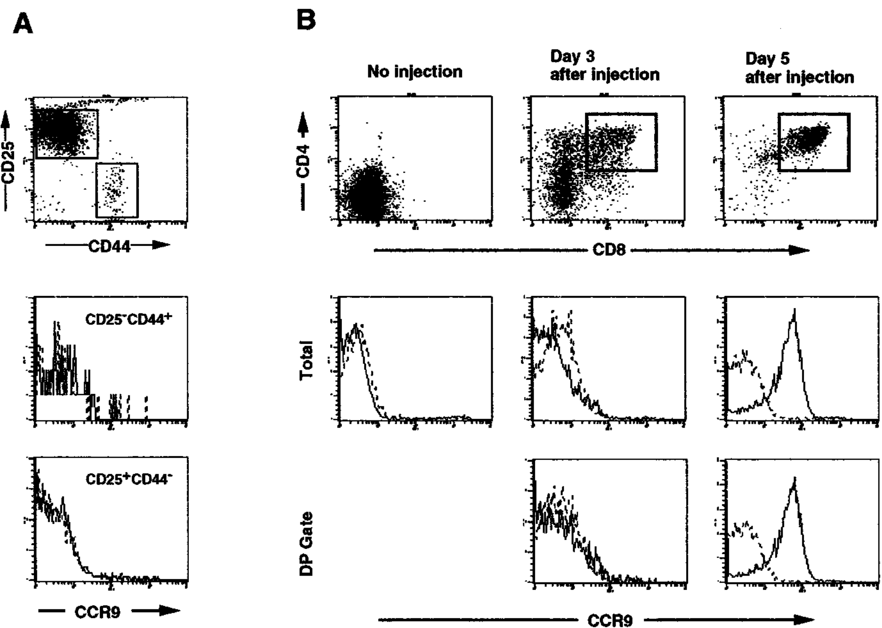


FIGURE 4. CCR9 expression on anti-CD3ε-stimulated and unstimulated Rag1-deficient thymocytes. *A*, Thymocytes from Rag1-deficient mice were stained with FITC-labeled anti-CD44, PE-labeled anti-CD25, and biotinylated anti-CCR9 plus av-Cy-Chrome. $CD25^-CD44^+$ and $CD25^+CD44^-$ gates were set, and CCR9 expression was analyzed on these subpopulations. *B*, Thymocytes were obtained from untreated or anti-CD3-injected Rag1-deficient mice 3 and 5 days after injection and were stained with FITC-labeled anti-CD8, PE-labeled anti-CD4, and biotinylated anti-CCR9 plus av-CyChrome. For staining of thymocytes from anti-CD3-injected Rag1-deficient mice, CCR9 expression on gated $CD4^+CD8^+$ cells was determined.

CCL25 appears to be an especially effective chemoattractant for $\gamma\delta TCR^+$ thymocytes.

We next examined CCR9 expression on γδ T cells from adult and fetal thymus. Approximately one-half of γδ T cells from adult thymus express CCR9 (Fig. 9*A*). CCR9 was also expressed on gestation day 17.5 $\gamma\delta TCR^+$ thymocytes, but not on gestation day 14.5 $\gamma\delta TCR^+$ thymocytes (Fig. 9*B*). Moreover, $V\gamma3^+$ thymocytes, which preferentially migrate to the skin, did not express CCR9, whereas most $V\gamma2^+$ thymocytes expressed CCR9 (Fig. 9*B*). Approximately 30–40% of lymph node and splenic γδ T cells expressed CCR9 (Fig. 9*A*). Most $CCR9^+\gamma\delta TCR^+$ lymph node T cells were $CD44^{low}$ and $CD45RB^{low}$ (Fig. 9*C*), suggesting that they were recent thymic emigrants (21, 22).

CCR9 expression on iIEL

As previously reported, both CCR9 and CCL25 mRNA are also expressed in the small intestine (Fig. 10*A*) (11–13). To determine the origin of CCR9 and CCL25 expression in the small intestine, we examined mRNA levels in B6 and $Rag1^{-/-}$ mice by Northern blotting (Fig. 10*A*). In $Rag1^{-/-}$ small intestines, which lack mature T and B lymphocytes, CCR9 expression was decreased, but

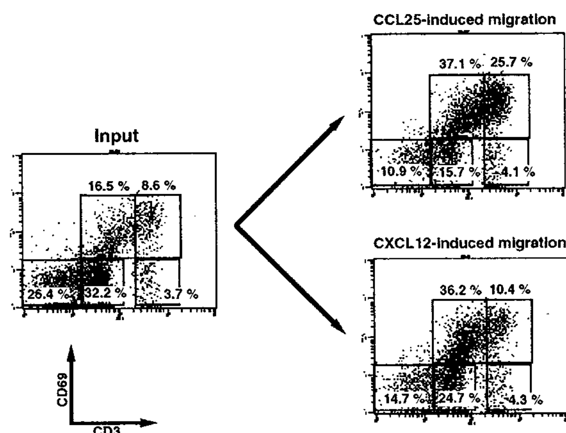
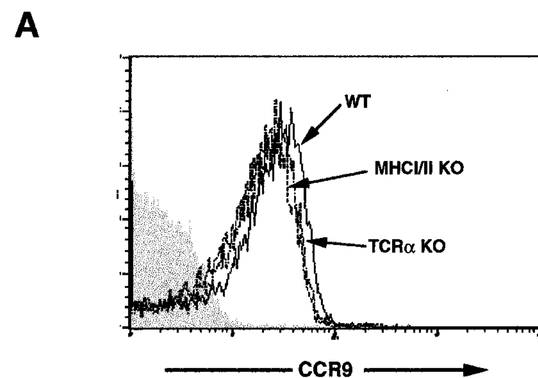


FIGURE 5. $CD3^{high}CD69^+$ thymocytes preferentially respond to CCL25. Chemotactic assay of thymocytes to CCL25 and CXCL12 was performed. Input and migrated cells were stained with anti-CD3 and anti-CD69 and analyzed by FACS. Input cell number was 1×10^6 per well. A total of $0.5 \pm 0.1 \times 10^4$ cells migrated in the absence of chemokine. A total of $24.3 \pm 4.1 \times 10^4$ cells migrated in response to CCL25. A total of $14.4 \pm 3 \times 10^4$ cells migrated in response to CXCL12.



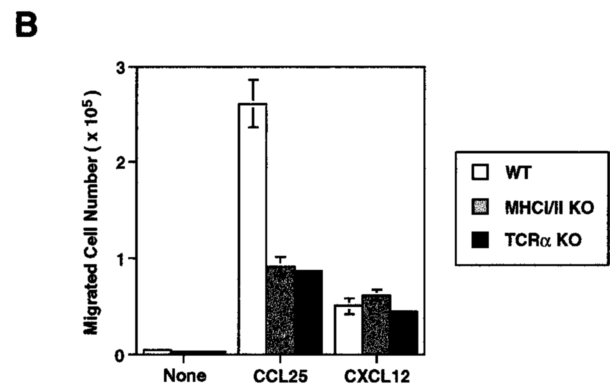


FIGURE 6. TCR signaling is not required for CCR9 expression but regulates CCL25 responsiveness. *A*, CCR9 expression on $CD4^+CD8^+$ thymocytes obtained from B6, MHC class I/II-deficient, or TCRα-deficient mice. *B*, Chemoattraction of thymocytes from B6, MHC class I/II-deficient, or TCRα-deficient mice to CCL25 or CXCL12. Each data point represents mean ± SD of duplicate cultures. The data shown are representative of three independent experiments.

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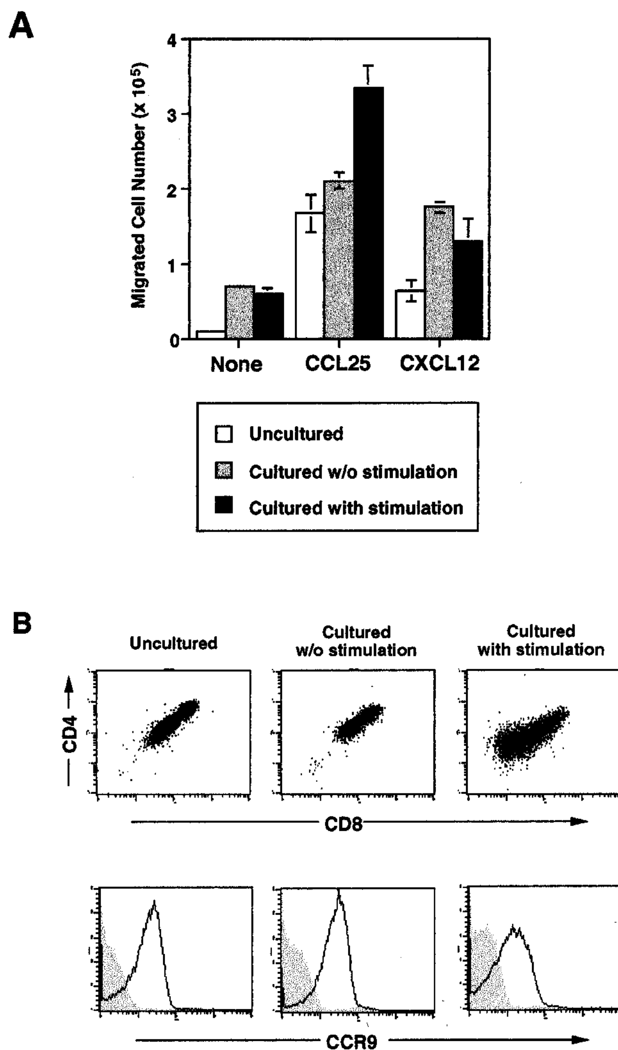

FIGURE 7. TCR signaling enhances CCL25 responsiveness. Thymocytes from MHC class I/II-deficient mice were cultured with or without plate-bound anti-CD3 (10 μg/ml) in serum-free medium for 24 h. Chemotaxis assays were performed on freshly prepared and cultured thymocytes. *A*, Enumeration of cells migrating to CCL25 or CXCL12. Each data point represents mean ± SD of duplicate cultures. The data shown are representative of three independent experiments. *B*, Analysis of CCR9 surface expression on unstimulated or anti-CD3-stimulated thymocytes.

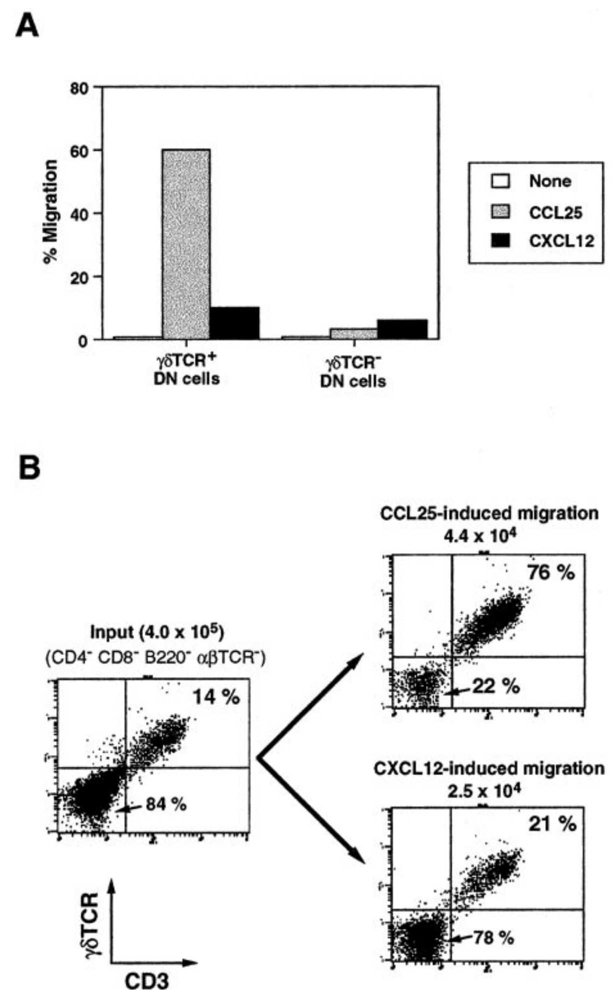

FIGURE 8. γδTCR$^+$ thymocytes respond to CCL25. *A*, CD4$^-$CD8$^-$B220$^-$αβTCR$^-$ thymocytes were enriched by magnetic separation, and chemotaxis assay was performed. *B*, γδTCR and CD3 expression were examined before and after chemotaxis. The data shown are from one of two experiments that gave similar results.

CCL25 expression was constant as compared with B6 mice (Fig. 10*A*). These data suggest that CCR9 is mainly expressed by mature lymphocytes, whereas CCL25 is produced by nonlymphoid cells. Interestingly, we could not detect CCR9 surface expression on either αβTCR$^+$ or γδTCR$^+$ iIEL (Fig. 10*B*), or on lamina propria lymphocytes (data not shown). We next purified αβTCR$^+$ and γδTCR$^+$ iIEL and examined CCR9 expression by semiquantitative RT-PCR analysis. CCR9 mRNA expression was observed in both αβTCR$^+$ and γδTCR$^+$ iIEL subsets, although γδTCR$^+$ iIEL expressed less CCR9 mRNA as compared with αβTCR$^+$ iIEL (Fig. 10*C*).

Discussion

In this study, we have characterized CCR9 surface expression on immature and mature T lymphocytes and correlated these findings with the ability of cells to migrate to CCL25, the only known ligand for CCR9. In αβ lineage T cells, CCR9 surface expression is first observed on DP thymocytes and is down-regulated during the transition of DP thymocytes to the mature CD4$^+$ or CD8$^+$ SP stage (Fig. 2). Interestingly, although thymocytes from gestation day 17.5 and Rag1$^{-/-}$ thymi 3 days after anti-CD3 stimulation contain DP cells, these cells did not express CCR9 on their surface (Figs. 3*B* and 4*B*). We observed that CCR9 mRNA is expressed in gestation day 14.5 thymocytes, and that thymocytes from gestation day 17.5 and adult mice express CCR9 mRNA at equivalent levels by Northern blot analysis (data not shown). These results indicate that CCR9 gene expression is induced at an earlier stage than we are able to detect using our Ab. One possible explanation for this discrepancy is that surface expression of CCR9 is regulated post-transcriptionally and does not correlate with mRNA levels.

Our results also indicate that the response of CCR9$^+$ cells to CCL25 is not simply dictated by the level of CCR9 surface expression. CD3^{high}CD69$^+$ thymocytes demonstrated enhanced CCL25-induced migration as compared with CD3^{low}CD69$^+$ thymocytes, even though they express lower levels of CCR9 (Fig. 5). In contrast, thymocytes from MHC class I/II$^{-/-}$ and TCR-α$^{-/-}$ mice, which fail to undergo positive selection and lack CD69$^+$ cells, showed reduced CCL25-induced migration, although they

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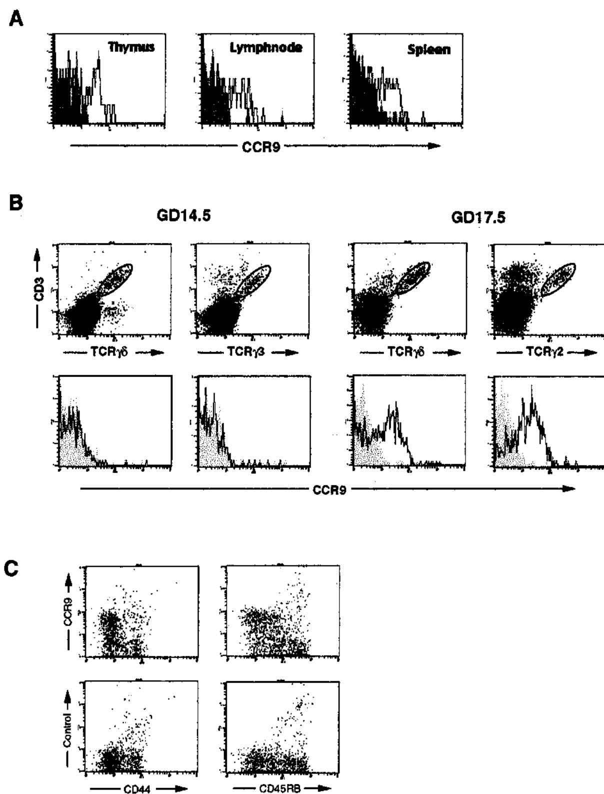

FIGURE 9. CCR9 expression on γδ T lymphocytes. *A*, Thymocytes, spleen cells, and lymph node cells were stained with FITC-labeled anti-TCRγδ, PE-labeled anti-CD3, and biotinylated anti-CCR9 plus av-CyChrome. $CD3^+TCR\gamma\delta^+$ populations were gated, and CCR9 expression was analyzed. *B*, Vγ2-TCR^+ fetal thymocytes express CCR9, but Vγ3-TCR^+ fetal thymocytes do not. Thymocytes from gestation day 14.5 and day 17.5 were stained with FITC-labeled anti-CD3, PE-labeled anti-TCRγδ, anti-Vγ2TCR, anti-Vγ3TCR, and biotinylated anti-CCR9 plus av-CyChrome. $CD3^+TCR\gamma\delta^+$ and $CD3^+V\gamma3TCR^+$ gestation day 14.5 thymocytes and $CD3^+TCR\gamma\delta^+$ and $CD3^+V\gamma2TCR^+$ gestation day 17.5 thymocytes were analyzed for CCR9 expression. *C*, $CD44^{low}$ and $CD45RB^{low}$ γδ T lymphocytes in lymph node preferentially express CCR9. Lymph node cells were stained with FITC-labeled anti-TCRγδ, PE-labeled anti-CD44 or anti-CD45RB, and biotinylated anti-CCR9 or control rabbit Ig plus av-CyChrome. $TCR\gamma\delta^+$ populations were gated, and CCR9 expression was analyzed in combination with CD44 or CD45RB expression.

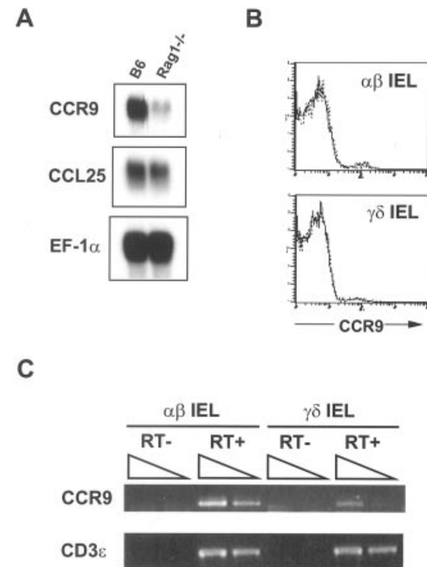

FIGURE 10. CCR9 expression on iIEL. *A*, CCR9 and CCL25 mRNA expression in small intestines from B6 and $Rag1^{-/-}$ mice. $Poly(A)^+$ RNA was analyzed by Northern blotting using mouse CCR9 cDNA as a probe. The same filter was stripped and rehybridized with mouse CCL25 probe or elongation factor-1α probe to assess loading. *B*, FACS analysis of CCR9 expression on $\alpha\beta TCR^+$ iIEL and $\gamma\delta TCR^+$ iIEL from small intestine. Isolated IEL were stained with FITC-labeled anti-TCRγδ, PE-labeled anti-TCRαβ, and biotinylated anti-CCR9 plus av-CyChrome. $\alpha\beta TCR^+$ or $\gamma\delta TCR^+$ populations were gated and analyzed for CCR9 expression. *C*, Semiquantitative RT-PCR analysis of CCR9 expression in $\alpha\beta TCR^+$ iIEL and $\gamma\delta TCR^+$ iIEL. cDNAs were prepared from purified cells, and serial dilutions (4-fold) of the cDNA template were subjected to PCR analysis using sets of primers to amplify CCR9 gene. CD3ϵ cDNA levels were analyzed for comparison of cDNA quantities in each sample. Purities of separated iIEL subsets were >97%.

expressed normal levels of CCR9 (Fig. 6). In vitro TCR stimulation of MHC class $I/II^{-/-}$ thymocytes significantly enhanced migration in response to CCL25, even though CCR9 surface expression was down-regulated (Fig. 7). Down-regulation of CCR9 expression was also observed by Zabel et al. (11) after activation of human PBLs by anti-CD3 stimulation. Collectively, these data indicate that the response of $CCR9^+$ thymocytes to CCL25 is enhanced by TCR signals during positive selection. Thus, CCL25 responsiveness is regulated by the expression of its receptor, CCR9, and by TCR signaling. These findings are consistent with previous results demonstrating that the activation state of T cells can influence their response to chemokines (23).

Initial studies showed that CCL25 is produced by thymic dendritic cells located at the corticomedullary junction (5). Subsequent in situ hybridization studies demonstrated that CCL25 mRNA is expressed by thymic epithelial cells in both the cortex and the medulla (10). In contrast, CXCL12 mRNA is expressed in the cortex, especially in the subcapsular region (24). During positive selection, DP thymocyte development proceeds from the $CD69^+CD3^{low}$ to the $CD69^+CD3^{high}$ and then the $CD69^-CD3^{high}$ stage. The response of thymocytes to CCL25 is enhanced by TCR stimulation, is maximal at the $CD69^+CD3^{high}$ stage, and is down-regulated at the $CD69^-CD3^{high}$ stage, whereas the response to CXCL12 is down-regulated at $CD69^+CD3^{high}$ SP stage (Fig. 5). Based on these data, we speculate that CCL25 may regulate the intrathymic trafficking of thymocytes in collaboration with other thymus-expressed chemokines including CXCL12.

CCL25 is also expressed by fetal thymic MHC class II^+ epithelial cells (25), and in situ hybridization studies indicate that CCL25 is expressed in the thymic anlage in day 12.5 embryos (26). A bone marrow cell subset of pre/pro-B cell phenotype ($B220^{low}CD24^-AA4.1^+NK1.1^-$), which may contain thymocyte precursors, migrates to CCL25 (27). CCL25 attracts fetal blood prothymocytes and $CD44^+CD25^-$ DN newborn thymocytes (26). Based on these observations, it has been suggested that CCL25/CCR9 interaction could play a role in the recruitment of T progenitors to thymus. However, we were unable to detect CCR9 surface expression on $CD44^+CD25^-$ DN thymocytes from fetal and adult mice (Figs. 3*A* and 4*A*), and $CD44^+CD25^-$ DN thymocytes from $Rag1^{-/-}$ mice did not respond to CCL25 (data not shown). It is possible that $CD44^+CD25^-$ DN cells from newborn mice express CCR9 at levels that are not detectable by our Ab. Alternatively, CCR9 expression on prothymocytes may be down-regulated rapidly after these cells enter the thymus.

Campbell et al. (28) proposed that CCL25 might function to retain cells in the thymus until they have fully matured because $CD4^+$ SP $CD69^-L\text{-selectin}^{high}$ thymocytes lose responsiveness to

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CCL25. However, we observed that a subset of peripheral CD8$^+$ T cells and CD44^{low}CD45RB^{low} $\gamma\delta$ T cells expresses CCR9 (Figs. 2*B* and 9*C*). Furthermore, a subset of naive (CD44^{low}CD62L^{high}) CD8$^+$ T cells preferentially responds to CCL25 (data not shown). These results indicate that CCR9 expression and CCL25 responsiveness are not sufficient to inhibit CD8$^+$ SP and $\gamma\delta$TCR$^+$ thymocyte emigration. Moreover, SP thymocytes accumulate in the thymus of pertussis toxin transgenic mice, suggesting that pertussis toxin-sensitive signaling responses are not essential for retention of thymocytes but instead may be required for thymocyte emigration (29).

Recent data suggest that chemokines and their receptors may play a role in the development and trafficking of $\gamma\delta$ lineage as well as $\alpha\beta$ lineage T cells (30). We found that a high percentage of $\gamma\delta$TCR$^+$ thymocytes and peripheral $\gamma\delta$ T cells expresses CCR9 and migrates in response to CCL25 (Figs. 8*A* and 9*A*). These data suggest that chemokines may regulate the development and function of $\gamma\delta$ lineage T cells as well as $\alpha\beta$ lineage T cells. CCR9 was expressed on ~80% of thymocytes bearing the Vγ2-TCR, which is the major population in adult thymus and peripheral lymphoid tissues (Fig. 9*B*). Notably, Vγ3-bearing fetal thymocytes, which are known to be the precursors of dendritic epidermal T cells, do not express CCR9 (Fig. 9*B*). In humans, CCR9 is not expressed on cutaneous lymphocyte Ag-positive memory CD4$^+$ and CD8$^+$ lymphocytes, which traffic to skin (11). The restricted expression pattern of CCR9 within different $\gamma\delta$ T lineage subsets suggests that CCR9 may regulate the migration of selected $\gamma\delta$ T lineage populations to specific sites such as the small intestine, in which CCL25 is known to be highly expressed.

In both mice and humans, CCL25 is highly expressed in the epithelial cells lining the small intestine, and, in humans, CCR9 is selectively expressed on the surface of intestinal homing T lymphocytes and mucosal lymphocytes in the small intestine (6, 11–13). We also observed that CCR9 is expressed in murine small intestine, as assessed by Northern blot analysis, and in both $\alpha\beta$TCR$^+$ and $\gamma\delta$TCR$^+$ iIEL subsets by RT-PCR (Fig. 10, *A* and *C*). However, we did not detect CCR9 surface expression with our Ab on murine iIEL and lamina propria lymphocytes from the small intestine (Fig. 10*B* and data not shown). Furthermore, in our experiments, iIEL did not migrate in response to 200 nM CCL25, a concentration that induces chemotaxis of thymocytes and peripheral CD8$^+$ T cells (data not shown). Based on these observations, we speculate that surface expression of CCR9 on iIEL may be down-regulated by CCL25 binding or cell activation, and/or the level of CCR9 expression may be too low to detect by our Ab. Rag1$^{-/-}$ small intestines lack mature lymphocytes but contain CD3$^-$CD8$\alpha\alpha^+$ iIEL (31) and cryptopatches (32). Fig. 10*A* shows that small amounts of CCR9 mRNA are expressed in Rag1$^{-/-}$ small intestine. These findings indicate that CCR9 may be expressed in the progenitor cells for mucosal lymphocytes and could potentially play a role in early mucosal T cell development and/or recruitment of precursor T cells to the intestine.

In conclusion, we have shown that during thymocyte development, CCR9 surface expression starts and is maximal at the DP stage and is down-regulated on mature SP thymocytes. DP thymocytes that have received activating signals through their TCRs exhibit enhanced CCL25-induced migration, suggesting that CCR9 may function in the process of intrathymic trafficking during positive selection. Among $\gamma\delta$ T cells, CCR9 is expressed on selective subsets, and about one-half of $\gamma\delta$TCR$^+$ thymocytes express CCR9 and migrate in response to CCL25. Thus, CCR9/CCL25 may be important for regulating the migration of specific subsets of $\gamma\delta$ T cells to particular sites. Collectively, these data suggest that CCR9 may play an important role in the development and trafficking of both $\alpha\beta$ and $\gamma\delta$ T cells.

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