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CCR7 Expression in Developing Thymocytes Is Linked to the CD4 versus CD8 Lineage Decision

Xinye Yin,* Ena Ladi,* Shiao Wei Chan,* Ou Li,* Nigel Killeen,† Dietmar J. Kappes,‡ and Ellen A. Robey2*

During thymic development, T cell progenitors undergo positive selection based on the ability of their T cell Ag receptors (TCR) to bind MHC ligands on thymic epithelial cells. Positive selection determines T cell fate, in that thymocytes whose TCR bind MHC class I (MHC-I) develop as CD8-lineage T cells, whereas those that bind MHC class II (MHC-II) develop as CD4 T cells. Positive selection also induces migration from the cortex to the medulla driven by the chemokine receptor CCR7. In this study, we show that CCR7 is up-regulated in a larger proportion of CD4+CD8+ thymocytes undergoing positive selection on MHC-I compared with MHC-II. Mice bearing a mutation of Th-POK, a key CD4/CD8-lineage regulator, display increased expression of CCR7 among MHC-II-specific CD4+CD8+ thymocytes. In addition, overexpression of CCR7 results in increased development of CD8 T cells bearing MHC-II-specific TCR. These findings suggest that the timing of CCR7 expression relative to coreceptor down-regulation is regulated by lineage commitment signals. The Journal of Immunology, 2007, 179: 7358–7364.

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that the differential expression of CCR7 in CD4+CD8+ thymocytes is dependent on the key lineage commitment transcription factor Th-POK. Finally, we provide evidence that overexpression of CCR7 in MHC-II-selected thymocytes leads to increased CD8 T cell development. These results are consistent with a model in which the timing of CCR7 expression relative to pre-T cell receptor (TCR) down-regulation is regulated by lineage commitment signals. These results also raise the possibility that differential expression of CCR7 in CD4+CD8+ thymocytes serves to amplify signaling differences generated upon MHC-I and MHC-II recognition and help to reinforce the CD4/CD8-lineage choice.

Materials and Methods

Mice

All mice were housed and bred in pathogen-free conditions at the American Association of Laboratory Care-approved animal facility at Life Sciences Division of the University of California, Berkeley. All mutant mouse strains were on the C57BL/6J background unless otherwise indicated. The following mice were bred to AND TCR-transgenic mice. CCR7-transgenic mice (25) were bred with MHC-I-deficient (I-A<sup>2m</sup>−/−) mice (32); DO11.10 on the BALB/c background (33); and MHC-I-deficient (β2m<sup>−−</sup>) mice (34) and MHC-II-deficient (I-A<sup>b</sup>−/−) mice (35). TCRβ-deficient mice (36), F5 TCR rag1<sup>−/−</sup> mice (37), and AND TCR rag1<sup>−/−</sup> mice (38) were obtained from The Jackson Laboratory. Th-POK<sup>−/−</sup> mutant mice (1) were bred to AND TCR-transgenic mice. CCR7-transgenic mice (25) were bred with AND<sup>+/+</sup> Rag1<sup>−/−</sup> or 5CCR7<sup>+/−</sup> mice to generate CCR7<sup>+/−</sup> Rag1<sup>−/−</sup> mice and with AND<sup>+/+</sup> Rag1<sup>−/−</sup> Rag<sup>+/−</sup> mice to generate AND<sup>+/−</sup> or AND<sup>−/−</sup> mice. MHC-I<sup>-/-</sup> or MHC-II<sup>-/-</sup> mice were used to study the effect of CCR7<sup>+/−</sup> in the absence of MHC-I or MHC-II.

Abs and flow cytometry

The CCL19-Fc fusion protein for detecting CCR7 expression was a gift from Dr. J. Cyster (University of California, San Francisco, CA). FITC-anti-mouse TCRβ, FITC-anti-mouse HSA, Cy5-anti-mouse CD8, and FITC-anti-mouse CD69 were purchased from eBioscience. PETR-anti-mouse CD4 was obtained from Invitrogen Life Technologies. PE-anti-human Fc receptor blocking serum was purchased from The Jackson Laboratory. The procedure for CCR7 staining has been described previously (39). Briefly, 1 million cells were washed twice and incubated with 2% normal rat and mouse serum for 30 min. The PE-conjugated anti-human Fc receptor blocking serum was then added and incubated for 20 min. The cells were washed twice followed by addition of anti-CD4 PETR, anti-CD8 PECy5, and anti-mouse TCR Abs and flow cytometry analysis of thymocytes from P14 TCR-transgenic mice. CCR7 and αβTCR levels on gated CD4+CD8+ and CD4+CD8+ populations are shown. Note that the mean fluorescence intensity of the CCR7 population for CCR7 and TCRα are lower for CD4+CD8+ compared with CD4+CD8+ thymocytes (9.1 vs 13.2 for CCR7 and 10.8 vs 12.9 for TCRα).

A C, CCR7 expression in gated CD69<sup>+</sup>CD4+CD8+ thymocytes from wild-type mice, MHC-II-deficient (I-A<sup>b</sup>−/−) mice (35) and MHC-I-deficient mice (β2m<sup>−−</sup>) and CD8<sup>−/−</sup> mice (9.1 vs 13.2 vs 19.0 for CCR7 and 10.8 vs 12.9 vs 22.2 for TCRα).

B, Four-color flow cytometric analysis of thymocytes from P14 TCR-transgenic mice. CCR7 and αβTCR levels on gated CD4+CD8+ and CD4+CD8+ populations are shown. Note that the mean fluorescence intensity of the CCR7 population for CCR7 and TCRα are lower for CD4+CD8+ compared with CD4+CD8+ thymocytes (9.1 vs 13.2 for CCR7 and 10.8 vs 12.9 for TCRα).

C, C, CCR7 expression in gated CD69<sup>+</sup>CD4+CD8+ thymocytes from wild-type mice, MHC-II-deficient (I-A<sup>b</sup>−/−) mice (35) and MHC-I-deficient mice (β2m<sup>−−</sup>) and CD8<sup>−/−</sup> mice (9.1 vs 13.2 vs 19.0 for CCR7 and 10.8 vs 12.9 vs 22.2 for TCRα).

FIGURE 1. Differential up-regulation of CCR7 in CD4+CD8+ thymocytes undergoing MHC-I vs MHC-II selection. A, CCR7 expression on gated CD4+CD8+ thymocytes from wild-type and TCR-transgenic mice. Representative flow cytometry profiles are shown in the left panel. Representative CD4+CD8+ gates are shown in Figs. 1, B and C, 2C, and 3. Compiled results from individual mice are shown in the right panel. All TCR-transgenic mice were rag deficient except DO11.10 mice. B, Four-color flow cytometric analysis of thymocytes from mice receiving TCR signals. Representative flow cytometry profiles are shown in the left panels. Solid line (---) represents CCR7 staining and dotted line (----) represents background control in which thymocytes were stained with all of the reagents except CCL19-Fc. Compiled results from individual mice are shown in the right panel.
anti-CD69 FITC. The thymocytes treated with all of the reagents mentioned above except CCL19-Fc were used as background control. Flow cytometry was performed with an ELITE FACS machine (Beckman Coulter). Data analysis was performed with FlowJo software (Tree Star).

**In vitro chemotaxis assay**

This method was based on a previously published procedure (25) and performed in RPMI 1640 medium containing 10% FBS. Thymocytes (3 x 10⁶ cells) in 0.1 ml of medium were placed in the upper chambers of the Transwells with 3-μm pore size polycarbonate membrane (Corning Costar). In brief, 0.6 ml of medium containing 100 nM CCL21 (R&D Systems) was added to the bottom chamber of the Transwell. The same volume of medium without CCL21 was also added to the bottom chamber to serve as nonspecific migration control. After incubation for 1 h at 37°C with 5% CO₂, cells that migrated into the lower chambers were collected, counted, stained with anti-CD4 and anti-CD8 Abs, and analyzed by flow cytometry. The number of migrated cells was calculated by subtracting the number of migrated cells without chemokine addition from the number of cells migrated in the presence of CCL21. The percentage of the migrated CD4⁺CD8⁺ thymocytes was expressed as a percentage of migrating CD4⁺CD8⁺ cells compared with the starting population. Control experiments in which CCL21 was added to both the upper and lower chamber were performed to distinguish chemokinesis from chemotaxis. No migration over background was observed in these control experiments.

**Results**

**Differential expression of CCR7 in CD4⁺CD8⁺ thymocytes undergoing positive selection via MHC-I vs MHC-II**

CCR7 is expressed on mature CD4 and CD8 T cells, is absent on the bulk of CD4⁺CD8⁺ thymocytes, and is up-regulated on CD4⁺CD8⁺ thymocytes during positive selection (20, 21). To examine the relationship between CCR7 induction and positive selection in more detail, we analyzed the expression of CCR7 on thymocytes by flow cytometry in a number of different TCR-transgenic models (Fig. 1A). Consistent with previous reports, CCR7 was significantly up-regulated in CD4⁺CD8⁺ thymocytes from all of the positive-selecting TCR-transgenic models examined (1.5–26%) compared with wild-type (0.51–1.2%) or compared with a TCR transgenic on a nonselecting background (5CC7 B10) (0.13–1.0%). Interestingly, CD4⁺CD8⁺ thymocytes from mice bearing MHC-I-specific TCR transgenes (F5, OT1, and P14) had a higher percentage of CCR7⁺ cells (12–26%) compared with mice expressing MHC-II-specific TCR transgenes (DO11.10, OT2, AND,

**FIGURE 2.** MHC-I-selected CD4⁺CD8⁺ thymocytes are more responsive to CCR7 ligands compared with MHC-II-selected CD4⁺CD8⁺ thymocytes. Total thymocytes isolated from B6, F5 TCR rag1⁻, and AND TCR rag1⁻ mice (A) and B6, MHC-I-deficient and MHC-II-deficient mice (B) were placed in the upper compartment of Transwell chambers containing CCL21 in the bottom compartment. After 1 h of incubation at 37°C, cells that had migrated into the lower chamber were analyzed by flow cytometry. The responsiveness of CD4⁺CD8⁺ thymocytes to CCL21 was calculated as the percentage of the migratory CD4⁺CD8⁺ thymocytes over total input thymocytes. A, Two independent experiments. B, Three independent experiments. C, Representative flow cytometric analysis showing gates used to define CD4⁺CD8⁺ thymocytes in starting and migrated populations.

**FIGURE 3.** The CD4-CD8-lineage switch in Th-POK mutant mice is associated with increased CCR7 expression on CD4⁺CD8⁺ thymocytes. Representative flow cytometry profiles of CCR7 expression on gated CD4⁺CD8⁺ thymocytes from AND TCR +/⁻ Th-POK +/⁻ and AND TCR +/⁻ Th-POK +/⁻ mice are displayed in left panel. Solid line (—) represents CCR7 staining. Dotted line (······) represents background control. The gate used to define CD4⁺CD8⁺ is indicated in bold. Compiled data of CCR7 expression from AND TCR +/⁻ Th-POK +/⁻ and AND TCR +/⁻ Th-POK +/⁻ mice are presented in the right panel. * and **, The range of values of the percentage of CCR7⁺CD4⁺CD8⁺ thymocytes among MHC-I- and MHC-II-selected CD4⁺CD8⁺ thymocytes from Fig. 1.
Similar results were obtained using mixed bone marrow chimeras in which TCR-transgenic thymocytes represented fewer than 5% of all thymocytes (data not shown). This indicates that the differential expression of CCR7 observed in TCR-transgenic mice was not due to the abnormally high frequency of positively selecting thymocytes in intact TCR-transgenic mice.

To rule out the possibility that the increase in CCR7 in MHC-I-specific CD4\(^+\)/CD8\(^+\) thymocytes was due to contaminating mature CD4\(^-\)/CD8\(^+\) thymocytes, we performed four-color flow cytometry to examine the levels of CCR7 and TCR on CD4\(^+\)/CD8\(^+\) and CD4\(^-\)/CD8\(^+\) thymocytes (Fig. 1B). We find that CD4\(^+\)/CD8\(^+\) thymocytes from P14 TCR-transgenic mice have a population of CCR7\(^+\) cells with a lower mean fluorescence intensity for both CCR7 (9.1 vs 13.2) and TCR (10.8 vs 12.9), compared with CD4\(^-\)/CD8\(^+\) thymocytes. This confirms that the CCR7\(^+\) thymocytes within the CD4\(^+\)/CD8\(^+\) gate are distinct from mature CD4\(^-\)/CD8\(^+\) thymocytes and suggests that they may represent a transitional population.

To confirm these observations with a diverse TCR repertoire, we also examined CCR7 expression in thymocytes derived from MHC-I-deficient (β\(_2\)m\(^-\)) or MHC-II-deficient (I-\(\alpha\)β\(^-\)) mice (Fig. 1C). For these analyses, we focused on CD69\(^+\)/CD4\(^+\)/CD8\(^+\) thymocytes, which are enriched for cells undergoing positive selection. CD69\(^+\)/CD4\(^+\)/CD8\(^+\) thymocytes from MHC-II-deficient mice, which are largely composed of thymocytes undergoing selection via MHC-I, exhibited a high percentage (16–25%) of CCR7\(^+\) cells, similar to that seen among CD4\(^+\)/CD8\(^+\) thymocytes bearing MHC-I-specific TCRs (12–26%). In contrast, CD69\(^+\)/CD4\(^+\)/CD8\(^+\) thymocytes from MHC-I-deficient mice, consisting largely of thymocytes being selected via MHC-II had a low percentage of CCR7\(^+\) cells (3.2–7.2%), similar to that seen in CD4\(^+\)/CD8\(^+\) thymocytes bearing MHC-II-specific transgenic TCRs. The low percentage of CCR7\(^+\) cells among CD69\(^+\)/CD4\(^+\)/CD8\(^+\) thymocytes from wild-type mice may reflect the fact that wild-type mice have more thymocytes selected on MHC-II compared with MHC-I. An alternative gating strategy (data not shown) indicated that CCR7 up-regulation correlated with CD8 down-regulation in thymocytes being selected on MHC-II. In contrast, thymocytes being selected on MHC-I contained CCR7 expression on thymocytes that retained substantial CD4 expression. Together, these results suggest that for thymocytes undergoing selection via MHC-I, up-regulation of CCR7 occurs before coreceptor down-regulation is complete.

To determine whether the differential expression of CCR7 in MHC-I- vs MHC-II-selected CD4\(^+\)/CD8\(^+\) thymocytes correlates with...
with their differential migratory response to CCR7 ligands, we performed in vitro chemotaxis assays. Thymocytes from TCR-transgenic mice or MHC-deficient mice were placed in the upper chamber of the Transwell, while the CCR7 ligand CCL21 was placed in the lower chamber. After 1 h, the number of migrating CD4+ CD8+ thymocytes was quantitated using flow cytometry. A higher proportion of MHC-I-selected TCR-transgenic (F5) CD4+ CD8+ thymocytes migrated in response to CCL21 compared with CD4+ CD8+ thymocytes bearing a MHC-II-specific TCR transgene (AND) (Fig. 2A). Similarly, a higher proportion of CD4+ CD8+ thymocytes from MHC-II-deficient mice migrated in response to CCL21 compared with CD4+ CD8+ thymocytes from MHC-I-deficient mice (Fig. 2B). Interestingly, the migrated CD4+ CD8+ population from F5 TCR-transgenic thymocytes expresses intermediate levels of CD4, consistent with a population that is in transition from CD4+ CD8+ to CD4- CD8+ (Fig. 2C). These results are consistent with the differential CCR7 expression pattern in MHC-I- vs MHC-II-selected CD4+ CD8+ thymocytes and indicate that this difference in surface expression of CCR7 corresponds to a different functional response to CCR7 ligands.

**CD4- to CD8-lineage switch in Th-POK mutant mice is associated with increased CCR7 expression on CD4+ CD8- thymocytes**

To address whether the differential expression of CCR7 during MHC-I- vs MHC-II-specific positive selection is related to lineage commitment, we examined the relationship between CCR7 and Th-POK, a zinc finger transcription factor that regulates CD4/CD8-lineage commitment. Th-POK expression is up-regulated in thymocytes during CD4 differentiation, and a spontaneous point mutation in Th-POK mutant mice results in redirection of MHC-II-restricted thymocytes to the CD8 lineage (1-4). We examined the expression of CCR7 in thymocytes of Th-POK+/− mice bearing a TCR transgene specific for MHC-II (AND). As previously shown (1), thymocytes from AND+/− Th-POK−/− mice have an increased proportion of mature CD8+ CD4− thymocytes and a decrease in mature CD4+ CD8− thymocytes (Figs. 3 and 4). Strikingly, this change in CD4/CD8 development is associated with an increase in the proportion (13-19%) of CD4+ CD8− thymocytes expressing CCR7, comparable to the percentage seen among CD4+ CD8− thymocytes selected by MHC-I (12-26%) (Fig. 4). These results further illustrate the link between lineage commitment and CCR7 expression. Moreover, the correlation between CD8-lineage commitment and CCR7 expression on CD4+ CD8− thymocytes shown here suggests that Th-POK may play a role in the differential expression of CCR7 in MHC-I- vs MHC-II-selected CD4+ CD8− thymocytes.

**Overexpression of CCR7 on developing thymocytes leads to a partial lineage switch from CD4 to CD8**

Chemokine signaling can modulate TCR signaling in mature T cells (40, 41). Because the strength and duration of TCR signaling may be a determinant of the CD4- vs CD8-lineage choice, this raises the possibility that the differential expression of CCR7 in CD4+ CD8+ thymocytes may differentially modulate TCR signaling during MHC-I vs MHC-II selection and consequently influence CD4/CD8-lineage choice. If this is the case, then overexpression of CCR7 in MHC-II-selected thymocytes may redirect MHC-II-selected thymocytes to CD8 line fate. To explore this possibility, we crossed CCR7-transgenic mice with MHC-II-specific TCR-transgenic mice (AND). Although AND mice had very few mature CD8+ CD4− cells, overexpression of CCR7 led to a significant increase in both the percentage and number of mature CD8+ thymocytes (TCRβhighCD8− CD4− or HSAlowCD8− CD4−) (Fig. 4, A and B). CCR7 overexpression also led to a decrease of percentage and number of mature CD4+ thymocytes (TCRβhigh CD4+ CD8+ or HSAlowCD4+ CD8+) (Fig. 4, A and B). The effect of the CCR7 transgene on the proportion of mature CD4 and CD8 T cells was also observed in the spleen (Fig. 4C). In addition, overexpression of CCR7 in another MHC-II-specific TCR-transgenic model (5CC7B7.10.A) led to an increase in both the proportion and total number of TCRβhighCD8− CD4− thymocytes (Fig. 5).
4B), although no obvious decrease of TCRβhighCD4+CD8− thymocytes was found in these mice. The increase in CD8 T cell development was not the result of selection using endogenous TCR, since comparable results were obtained with the AND TCR on both rag− and rag+ backgrounds (Fig. 4, A and B). Together these results suggest that overexpression of CCR7 in TCR-transgenic mice leads to a modest, but highly reproducible, increase in the development of CD8 T cell-bearing MHC-II-specific TCRs.

To test the effect of overexpression of CCR7 on lineage commitment in mice with a diverse TCR repertoire, we crossed CCR7-transgenic mice with MHC-I-deficient mice (β2m−/−). Consistent with the observations in MHC-II-specific TCR-transgenic mice, overexpression of CCR7 in β2m−/− mice also led to an increase in TCRβhighCD8+CD4− and HSAlowCD8+CD4− thymocytes and this was accompanied by a decrease in TCRβhighCD4+CD8− and HSAlowCD4+CD8− thymocytes (Fig. 5). This result confirms the observations from TCR-transgenic mice. Although we cannot rule out that factors such as altered negative selection or thymic egress could account for some of the differences in CD4 and CD8 development reported here, we favor the interpretation that CCR7 overexpression causes some thymocytes bearing MHC-II-specific TCR to chose the CD8 lineage, rather than the CD4 lineage. We did not find evidence for a reciprocal change in the ratio of CD4/CD8 T cells when crossing CCR7 knockout mice with MHC-I-restricted TCR-transgenic mice or MHC-II knockout mice (data not shown). This may be because other chemokine receptors that turn on during positive selection, such as CCR4 or CCR8, can compensate for the loss of CCR7 (20, 42).

Discussion
Positive selection leads to CD4- vs CD8-lineage commitment with associated changes in expression of coreceptors and transcription factors. Positive selection also regulates thymocyte migration patterns; however, no correlation has been reported between lineage commitment and migration in the thymus. In this study, we report that positive selection via MHC-I leads to CCR7 up-regulation on a higher proportion of CD4+CD8+ thymocytes compared with positive selection via MHC-II. This differential expression of CCR7 correlates with the activity of the transcription factor Th-POK, a key regulator of CD4/CD8-lineage commitment. We also provide evidence that enforced expression of CCR7 in thymocytes leads to increased development of CD8 T cells bearing MHC-II-specific TCRs. Together our results suggest that lineage commitment signals control the extent of CCR7 expression in CD4+CD8+ thymocytes and raise the possibility that differential expression of CCR7 in MHC-I- and MHC-II-selected CD4+CD8+ thymocytes may help to reinforce the CD4- vs CD8-lineage choice.

CCR7 is highly expressed in mature T cells of both the CD4 and CD8 lineage, but not in nonselected CD4+CD8+ thymocytes (20, 21). Thus, the differential expression of CCR7 that we observe in CD4+CD8+ thymocytes selected on MHC-I vs MHC-II is likely to reflect a difference in the timing of CCR7 induction relative to coreceptor down-regulation. Although the basis for this differential regulation is not yet clear, an important clue comes from our analysis of Th-POK mutant mice. Whereas few (4–8%) of the CD4+CD8+ thymocytes bearing the MHC-II-specific AND TCR express CCR7, in mice with a loss-of-function mutation in Th-POK this percent increases ~3-fold (13–22%). Th-POK expression in MHC-II-selected thymocytes could cause a delay in turning on CCR7 expression and/or may lead to more rapid coreceptor down-regulation following CCR7 induction. In either case, the result would be that MHC-I-selected thymocytes remain CD4+CD8− as they turn on CCR7, whereas for MHC-II-selected thymocytes CCR7 induction occurs co-coordinately with CD8 down-regulation.

CCR7 is involved in multiple functions during T cell development, including regulating outward migration of early thymic progenitors (22), migration of positive selected thymocytes from the cortex to the medulla (21), thymic emigration (43), and homing of mature T cells to the lymph node (44). Our study suggests a possible additional function for CCR7 in CD4- vs CD8-lineage commitment. In particular, the observation that enforced CCR7 expression in thymocytes bearing MHC-II-specific TCR leads to increased CD8-lineage development implies that differential CCR7 expression at the CD4+CD8+ stage helps to reinforce the CD4- vs CD8-lineage choice.

How might the differential expression of CCR7 affect the CD4- vs CD8-lineage choice? One possibility is suggested by a popular “quantitative signaling model.” According to this model, weak or transient TCR signals favor the CD8 lineage, whereas moderate or prolonged signals favor the CD4 lineage (11–14). Perhaps induction of CCR7 during positive selection of MHC-I-specific thymocytes leads to migration toward CCR7 ligands, thus disrupting interactions with cortical thymic epithelial cells and reinforcing the transient nature of the TCR signal generated upon MHC-I recognition. In contrast, thymocytes being selected via MHC-II would undergo CCR7 up-regulation only after they have completed the positive selection process. Another possibility is that up-regulation of CCR7 in CD4+CD8+ thymocytes being selected on MHC-I could attract thymocytes to local sources of CCR7 ligands in the cortex (Ref. 22 and F. E. Lati, T. Schwickert, T. Chatanova, H. Aaron, N. Killeen, and E. A. Riley, manuscript in preparation) where they may receive additional signals reinforcing their CD8 fate. Future studies comparing the migration patterns, cell interactions, and signaling events during the selection of MHC-I- and MHC-II-specific thymocytes should shed light on the relationship between thymocyte migration and the development of CD4- and CD8-lineage T cells.

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Disclosures
The authors have no financial conflict of interest.

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