Characterization of CCR9 Expression and CCL25/Thymus-Expressed Chemokine Responsiveness During T Cell Development: CD3<sup>hi</sup>CD69<sup>+</sup> Thymocytes and γ<sub>δ</sub>TCR<sup>+</sup> Thymocytes Preferentially Respond to CCL25

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Characterization of CCR9 Expression and CCL25/Thymus-Expressed Chemokine Responsiveness During T Cell Development: CD^3^high^CD69^+^ Thymocytes and γδTCR^+^ Thymocytes Preferentially Respond to CCL25

Shoji Uehara,* Kaimei Song,† Joshua M. Farber,† and Paul E. Love2*

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 CD^4^-CD^8^- (double-negative) thymocytes, but not on immature CD^4^+CD^8^- (double-negative) thymocytes. CCR9 was down-regulated during the transition of double-negative thymocytes to the CD^4^+ or CD^8^+ (single-positive) stage, and only a minor subset of CD^8^+ lymph node T cells expressed CCR9. All CCR9^+ thymocyte subsets migrated in response to CCL25; however, CD^69^+ thymocytes demonstrated enhanced CCL25-induced migration compared with CD^69^- 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 γδTCR^+ thymocytes and peripheral γδTCR^+ 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 αβTCR^+ and γδTCR^+ 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, 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 αβ and γδTCR^+ thymocytes and that these cells are specifically responsive to CCL25. Moreover, in αβ 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 αβ and γδ 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 X II^-/- (β^-microglobulin^-/- X A^-/-) (16).

Generation of anti-murine CCR9 Ab
Rabbit polyclonal Ab against murine CCR9 was prepared according to standard methods. A 16-aa NH2-terminal peptide of murine CCR9 (CM FDDFSYDSTASTD) 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).

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Validation of anti-murine CCR9 Ab

Mouse CCR9 cDNA was amplified from mouse thymocyte cDNA by PCR using the following EcoRI site containing primers: forward primer, 5′-CCCGGATTCATGCCCCTCGAGGCTGATTGGCC-3′; reverse primer, 5′-CCGGAGCTCCAAAAAAGGACCATTGCCCCT-3′. The PCR fragment was inserted into the EcoRI site of the retroviral vector pCDR4 (R), which was kindly provided by T. Murphy, Washington University School of Medicine (St. Louis, MO). pCDR4 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-αβTCR, anti-γ-δTCR, anti-Vγ2TCR, and anti-Vγ3TCR mAbs. Unconjugated anti-FcyRII (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 CCXCL12 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 × 10^6 cells/ml in RPMI 1640 plus 0.5% BSA, and chemotaxis assays were performed 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 hydridoma serum-free medium (Life Technologies, Rockville, MD). A total of 4 × 10^5 thymocytes was resuspended in 2 ml of hydridoma serum-free medium and plated in uncoated or anti-CD3-coated wells for 20 h at 37°C, 5% CO2. After incubation, thymocytes were pelleted, washed, and resuspended at 1 × 10^6 cells/ml in RPMI 1640 with 0.5% BSA, and chemotaxis assays were performed as described above.

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 NH2-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. 1A), or with vector containing CCR9 sequences in the antisense orientation (data not shown). Similar results were obtained with NIH3T3 cells transfected using murine CCR9-encoding retrovirus (data not shown). In addition, the anti-CCR9 Ab did not react with mouse CCR6-, CCR8-, or CCR3-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. 1B). Incubation with CCXCL12 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.
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. 3B). Interestingly, although thymocytes from gestation day 17.5 contain DP cells, these cells did not express CCR9 (Fig. 3B). 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 \(\mu\)g 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. 4B). Detectable CCR9 surface expression was only observed at day 5 when >90% of thymocytes were DP (Fig. 4B). 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.

CCL25 at 4°C had no effect on CCR9 staining (Fig. 1B). 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\(^{low}\)CD8\(^+\)) thymocytes (Fig. 2A). Most DN cells did not express CCR9 (Fig. 2A), and analysis of immature DN thymocyte subsets (distinguished on the basis of CD25 and CD44 expression) failed to reveal detectable CCR9 surface expression (Fig. 3A). 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. 2B). 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

**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 \(\times\) 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.

**FIGURE 2.** CCL25 at 4°C had no effect on CCR9 staining (Fig. 1B). These results suggest that CCL25 may induce down-modulation of CCR9 on thymocytes.

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-\(\alpha\beta\)-mice. MHC class I/II\(^{-/-}\) and TCR-\(\alpha\beta\)-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-\(\alpha\beta\)-mice, thymocytes from MHC class I/II\(^{-/-}\) and TCR-\(\alpha\beta\)-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. 7A). Migration to CXCL12 was unchanged by TCR stimulation. Although migration to CCL25 was enhanced in cells stimulated by TCR cross-linking,
surface levels of CCR9 were down-regulated (Fig. 7B). 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 γδTCR⁺ cells in addition to immature αβ 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 αβTCR⁺ cells. Migrated cells were stained with anti-CD3 and anti-γδTCR and examined by FACS (Fig. 8B). In the absence of chemokine, <1% of DN cells migrated in this assay (Fig. 8A). CCL25 induced the chemotaxis of 60% of γδTCR⁺ thymocytes but only 3% of γδTCR⁻ DN cells. γδTCR⁺ thymocytes were less responsive to CXCL12, as only 10% of γδTCR⁺ thymocytes migrated to this chemokine. Thus,
CCL25 appears to be an especially effective chemoattractant for γδ TCR thymocytes. We next examined CCR9 expression on γδ TCR thymocytes from adult and fetal thymus. Approximately one-half of γδ TCR thymocytes from adult thymus express CCR9 (Fig. 9A). CCR9 was also expressed on gestation day 17.5 γδ TCR thymocytes, but not on gestation day 14.5 γδ TCR thymocytes (Fig. 9B). Moreover, Vγ3 thymocytes, which preferentially migrate to the skin, did not express CCR9, whereas most Vγ2 thymocytes expressed CCR9 (Fig. 9B). Approximately 30–40% of lymph node and splenic γδ T cells expressed CCR9 (Fig. 9A). Most CCR9+ γδ TCR+ lymph node T cells were CD44low and CD45RBlow (Fig. 9C), 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. 10A) (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. 10A). In Rag1−/− small intestines, which lack mature T and B lymphocytes, CCR9 expression was decreased, but...
CCL25 expression was constant as compared with B6 mice (Fig. 10A). 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 /H9251/H9252 TCR or /H9253/H9254 TCR iIEL (Fig. 10B), or on lamina propria lymphocytes (data not shown). We next purified /H9251/H9252 TCR and /H9253/H9254 TCR iIEL and examined CCR9 expression by semiquantitative RT-PCR analysis. CCR9 mRNA expression was observed in both /H9251/H9252 TCR and /H9253/H9254 TCR iIEL subsets, although /H9253/H9254 TCR iIEL expressed less CCR9 mRNA as compared with /H9251/H9252 TCR iIEL (Fig. 10C).

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 /H9251/H9252 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. 3B and 4B). 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. CD3highCD69+ thymocytes demonstrated enhanced CCL25-induced migration as compared with CD3lowCD69+ thymocytes, even though they express lower levels of CCR9 (Fig. 5). In contrast, thymocytes from MHC class I/II−/− and TCR−/− mice, which fail to undergo positive selection and lack CD69+ cells, showed reduced CCL25-induced migration, although they
expressed normal levels of CCR9 (Fig. 6). In vitro TCR stimulation of MHC class 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 thymocytes to CCL25 is enhanced by TCR signals during positive selection. Thus, CCL25 retention in the thymus until they have fully matured because CD44+/−/− thymocytes did not. Thymocytes from gestation day 14.5 and day 17.5 were stained with FITC-labeled anti-CD3, PE-labeled anti-CD44 or anti-CD45RB, and biotinylated anti-CCR9 plus av-CyChrome. CD3− TCRγδ+ and CD3− TCRγδ− gestation day 14.5 thymocytes and CD3− TCRγδ+ and CD3− TCRγδ− gestation day 17.5 thymocytes were analyzed for CCR9 expression. CD44− and CD45RB− y6 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. TCRγδ+ populations were gated, and CCR9 expression was analyzed in combination with CD44 or CD45RB expression.

CD69+ CD3low to the CD69+ CD3high and then the CD69− CD3high stage. The response of thymocytes to CCL25 is enhanced by TCR stimulation, is maximal at the CD69+ CD3high stage, and is down-regulated at the CD69− CD3high stage, whereas the response to CXCL12 is down-regulated at CD69− CD3high 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−/−/−CD4−/−/−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/CCL9 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. 3A and 4A), and CD44+ CD25− DN thymocytes from Rag1−/− mice did not respond to CCL25 (data not shown). It is possible that CD44+ CD25− DN thymocytes 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-selectinhigh thymocytes lose responsiveness to...
CCR9. However, we observed that a subset of peripheral CD8⁺ T cells and CD44lowCD45RBlow γδ T cells expresses CCR9 (Figs. 2B and 9C). Furthermore, a subset of naive (CD44lowCD62Lhigh) 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 γδ TCR⁺ thymocyte emigration. Moreover, SP thymocytes accumulate in the thymus of pertussis toxin transgenic mice, suggesting that pertussis toxin-sensitizing 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 γδ lineage as well as αβ lineage T cells (30). We found that a high percentage of γδTCR⁺ thymocytes and peripheral γδ T cells expresses CCR9 and migrates in response to CCL25 (Figs. 8A and 9A). These data suggest that chemokines may regulate the development and function of γδ lineage T cells as well as αβ 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. 9B). Notably, Vγ3-bearing fetal thymocytes, which are known to be the precursors of dendritic epidermal T cells, do not express CCR9 (Fig. 9B). 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 γδ T lineage subsets suggests that CCR9 may regulate the migration of selected γδ 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 αβTCR⁺ and γδTCR⁺ 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. 10B 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 peripher al 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⁻CD8α⁻γδ iIEL (31) and cryptopatches (32). Fig. 10A 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 γδ T cells, CCR9 is expressed on selective subsets, and about one-half of γδTCR⁺ thymocytes express CCR9 and migrate in response to CCL25. Thus, CCR9/CCL25 may be important for regulating the migration of specific subsets of γδ T cells to particular sites. Collectively, these data suggest that CCR9 may play an important role in the development and trafficking of both αβ and γδ T cells.

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