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Murine CCR9, a Chemokine Receptor for Thymus-Expressed Chemokine That Is Up-Regulated Following Pre-TCR Signaling

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Chemokines are likely to play an important role in regulating the trafficking of developing T cells within the thymus. By using anti-CD3ε treatment of recombinase-activating gene 2 (Rag2<sup>−/−</sup>) mice to mimic pre-TCR signaling and drive thymocyte development to the double positive stage, we have identified murine GPR-9-6 as a chemokine receptor whose expression is strongly induced following pre-TCR signaling. GPR-9-6 mRNA is present at high levels in the thymus, and by RT-PCR analysis its expression is induced as normal thymocytes undergo the double negative to double positive transition. Furthermore we show that TECK (thymus-expressed chemokine), a chemokine produced by thymic medullary dendritic cells, is a functional ligand for GPR-9-6. TECK specifically induces a calcium flux and chemokinesis of GPR-9-6-transfected cells. In addition, TECK stimulates the migration of normal double positive thymocytes, as well as Rag2<sup>−/−</sup> thymocytes following anti-CD3ε treatment. Hence, GPR-9-6 has been designated as CC chemokine receptor 9 (CCR9). Our results suggest that TECK delivers signals through CCR9 important for the navigation of developing thymocytes.

exception, all chemokines contain four cysteine residues linked by intramolecular disulfide bonds. Four chemokine subfamilies have been defined based on the spacing of the two NH₂-terminal cysteines with the majority falling into the CXC (α) or CC (β) family. Although there is some redundancy in the recognition of chemokines by multiple receptors, distinct CXC and CC chemokine receptor subfamilies (CXCRR and CCR) have also been defined based on structural homology and specific binding to the corresponding chemokine class.

Developing thymocytes express multiple chemokine receptors and have been shown to respond to chemokines that are produced within the thymus. For example, thymocytes express CXCR4 and migrate in response to its ligand SDF-1 (stromal cell-derived factor-1), which is ubiquitously expressed but present at high levels in the thymus (17–20). Additional chemokine receptor-ligand pairs present in the thymus include: CCR4, which binds TARC (thymus and activation-regulated chemokine) and MDC (macrophage derived chemokine) (21–23); CCR5 and MIP-1β (macrophage inflammatory protein) (24); CCR7 and ELC (EBI-ligand chemokine) (25, 26); and CCR8 and TARC (22, 27, 28). In some cases, these chemokine receptors have been shown to be differentially expressed at specific stages of thymocyte development. For example, DN and DP thymocytes are reported to express higher levels of CXCR4 (relative to SP cells) and show greater responsiveness to SDF-1 (17), whereas DP and SP thymocytes express greater levels of CCR5 and are responsive to MIP-1β (24). Finally, CCR7 expression increases and chemotaxis to ELC is acquired as thymocytes mature to SP cells (17, 26).

One CC chemokine hypothesized to play a role in the trafficking of developing thymocytes is TECK (thymus-expressed chemokine). Originally identified by random sequencing of a recombinase-activating gene 1 (Rag1−/−) thymus cDNA library, TECK is specifically expressed in the thymus and small intestine, both sites of T cell development (29). TECK efficiently stimulates the chemotaxis of thymocytes and thymic DC (29, 30). Furthermore, in situ hybridization studies for TECK mRNA or protein indicate that this chemokine is specifically produced by medullary DC of the adult thymus (29). In addition, recent RT-PCR analysis indicates that TECK is expressed by fetal thymic epithelial cells, suggesting that it may also function to direct the migration of early T cell progenitors to the thymic rudiment (30). Thus it has been proposed that TECK is likely to regulate the migration of developing thymocytes (29).

To identify regulatory molecules important for thymocyte development, we have performed a genetic screen to identify transcripts induced by pre-TCR stimulation. Rag2−/− mice (which lack recombination activating genes required for TCR locus rearrangement) do not express the pre-TCR and therefore contain only immature DN thymocytes (31). However, development of DN thymocytes to the DP stage can be experimentally induced in a highly synchronous manner by treatment with anti-CD3ε mAb in vivo (31–33). cDNA from stimulated and unstimulated Rag2−/− thymocytes was isolated and applied to a PCR-based subtractive hybridization. From this screen we isolated a cDNA clone corresponding to the murine homologue of an orphan chemokine receptor (GPR-9-6). Interestingly, GPR-9-6 is most homologous to CCR7, known to be important for the trafficking of peripheral T cells and DC in the lymph node and spleen (34). Here, we report that murine GPR-9-6 mRNA is strongly induced (>10-fold) following anti-CD3 treatment of Rag2−/− thymocytes and is expressed at high levels in normal murine thymocytes. Analysis of sorted thymocyte subsets has confirmed that GPR-9-6 expression is up-regulated at the DN to DP transition. Furthermore, we have determined that the chemokine TECK is a functional ligand for GPR-9-6, and that DP and SP thymocytes and anti-CD3-stimulated Rag2−/− thymocytes migrate in response to TECK. Another group also recently reported the cloning of murine and human GPR-9-6 cDNAs and the identification of TECK as a specific ligand (35). Our results are consistent with theirs, but include a detailed analysis of the expression pattern of GPR-9-6 in murine thymocytes, as well as the migratory response of specific thymocyte subpopulations to TECK. As TECK is produced by thymic DC and fetal thymic epithelial cells, the results reported below suggest that CCR9 (GPR-9-6) may transmit signals to T cell progenitors that are important for their trafficking during development in the thymus. Thus, our analysis supports the emerging view (36) that the controlled expression of different chemokine receptors at specific stages of thymocyte differentiation serves to regulate the trafficking of developing T cells.

Materials and Methods

Isolation of murine GPR-9-6 cDNA fragment (CCR9) by PCR-based subtractive hybridization

Rag2−/− mice were housed in a specific pathogen free (SPF) facility. Four-week-old Rag2−/− mice were injected i.p. with 150 μg of anti-CD3ε mAb (145-2C11) diluted in 300 μL PBS or PBS alone. 145-2C11 was affinity purified on protein G sepharose (Pharmacia Biotech, Piscataway, NJ). Sixteen hours after mAb treatment, pooled thymocytes from each group were collected by disaggregation through a wire mesh. Total RNA was isolated by the CsCl gradient centrifugation, and poly(A)+ RNA prepared using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA). cDNA was synthesized using an oligo(dT)-based primer and preamplified for 19 cycles using the SMART kit (Clontech, Palo Alto, CA) under conditions designed to give full-length cDNA products and to minimize distortion of the relative abundance of individual clones. The preamplified cDNA was used for suppression subtractive hybridization with the Clontech PCR Select kit according to the manufacturer’s instructions and screened essentially as described (37), except that the resulting difference products were subcloned into pBluescript (Stratagene, La Jolla, CA). Sequencing reactions were performed with primers specific to the T3 and T7 promoters, using the dye Rhodamine PCR mix and analyzed on an ABI model 377 (Applied Biosystems, Foster City, CA) automated sequencer. Following homology searches against the National Center for Biotechnology Information (NCBI) Nonredundant and EST databases using the BLAST 2.0 algorithm, one clone (2B7) matched two nonoverlapping murine thymus-derived ESTs (accession nos. AA110666 and AA863796) and was chosen for further study. As the assembled contig (Sequencer 3.0 software, Gene Codes, Ann Arbor, MI) lacked an open reading frame, rapid amplification of cDNA ends (RACE) PCR was performed using the Marathon cDNA amplification kit (Clontech) according to the manufacturer’s instructions. 5’-RACE using the primer 5’-GTAGCAGGGAGCCTCAGAAGGAAAGGCC-3’ produced an ~2.5-kb fragment; sequencing revealed it to be the murine homologue of the orphan human chemokine receptor GPR-9-6 (accession no. U45982). A cDNA fragment containing the entire open reading frame of murine GPR-9-6 was then generated using KlenTaq polymerase (Clontech) to amplify normal thymus cDNA with the following forward and reverse primers: 5’-CAGCCGACTGCAAGTTCTCCATCC-3’ and 5’-TGTGCAAGGCTGGCCTGCTTTTGC-3’. Six independent clones were subcloned into pBluescript using internal PsI sites and sequenced completely to obtain a consensus sequence.

Northern blot analysis

Total RNA was isolated from thymi of 4-wk-old PBS or anti-CD3ε treated Rag2−/− mice, or tissues of 6-wk-old C57BL/6 mice using RNA STAT-60 (Tel-Test, Friendswood, TX). RNA (8 μg) from Rag2−/− thymi, Rag2−/− TAP−/− OT-1 transgenic (nonselecting thymi; see Ref. 37), or Rag2−/− OT-1 transgenic (selecting) or were separated on a 1% agarose formaldehyde gel, transferred to a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) and analyzed according to the manufacturer’s instructions. For multi-tissue Northern blots, 10 μg RNA from the indicated tissues were loaded per lane. To analyze mCCR9 (murine CCR9) expression in activated splenocytes, C57BL/6 splenocytes were cultured at 2 × 10⁶ cells/ml in RPMI 1640 10% FCS (Gemini-BioProducts, Calabasas, CA) alone or in the presence of 2.5 μg/ml Con A (Calbiochem, La Jolla, CA), or 5 μg/ml LPS (Dîfo, Detroit, MI), and analyzed at 0, 24, and 48 h. Murine cDNA probes included the 1.4-kb PsI fragment of mCCR9, an ~450-bp fragment
from the 5′ coding region of mCCR7, CD69, and EFlα as a loading control.

These were radio labeled by random priming (Life Technologies, Gaithersburg, MD) using [α-32P]dATP (DuPont, Boston, MA).

**RT-PCR analysis of sorted thymocyte subsets and fetal thymus**

Thymocytes from 6 to 8-week-old C57BL/6 mice were sorted using a flow cytometer into DN, DP, CD4 SP, and CD8 SP fractions. RNA was prepared from sorted cells and used to synthesize cDNA as previously described (38). Three-fold serial dilutions of cDNA from each population were normalized according to the hypoxanthine phosphoribosyltransferase (HPRT) signal. Amplification of HPRT was performed using similar conditions with primers specific to exons 7/8 and 9 (38), except that an annealing temperature of 55°C was used. PCR of CCR9 was performed for 30 cycles (94°C for 1 min, 68°C for 45 s, and 72°C for 1 min) using the forward primer 5′-CCCCAGTTCTGTTACTGAGTCTG-3′, and the reverse primer being the same reverse primer listed above for RACE PCR of mCCR9. PCR products were separated on a 1% agarose gel, transferred to a nylon membrane, and visualized by Southern blotting. For mCCR9, the 270-bp 2B7 probe was used; for HPRT a probe was generated with the resulting constructs verified by DNA sequencing. An HA marker (hCD2). Generation of retrovirus, infection of cells, and enrichment for infected cells by panning for hCD2-positive cells was performed as for sorted thymocyte subsets, except that 28 cycles were used.

**Generation of mCCR9 and mCCR7 expression constructs**

PCR amplification was performed using Pfu Turbo polymerase (Stratagene), with the resulting constructs verified by DNA sequencing. An HA (hemagglutinin) epitope tag was added to the NH2 terminus of mCCR9 at two different sites: the initiator methionine present in our mCCR9 cDNA clone, and the methionine at amino acid 13, predicted to be the start site in the reported human genomic GPR-9-6 (hCCR9) clone. These constructs were generated using 5′ primers designed to contain a BamHI restriction enzyme site, and a 3′ primer in the mCCR9 coding sequence. The 5′-tagged cDNA fragments of the resulting PCR products were ligated to 3′ mCCR9 coding sequences in pBluescript at the unique EcoRI site for HA-CCR9.1 or the NotI site for HA-CCR9.2 (because this 5′ primer contained an NcoI site). The initiator methionine for these constructs is indicated by bold lettering: HA-CCR9.1 forward primer; 5′-AAGGGATCCGCTACCTC

TATCCTCTATGTGGTCATGTTATGGATGTCAGTATGACGC

TTATGACCTC-3′; HA-CCR9.2 forward primer; 5′-AAGGGATCCACCA

TGCTATCTCATTACGATGGTTGCAGATGTGACGCACAGA

CTCCAAAGG-3′; and reverse primer; 5′-GGTCTAAGCTACTTCT

CTCTTTG-3′.

A construct spanning the coding region of mCCR7 (accession no. L31580) was generated by PCR amplification of overlapping 5′ and 3′ fragments that were then joined using a unique HindIII site. For the 5′ fragment an EcoRI restriction site was introduced. PCR primers used were: 5′-CCCTG-3′ and 5′-GTATTTCTCAGCGACACGACC-3′ and 5′-GATGCATAGGAGCAGCATCC-3′; 3′-CCR7, 5′-GCGAAGCTTC

GGATCTTCCGCG-3′ and reverse primer; 5′-GGTACGCTGATTATGACGCACCC-3′.

The 1.4-kb untagged mCCR9 cDNA, each HA-tagged mCCR9 cDNA, and mCCR7 were then subcloned from pBluescript into the retroviral expression vector pMl (39, 40). Subcloning of mCCR9 constructs utilized BamHI and EcoRI sites, whereas mCCR7 was subcloned using BamHI and XhoI/SalI sites.

Isolation and flow cytometry of L.1.2 cells expressing mCCR9 and mCCR7

The murine pre-B cell line L.1.2 (41) was maintained in RPMI 1640 containing 10% FCS, 2 mM glutamine, 25 mM HEPES, 50 units/ml penicillin, and 100 μg/ml streptomycin. L.1.2 cells were retrovirally infected with each chemokine receptor-pMi construct. pMi contains an internal ribosome entry site (IRES), allowing cells expressing the chemokine receptor transgene to be identified by the IRES-driven human CD2 marker (hCD2). Generation of retrovirus, infection of cells, and enrichment for positively transfected cells by panning for hCD2-positive cells was performed as previously described (39, 40). Following panning, L.1.2 clones expressing each chemokine receptor construct were obtained by limiting dilution. For flow cytometry, retrovirally infected L.1.2 cells were stained using PE-anti-hCD2 (PharMingen, San Diego, CA), or the anti-HA mAb HA.11 (Berkeley Antibody, Berkeley, CA) followed by FITC-goat anti-mouse IgG (PharMingen). L.1.2 cells were stained in the presence of the rat anti-Fc receptor mAb 2G4.2 (42). Flow cytometry was performed on a FacsCan or FacsCalibur machine, and analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

Chemokines and calcium fluorimetry

Recombinant murine 6Cine (SLC), Mip-3β (EHC), and TECK were from R&D Biosystems (Minneapolis, MN), while MDC was a gift from Dr. Jason Cyster (University of California, San Francisco). For calcium fluorimetry, L.1.2 cells were loaded with 20 μg/ml Indo-1-AM (Molecular Probes, Eugene, OR, or Sigma, St. Louis, MO) in the dark at 37°C in the presence of DMEM, 3% FCS, and 4 mM Probenecid (Sigma) using standard procedures (43). Mobilization of calcium in response to a dose range of chemokines or ionomycin was measured using a FACStar® flow cytometer; listmode files were collected using Lysis II Software (Becton Dickinson). Data were later analyzed using ReproMan (True-Facts Software, San Diego, CA) and Mtime 2.0 software (Phoenix Flow Systems, San Diego, CA).

**Chemotaxis assays**

Migration assays were performed using transwell polycarbonate membranes (24-well format, 6.5 μm diameter, 5 μm pore size) from Costar (Cambridge, MA). Transwells were preincubated overnight in assay medium consisting of RPMI 1640, 0.5% FCS, and 1% BSA. For assays using Rag2−/− thymocytes, 4-week-old mice were treated with mAb 2C11 or PBS as described above, with their thymocytes isolated 20 h or 3 days post-mAb stimulation. In chemotaxis assays, 5 × 103 L.1.2 cells or 1 × 106 thymocytes in 100 μl were added to the upper chamber and allowed to migrate to chemokines diluted in 600 μl assay medium in the lower chamber. Following incubation for 4 h at 37°C and 8% CO2, the number of transmigrated cells in the lower chamber was determined by counting with a hemocytometer. Migrated C57BL/6 thymocytes or thymocytes of Rag2−/− mice treated with 2C11 mAb for 3 days were stained with anti-CD4-PE and anti-CD8-FITC (PharMingen). Rag2−/− thymocytes 20-h post-2C11 treatment were stained with anti-CD44-PE and anti-CD25-FITC mAb. All of these mAbs were from Pharmingen. Statistical significance was assessed using the Mann-Whitney U test as described (37). The percentage of each thymocyte subpopulation (DN, DP, CD4 SP, or CD8 SP) that responded to TECK or ELC was calculated as follows: the absolute number of cells migrating in the absence of chemokine was subtracted from the absolute number of cells from that population migrating in the presence of chemokine. This number was then divided by the absolute number of input cells for a given population.

**Results**

**Identification of mCCR9 as a chemokine receptor induced by anti-CD3e stimulation of Rag2−/− thymocytes**

To identify regulatory molecules induced during the DN to DP transition of thymocyte development, we performed subtractive hybridization using cDNA from anti-CD3e-stimulated and unstimulated Rag2−/− thymocytes. We and others have found that thymocytes of Rag2−/− mice treated with a single i.p. injection of the anti-CD3e mAb 2C11 underlie the DN to DP transition in a synchronous and ordered fashion. Stimulated thymocytes start to acquire surface expression of the activation marker CD69 within 2 h, decrease CD25 within 16 h, and start to express CD8 by 2–3 days, with ~50% of cells becoming CD4−/CD8+ by day 4 (32, 33, 44). Cellular proliferation is also rapidly induced, such that cell numbers increase 2-fold by 16 h, 10-fold in 4 days, and 100-fold in 1 wk.

We performed subtractive hybridization using cDNA from pooled Rag2−/− thymocytes 16 h post-anti-CD3e mAb treatment. At this time point, >80% of thymocytes become CD69+ and progress from being CD44−/CD25+ to CD44+CD25− (data not shown), as normally occurs following pre-TCR signaling (1). Among the cDNAs obtained following subtractive hybridization, one cDNA clone (clone 2B7) was of interest because it only matched two overlapping murine thymus-derived ESTs, although it lacked an open reading frame. Northern blot analysis showed that this clone was derived from an ~3-kb mRNA whose expression was strongly induced following anti-CD3e stimulation of Rag2−/− thymocytes, and showed high level expression in normal murine thymus. RACE PCR to identify the full-length coding sequence revealed that 2B7 corresponded to the murine homologue of the murine thymus-derived ESTs derived from pre-B cells.
up-regulation (5-fold) was also observed in RNA from Rag2 stimulated, and was further up-regulated by 48 h, to a level comparable to that observed in normal C57BL/6 thymocytes. Some up-regulation (5-fold) was also observed in RNA from Rag2−/− thymocytes only 7 h after anti-CD3ε treatment, a time point at which the majority thymocytes express CD69 (data not shown). To confirm that mCCR9 is induced as thymocytes mature to the DP compartment, we performed Northern blot analysis using RNA from OT-1 TCR transgenic thymocytes on a nonselecting (Rag2−/− TAP1−/−) or selecting (Rag2−/−) background. In the former, thymocytes are arrested at the DP stage due to lack of the MHC class I/peptide ligand (37). As shown in Fig. 1B, mCCR9 expression is markedly up-regulated in a nonselecting background containing mostly DP cells, relative to Rag2−/− DN thymocytes. In addition, Northern blot analysis of RNA from multiple tissues revealed that mCCR9 expression was restricted to the lymphoid system (Fig. 1C), with very high level expression in the thymus and much lower levels in lymph node, spleen, and small intestine, another site of T cell development (45). Finally, by Northern blot analysis we failed to detect any CCR9 expression following stimulation of murine splenocytes with Con A or LPS, suggesting that CCR9 expression is not induced by activation of peripheral T or B cells (data not shown).

To verify that the CCR9 mRNA we detected was predominantly derived from thymocytes and not stromal cells, we analyzed expression of mCCR9 using a Northern blot containing RNA from separated C57BL/6 thymocyte and stromal cell material (37). Because the mCCR9 hybridization signal observed was similar to that seen using a CD4 (thymocyte-specific) probe (data not shown), we conclude that mCCR9 is present at significant levels in murine thymocytes. However, these studies do not exclude the possibility that mCCR9 is also expressed in thymic stromal cells. Finally, we analyzed CCR9 expression in several hematopoietic cell lines (data not shown). Notably, CCR9 was not detected in the murine DN pre-T cell lines NCKA and KFF (46) by Northern blot analysis, but was detected in the DP thymoma line AKR1 (47), albeit at low levels. In addition, the following cell lines were negative for CCR9 mRNA: the B cell lymphoma WEHI 231, the pre-B line NAG8/7 (48), a bone marrow-derived macrophage line BM12 (49), and a bone marrow-derived DC line JawsII (50). Together these results strongly suggested that CCR9 expression is induced in thymocytes as they mature from the DN to the DP compartment.

For comparison, we evaluated the expression of CCR7 under the same conditions. Although CCR7 mRNA expression was also induced at low levels by anti-CD3ε treatment of Rag2−/− thymocytes (data not shown), it was unlike CCR9 in that it was present at higher levels in spleen and lymph node, relative to the thymus (Fig. 1C), consistent with previous reports (51, 52). Strong up-regulation of CCR7 expression and responsiveness to its ligand ELC have been shown to occur as DP thymocytes mature to SP cells (17, 26) and following peripheral T cell activation (53). Although some increased CCR7 expression appears to occur at the DN to DP transition (17), the CCR7 expression pattern differs significantly from CCR9, suggesting that the latter may play a role in earlier stages of thymocyte development.

To confirm that CCR9 expression is induced as normal murine thymocytes undergo the DN to DP transition, we analyzed mCCR9 expression using cDNA from sorted thymocyte subsets (Fig. 2A). Quantitative RT-PCR analysis showed that CCR9 expression was 5- to 10-fold higher in DP thymocytes relative to DN cells, and then decreased with maturation to the CD4 SP or CD8 SP compartment. This result was confirmed using cDNA from two independent thymocyte sorts. Some amount of CCR9 mRNA expression in DN cells was expected, because this population also includes thymocytes that have received a pre-TCR signal and have initiated the DN to DP transition (1, 54). This result is consistent with our Northern blot analysis which showed that CCR9 expression is rapidly induced by anti-CD3ε treatment of Rag2−/− mice.

**FIGURE 1.** Northern blot analysis of mCCR9 expression. A, mCCR9 expression is up-regulated in anti-CD3ε-stimulated Rag2−/− thymocytes. Thymocyte RNA was isolated from pooled untreated Rag2−/− mice (unstim.); Rag2−/− mice treated with 2C11 mAb for 16, 48, or 72 h; or control C57BL/6 mice. The same blot was successively hybridized with probes for CCR9 (top panel) or EF1α as a loading control (bottom panel). Markers for the migration of 28S and 18S rRNA are shown. B, CCR9 is expressed at comparable levels in OT-1 transgenic Rag2−/− TAP1−/− (nonsel., lane 2), OT-1 Rag2−/− (sel., lane 3), and normal C57BL/6 thymocytes. RNA from Rag2−/− thymocytes (unstim., lane 1) is included for comparison. C, Analysis of CCR9 and CCR7 expression in different murine tissues. Although the EF1α signal varied among some tissues, ethidium bromide staining of the hybridization membrane showed a comparable amount of RNA in each lane (data not shown).

Analysis of mCCR9 expression during T cell development and in murine tissues

Using the full-length cDNA as a probe, we performed Northern blot analysis to determine the time course of CCR9 up-regulation following anti-CD3ε stimulation of Rag2−/− mice (Fig. 1A). CCR9 mRNA was strongly induced within 16 h of anti-CD3ε stimulation, and was further up-regulated by 48 h, to a level comparable to that observed for normal C57BL/6 thymocytes. Some up-regulation (5-fold) was also observed in RNA from Rag2−/− thymocytes only 7 h after anti-CD3ε treatment, a time point at which the majority thymocytes express CD69 (data not shown). To confirm that mCCR9 is induced as thymocytes mature to the DP compartment, we performed Northern blot analysis using RNA from OT-1 TCR transgenic thymocytes on a nonselecting (Rag2−/− TAP1−/−) or selecting (Rag2−/−) background. In the former, thymocytes are arrested at the DP stage due to lack of the MHC class I/peptide ligand (37). As shown in Fig. 1B, mCCR9 expression is markedly up-regulated in a nonselecting background containing mostly DP cells, relative to Rag2−/− DN thymocytes. In addition, Northern blot analysis of RNA from multiple tissues revealed that mCCR9 expression was restricted to the lymphoid system (Fig. 1C), with very high level expression in the thymus and much lower levels in lymph node, spleen, and small intestine, another site of T cell development (45). Finally, by Northern blot analysis we failed to detect any CCR9 expression following stimulation of murine splenocytes with Con A or LPS, suggesting that CCR9 expression is not induced by activation of peripheral T or B cells (data not shown).

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As early as gestational day 14. Shown to be produced by DC in the spleen. TECK is also present in Rag2 high level in the thymus and in small intestine, but not in the reported for the chemokine TECK (29), with TECK present at a

ingly, the pattern of CCR9 expression is quite similar to that

regulated by pre-TCR stimulation of murine thymocytes. In addi-

The results described above strongly suggest that CCR9 is up-

TECK stimulates a calcium flux in mCCR9-transfected cells

FIGURE 2. Analysis of CCR9 expression in sorted thymocyte subsets and whole fetal thymi. A. RT-PCR analysis of sorted adult thymocyte sub-

sets. Three-fold serial dilutions of cDNA from sorted subsets (DN, DP, CD4 SP, and CD8 SP) of thymocytes, or total thymocytes (Thy) were analyzed by PCR using primers for CCR9 (top panel) or HPRT as a nor-

malization control (bottom panel). Reaction products were visualized by Southern blotting. Purity of the sorted populations was verified by flow

cytometry and by RT-PCR analysis using primers for CD4 and CD8 (data not shown). B. RT-PCR analysis of cDNA from murine fetal thymus. cDNA isolated from whole fetal thymus lobes on gestational day 15, 16, or 17, or pooled lobes from gestational day 17–19 was analyzed by PCR for CCR9 or HPRT. The day of gestation is based on the plug date being day 1. Because of limited material, only the lower two of three-fold serial dilutions were analyzed for day 15 fetal thymus. Reaction products were visualized on ethidium bromide stained agarose gels.

(35), who did not observe differential expression of CCR9 in dif-

ferent thymocyte subpopulations. However, their analysis did not

include a quantitative titration of input cDNA, making it quite

possible that they could have missed the differential expression that we observed.

We also assessed the expression of mCCR9 during fetal thymo-
cyte development by performing RT-PCR analysis of fetal thymus mRNA (Fig. 2B). mCCR9 mRNA was detected in fetal thymus as early as day 15 of gestation, when only immature DN thymocytes are present. An increase in CCR9 expression was observed in two independent experiments using cDNA from day 17 fetal thymus, or pooled thymi from day 17–19, consistent with up-regulation following pre-TCR stimulation as significant numbers of DP cells are pooled thymi from day 17–19 was analyzed by PCR for

CCR9 or HPRT. The day of gestation is based on the plug date being day 1. Because of limited material, only the lower two of three-fold serial dilutions were analyzed for day 15 fetal thymus. Reaction products were visualized on ethidium bromide stained agarose gels.

(1A), and that CCR9 mRNA is present at high levels in non-

selecting thymy in which development is arrested at the DP stage (Fig. 1B). Our results differ from those reported by Zaballos et al. (35), and by Zack and Gruber (28), who have also reported differential expression of CCR9 in differ-

ent thymocyte subpopulations. However, their analysis did not

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cyte development, CCR9 might also function in the immigration of thymocyte progenitors into the thymus.

TECK stimulates a calcium flux in mCCR9-transfected cells

The results described above strongly suggest that CCR9 is up-

regulated by pre-TCR stimulation of murine thymocytes. In addition, CCR9 is detectable in early thymocyte progenitors. Interest-

ingly, the pattern of CCR9 expression is quite similar to that reported for the chemokine TECK (29), with TECK present at a high level in the thymus and in small intestine, but not in the spleen. TECK is also present in Rag2−/− thymus and fetal thymus as early as gestational day 14. Shown to be produced by DC in the medullary compartment of adult murine thymi (29) or fetal thymic MHC class II+ epithelial cells (30), TECK has been implicated as playing a role in thymocyte development. Similar to TECK, the expression pattern of CCR9 suggests a possible role in thymocyte development.

As a first step toward identifying a functional ligand for mCCR9, we generated L1.2 cells stably expressing two different NH2-terminal HA-tagged versions of mCCR9. The HA tag was either placed upstream of the initiator methionine encoded by the mCCR9 cDNA clone (mCCR9.1) or at amino acid 13, the homol-

ogous methionine residue predicted to be the translational start from the human genomic sequence (CCR9.2). Thus the HA tag allowed us to analyze the level of mCCR9 expression at the cell surface and to determine which methionine start codon would generate a stable protein. As shown in Fig. 3A, flow cytometry analysis demonstrated that L1.2 cells expressed significant levels of either HA-tagged version of mCCR9 at the cell surface, indicating that either methionine could initiate the translation of a stable protein. Importantly these results suggested that we had obtained a functional mCCR9 cDNA clone.

To identify a ligand for CCR9, we assessed the ability of several chemokines to stimulate a calcium flux in L1.2 cells expressing an untagged version of the full-length mCCR9 cDNA or mCCR7 as a control (Fig. 3). Like the HA-tagged mCCR9 constructs, the untagged mCCR9 or mCCR7 cDNA inserts were expressed using the retroviral vector pML, which contains an IRES followed by a cDNA encoding a tail-less form of human CD2 (hCD2). This makes it possible to identify transgene expressing cells by flow cytometry for the hCD2 reporter (39, 40). As shown, the mCCR9 and mCCR7 constructs were expressed at comparable levels in L1.2 cells (Fig. 3B). Because mCCR9 is expressed at high levels in developing thymocytes, we focused on chemokines reported to be present in the thymus. mCCR9 failed to mediate a significant calcium flux to ELC or SLC (over a concentration range of 1–50 nM), whereas mCCR7 transfectants responded well to both of these previously established ligands (Refs. 25 and 26; Fig. 3, C and D, and data not shown). Consistent with this observation, it has been reported by others that the human GPR-9-6 clone (hCCR9) fails to respond to ELC in calcium flux assays (25). Similar results have been reported for TARC (22). In addition mCCR9 transfec-
tants were unresponsive to MDC (Fig. 3D). Remarkably, TECK stimulates a strong and specific calcium flux in mCCR9 L1.2 cells, but not in untransfected or mCCR7-transfected L1.2 cells. This rapid response was dose sensitive, with over 90% or 50% of the transfected cells responding to 10 nM or 1 nM TECK, respectively (Fig. 3C). It has also been reported that TECK does not stimulate CCR6-transfected cells (55). Our results are consistent with the calcium flux analysis performed by Zaballos et al. (35) using CCR9 transfected HEK 293 cells, although we observed a somewhat more robust calcium flux in mCCR9-transfected L1.2 cells at lower concentrations of TECK. In addition, we have determined that L1.2 cells expressing either HA-tagged version of mCCR9 (Fig. 3A) mobilize intracellular calcium in response to TECK, achieving levels quite comparable to that of L1.2 cells expressing the full-length untagged mCCR9 cDNA (data not shown). Thus, the first 12 aa of mCCR9 are not essential for ligand binding.

Together, the results described above confirm that mCCR9 is a specific and functional receptor for TECK, a chemokine proposed to play a role in the migration of developing thymocytes (29).

TECK stimulates chemotaxis of mCCR9-transfected cells

We next tested the ability of mCCR9 to stimulate the chemotaxis of L1.2 cells in response to TECK. As shown in Fig. 4, we ob-
served a robust migration such that ~90% of CCR9 positive L1.2
cells migrated in response to TECK (left panel), but not to ELC (right panel). Conversely, CCR7 positive L1.2 cells responded to ELC, but not to TECK. Chemotaxis was distinguished from chemokinesis by inclusion of TECK in both the upper and lower compartment of the chemotaxis chamber (CCR9 L1.2 control, left panel). In contrast to previous studies reporting a maximal chemotactic response of thymocytes to TECK at 10 ng/ml (~1 nM; Refs. 29 and 30), we observed that mCCR9 transfectants and thymocytes (see below) required significantly higher concentrations of TECK (50 nM) to undergo chemotaxis. While Zaballos et al. (35) did not analyze chemotaxis in response to mTECK, they observed that a relatively high dose of hTECK (100 nM) was also required to stimulate efficient migration of hCCR9 HEK 293 cells. These results, together with the strong calcium flux observed in mCCR9 transfectants (even in the presence of 1 nM TECK, Fig. 3C), establish that TECK is a specific and functional ligand for mCCR9.

TECK attracts anti-CD3ε-stimulated Rag2−/− thymocytes and normal DP and SP thymocytes

We next analyzed the chemotactic activity of murine thymocytes in response to TECK. As shown in Fig. 5A, normal C57BL/6 thymocytes responded to TECK with 10-fold more thymocytes migrating to the bottom transwell chamber containing TECK, relative to the control. Like the L1.2 CCR9 transfectants, a lower concentration of TECK failed to stimulate the chemotaxis of normal thymocytes, consistent with the response being mediated by CCR9. Because we had identified CCR9 as a chemokine receptor upregulated following anti-CD3ε treatment of Rag2−/− thymocytes, we wished to determine whether responsiveness of Rag2−/− thymocytes to TECK could be similarly induced. We tested the chemotactic activity of Rag2−/− thymocytes 20 h after in vivo treatment with anti-CD3ε or PBS as control. Flow cytometry of stimulated thymocytes has consistently shown that at this time point ~90% of thymocytes have received a pre-TCR-like signal and down-regulated CD25 (data not shown), whereas Northern blot analysis has revealed that mCCR9 mRNA is present at high levels (Fig. 1A). We noted that even in the absence of chemokine, anti-CD3ε-stimulated thymocytes showed significantly more migratory activity than the PBS control, consistent with the notion that the migration of developing thymocytes is induced following pre-TCR signaling (Fig. 5A). Rag2−/− thymi likely contain a significant amount of TECK, that could stimulate the migratory activity of CCR9 positive cells. Furthermore, anti-CD3ε-treated Rag2−/− thymocytes acquired the ability to specifically respond to TECK in chemotaxis assays. This result was reproducible, and the
difference in the migration of anti-CD3e treated Rag2−/− thymocytes in the presence or absence of TECK was statistically significant ($p < 0.01$). We also tested the chemotactic activity of Rag2−/− thymocytes 3 days post-anti-CD3e treatment, a time point when $>90\%$ of cells are CD25+ with $5\%$ DP cells (data not shown). At this time point, the number of cells migrating ($\pm$ SEM) in the absence of chemokine was similar to unstimulated Rag2−/− thymocytes (2.3 $\times$ 10^4 $\pm$ 0.26), but was increased in response to TECK (1.0 $\times$ 10^5 $\pm$ 0.09). Hence, as for CCR9 L1.2 cells, normal C57BL/6 and anti-CD3e-stimulated Rag2−/− thymocytes showed specific migration in response to 100 nM TECK. Because TECK and CCR9 show a similar expression pattern at sites of T cell development, these results are consistent with CCR9 mediating the chemotaxis induced by TECK in developing thymocytes.

Finally, we studied the subpopulations of thymocytes responding to TECK or ELC by staining the cells that had migrated to the lower transwell chamber for CD4 and CD8 (Fig. 5B). A difference in the populations of C57BL/6 thymocytes migrating to TECK in comparison to ELC was readily apparent. While the flow cytometry profile of cells migrating to ELC showed an enrichment of CD4 SP and CD8 SP cells (right panel), consistent with previous reports (17, 26), both DP and SP thymocytes migrated in response to TECK (middle panel). We also calculated the percent of input cells ($\pm$ SEM) specifically migrating for each thymocyte subset in

**FIGURE 4.** TECK stimulates chemotaxis of CCR9-expressing L1.2 cells. Data are expressed as the mean percentage of input cells migrating to the lower transwell chamber in response to the indicated concentrations of TECK (left panel) or ELC (right panel). Cells used in the assay are indicated in the legend to the right. Chemotaxis of CCR9-positive cells was distinguished from chemokinesis by placing the indicated concentration of TECK in both the top and bottom chemotaxis chambers (CCR9 L1.2, Control). Vector-only L1.2 cells failed to respond to either chemokine (data not shown). The number of input cells was $5 \times 10^5$ per well. Similar chemotaxis experiments were performed three times. Results from a representative experiment performed in triplicate are shown ($\pm$SEM). For many conditions the SEM was $<2\%$ of input, and therefore is not visible on the graph.

**FIGURE 5.** Induction of chemotactic activity in murine thymocytes in response to TECK and ELC. A, TECK mediates chemotaxis of 2C11-stimulated Rag2−/− thymocytes and C57BL/6 thymocytes. Data are expressed as the mean number of cells ($\pm$ SEM) migrating in the absence of chemokine (none) or in the presence of 10 nM or 100 nM TECK. Rag2−/− mice were treated with PBS or the anti-CD3e mAb 2C11, and pooled thymocytes from each group were harvested for chemotaxis assays 20 h later. Input cell number was $1 \times 10^6$ per well. Each part of the experiment was repeated at least two times. Results from a representative experiment performed in triplicate are shown. B, Flow cytometry analysis of C57BL/6 thymocytes migrating in response to TECK and ELC. Thymocytes in the bottom wells of chemotaxis assay chambers were counted and stained with anti-CD4 and anti-CD8. The percentage of live gated cells in each population is shown. This experiment was performed twice in triplicate. Representative results are shown.
response to TECK or ELC, and thus confirmed that increased responsiveness to TECK is acquired with thymocyte maturation to DP cells (Table I). This correlates well with up-regulation of CCR9 expression at the DN to DP transition. However, although RT-PCR analysis showed that SP thymocytes express a lower amount of CCR9 mRNA relative to DP cells (Fig. 2A), both populations migrated similarly to TECK. It is possible that a significant amount of CCR9 protein may still be present in a subpopulation of SP thymocytes, which can only be determined with development of staining reagents for CCR9. After submission of this manuscript, Campbell et al. (36) reported a detailed analysis of the migration of specific populations of DP and SP thymocytes to several chemokines expressed in the thymus, including TECK. Consistent with our own results (Fig. 5 and Table I), DP and SP thymocytes responded to TECK (36). Interestingly, responsiveness to TECK was lost in the most mature population of CD4 SP thymocytes (CD69<sup>+</sup> L-selectin<sup>high</sup>), while migration to ELC and SLC was strong, suggesting that CCR9 is specifically down-regulated just prior to emigration from the thymus. Peripheral T cells (29, 36) and activated T cells (30) are unresponsive to TECK, consistent with a loss in CCR9 expression once thymocytes fully mature and emigrate to the periphery. This conclusion is supported by our Northern blot analysis of Con A-stimulated splenocytes, in which we failed to detect up-regulation of CCR9, although expression of CD69 mRNA was induced (data not shown). Together with the results of our chemotaxis assays, these results support the notion that CCR9 delivers signals to thymocytes in response to TECK that are important for T cell development.

**Table I. Migration of normal thymocyte subsets**

<table>
<thead>
<tr>
<th>Subset</th>
<th>100 nM TECK</th>
<th>100 nM ELC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN</td>
<td>4.8 ± 0.4</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>DP</td>
<td>17.3 ± 1.7</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>CD4 SP</td>
<td>18.5 ± 1.8</td>
<td>25.4 ± 3.2</td>
</tr>
<tr>
<td>CD8 SP</td>
<td>27.3 ± 2.3</td>
<td>40.6 ± 3.8</td>
</tr>
</tbody>
</table>

*Mean percentage ± SEM of the absolute number of input cells specifically migrating in response to TECK or ELC. See Materials and Methods for calculation. Data are from two separate experiments performed in triplicate and showing very similar results.

Discussion

Chemokines and their receptors are likely to be critical for the regulation of T cell development in the thymus at several different stages. Hematopoietic stem cells are first recruited to the thymus through a pertussis toxin-sensitive mechanism (30), consistent with this being a chemokine-dependent process. Thereafter, developmental proceeds in parallel with migration, such that thymocytes migrate from the outer to the inner cortex as they undergo the DN to DP transition, and then to the medulla following positive selection (5, 6). In addition, the involvement of chemokine receptors in the export of mature SP thymocytes explains the observations that thymocytes expressing a pertussis toxin transgene (56) or those deficient in G<sub>αi</sub> (57) fail to emigrate to the periphery. Finally, it has also been suggested that recirculation of mature peripheral T cells to the thymus may also be driven by chemotactants (17). Despite the biologic importance of these processes, little is known about the specific chemokines or the receptors that regulate the trafficking of developing thymocytes at these different stages.

In this report we have identified murine GPR-9-6 as a CC family chemokine receptor whose expression is up-regulated at the DN to DP transition of thymocyte development. We have shown that GPR-9-6 mRNA expression is rapidly induced following anti-CD3ε stimulation of Rag2<sup>−/−</sup> thymocytes (which mimics pre-TCR signaling), and that it is significantly increased as normal thymocytes mature to DP cells. In addition we have determined that GPR-9-6 is a specific and functional receptor for the chemokine TECK, and that anti-CD3ε-stimulated Rag2<sup>−/−</sup> thymocytes, as well as normal DP and SP cells, migrate in response to TECK. Based on sequence homology to other CC chemokine receptors and its specific ability to mediate a calcium flux and chemotaxis in response to TECK, we agree with the proposal by Zaballos et al. (35) to designate GPR-9-6 as CCR9. It has also been proposed that the chemokine receptor D6 should be designated as CCR9 (14, 58, 59), while the rat and human equivalents have also been published as CCR10 (60, 61). However, this receptor is highly promiscuous in its binding and expression pattern, and the human D6 equivalent fails to signal in transfected cells (59). Hence, GPR-9-6 has been designated here and by Zaballos et al. (35) as CCR9.

In contrast to our studies, Zaballos et al. (35) failed to observe differential expression of CCR9 by RT-PCR analysis of sorted thymocyte subsets. Notably, they evaluated CCR9 expression in CD25<sup>+</sup> DN cells, which comprise the majority of thymocytes in Rag2<sup>−/−</sup> mice due to absence of the pre-TCR (1, 31). This discrepancy from our results is likely explained by nonlinear amplification of a small amount of CCR9 mRNA present in their sorted DN thymocytes. In support of this, by Northern blot analysis we detected low level expression of CCR9 mRNA in Rag2<sup>−/−</sup> thymocytes, which was strongly up-regulated following anti-CD3ε stimulation (Fig. 1A), as well as strong expression of CCR9 mRNA in thymocytes arrested at the DP stage of development (Fig 1B). Moreover, we performed a quantitative RT-PCR analysis of normal thymocyte subsets using titrated cDNA from two independent sorts. We have also recently performed in situ hybridization studies which indicate that CCR9 mRNA is expressed in the majority of cortical thymocytes, and to a lesser extent in the medulla (in collaboration with T. Gurney and N. Landau, unpublished results).

Because TECK is produced by medullary DC in the adult thymus (29), our results suggest that up-regulation of CCR9 following pre-TCR assembly may be an important mechanism regulating the cortico-medullary migration of developing thymocytes. Another possibility proposed by Campbell et al. (36) is that TECK may function to retain cells in the thymus until they have fully matured, since CD4 SP CD69<sup>−</sup> L-selectin<sup>high</sup> thymocytes lose responsiveness to TECK. Finally, TECK may also be expressed by fetal thymic MHC class II<sup>+</sup> epithelial cells, suggesting that it could also play a role in the recruitment of early thymocyte progenitors (30). In support of this notion, we have detected CCR9 expression in fetal liver, as well as in fetal thymus tissue as early as day 15 of gestation. Thus, CCR9 and its ligand TECK may regulate the trafficking of developing thymocytes at two different stages: during emigration of hematopoietic precursors to the fetal thymus, and later following induction of the DN to DP transition.

In the family of chemokine receptors CCR9 is phylogenetically grouped with CCR6 and CCR7, suggesting a similarity in function (34, 35). In this regard it is interesting that CCR6 and CCR7 are predominantly expressed in lymphoid tissues, and that they share the feature of having introns in their 5′ coding sequence, which is uncharacteristic of most chemokine receptors (34, 52, 62). Both CCR6 and CCR7 appear to play important roles in regulating the trafficking of different subsets of T cells and DC in the periphery (63). For example, liver and activation-regulated chemokine (LARC; MIP-3α), a unique ligand for CCR6, specifically stimulates the migrational arrest of memory CD4<sup>+</sup> T cells under rolling flow conditions (64). Recent evidence indicates that CCR7 may be essential for the homing of naive peripheral T cells to lymph nodes.
and the splenic white pulp (26, 63). In particular, T cells of mice with the spontaneous P1t mutation (paucity of lymph node T cells) fail to migrate to the T cell-rich zones of peripheral lymphoid organs (65, 66), a phenotype attributed to the absence of the CCR7 ligand, SLC, in these mice (67). CCR6 and CCR7 are also expressed by specific subsets of DC. While CCR6 is present on immature DC derived from hematopoietic but not monocyte precursors, DC activation and maturation is accompanied by a loss of CCR6 expression with sharp up-regulation of CCR7 (68, 69). Thus it has been suggested that CCR7 may be important for the colocalization of T cells and Ag-presenting DC in peripheral lymphoid tissues (63). Interestingly TECK has also been reported to have chemotactic activity for activated DC and macrophages (29), suggesting that CCR9 could play a role in the association of these cell types with developing thymocytes.

While several chemokines and their receptors are expressed within the thymus, their specific contribution to T cell development remains unclear. Studies of mice deficient in SDF-1 or its receptor, CXCR4, demonstrated that chemokines can play essential roles in lymphoid development, as either of these mouse mutants shows a severe defect in early B cell development prior to the pre-B stage (112). However, high level expression of SDF-1 and CXCR4 in the thymus, thymocyte development proceeds normally in their absence (72). In addition CCR5 is expressed on developing thymocytes, but its absence does not appear to impair T cell development. Humans with homozygous CCR5 mutations have the benefit of being resistant to HIV infection, but appear to have normal immune function (73). Finally, TECK has been shown to specifically stimulate the migration of fetal thymic precursors in chemotaxis assays and to be produced by MHC class II* fetal thymus epithelial cells (30). Together with our observation that CCR9 is expressed in the fetal liver and in day 15 fetal thymus, these results implicate a role for TECK and CCR9 in the recruitment of fetal thymocyte precursors. However, in one report an anti-TECK antiserum (which neutralized chemotaxis of thymic precursors to TECK) failed to inhibit colonization of fetal thymus lobes (30). The role of TECK later in thymocyte development was not addressed in this study. Although it cannot be excluded that the amount of anti-TECK antiserum present in the fetal thymus lobes was insufficient to fully neutralize chemokine activity, it is also possible that TECK has some redundancy of function with other chemokines. As for other important regulatory molecules, it is likely that some chemokines and their receptors may serve redundant functions in T cell development. However, the tight regulation of chemokine receptor expression at discreet stages of thymocyte development strongly suggests that different chemokines serve specific functions during T cell differentiation (36).

In addition to stimulating migration, there is evidence that chemokine receptors may function to deliver signals for lymphocyte proliferation and survival (14). For example, the CC chemokine RANTES has been shown to stimulate T lymphocyte activation and proliferation (74), whereas SDF-1 was initially cloned by Nagasawa et al. (75) as a pre-B-cell growth stimulating factor (PBSF). Studies of the ability of SDF-1 to drive the proliferation of immature DN thymocytes have not been reported. A role for chemokine receptors in promoting cell survival was suggested by the observation that the chemokine I-309, (which binds CCR8), protects murine T cell lymphomas from dexamethasone-induced apoptosis (76). Our studies show that CCR9 expression is strongly induced following pre-TCR stimulation, a time of intense cellular proliferation (Figs. 2 and 3, and Ref. 1). Having identified TECK as a specific ligand, we are now in a position to analyze the function of CCR9 with regard to its ability to support thymocyte proliferation or survival, and to analyze the function of CCR9 in the regulating the trafficking of developing thymocytes.

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