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Formation of the Intrathymic Dendritic Cell Pool Requires CCL21-Mediated Recruitment of CCR7 + Progenitors to the Thymus

Emilie J. Cosway,* Izumi Ohigashi,† Karin Schauble,‡ Sonia M. Parnell,* William E. Jenkinson,* Sanjiv Luther,‡ Yousuke Takahama, † and Graham Anderson*

During αβ T cell development in the thymus, migration of newly selected CD4+ and CD8+ thymocytes into medullary areas enables tolerance mechanisms to purge the newly selected αβ TCR repertoire of autoreactive specificities. Thymic dendritic cells (DC) play key roles in this process and consist of three distinct subsets that differ in their developmental origins. Thus, plasmacytoid DC and Sirpα+ conventional DC type 1 (cDC1) are known to arrive intrathymically from immature progenitors, the precise nature of such thymus-colonizing progenitors and the mechanisms controlling their thymus entry are unclear. In this article, we report a selective reduction in thymic cDC1 in mice lacking the chemokine receptor CCR7. In addition, we show that the thymus contains a CD11c+MHC class II+Sirpα+ cDC progenitor population that expresses CCR7, and that migration of these cells to the thymus is impaired in Ccr7−/− mice. Moreover, thymic cDC1 defects in Ccr7−/− mice are mirrored in plt/plt mice, with further analysis of mice individually lacking the CCR7 ligands CCL21Ser (Ccl21a−/−) or CCL19 (Ccl19−/−) demonstrating an essential role for CCR7-CCL21Ser during intrathymic cDC1 development. Collectively, our data support a mechanism in which CCR7-CCL21Ser interactions guide the migration of cDC progenitors to the thymus for correct formation of the intrathymic cDC1 pool. The Journal of Immunology, 2018, 201: 000-000.

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Abbreviations used in this article: cDC1, conventional DC type 1; cDC2, conventional DC type 2; DC, dendritic cell; MHC II, MHC class II; mTEC, medullary thymic epithelial cell; pDC, plasmacytoid DC; pre-cDC, cDC-committed progenitor; Treg, T regulatory; WT, wild type; YG, yellow/green.

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impaired in thymocyte deletion (25). Moreover, ligands for both CCR2 (CCL2) and CCR9 (CCL25) are expressed by thymic stromal cells (26–28), highlighting the importance of thymic microenvironments in the control of thymic DC. Although these studies are important, as they explain how pDC and cDC2 are able to contribute to the intrathymic DC pool, the mechanisms that control intrathymic Sirps−/− DC1 (20) are less clear. Indeed, although several studies have studied DC potential within thymic cells (21, 22, 29, 30), direct examination of the mechanisms regulating thymic cDC1 has been hindered by limitations in the identification of cDC-committed progenitors in the thymus. In contrast, stages of cDC development in peripheral lymphoid tissues are well defined (20, 31), and cDC-committed progenitors (pre-cDC) have been identified at multiple sites, including spleen and bone marrow (32–34). For example, in cell transfer experiments analyzing splenic DC development, pre-cDC with a Lin−CD11c+MHC II−Flt3−Sirpα−/low phenotype were shown to selectively give rise to cDC progeny but not pDC or monocytes (32). Importantly, however, although such pre-cDC have been identified in peripheral tissues, their presence in the thymus has not been examined. Consequently, mechanisms regulating the entry of DC progenitors into the thymus, and the possible requirement for particular chemokine receptors in this process, have not been addressed.

In this study, we have examined development of the intrathymic DC pool in the adult mouse thymus. We find that the thymus contains a population of Lin−CD11c+MHC II+Flt3−Sirpα−/low pre-cDC that expresses the chemokine receptor CCR7. In adult Ccr7−/− mice, we show that a selective reduction in cDC1 correlates with a reduction in thymic pre-cDC, with short-term in vivo homing assays indicating a reduced ability of Ccr7−/− pre-cDC to enter the thymus. Finally, by analyzing mice lacking expression of individual CCR7 ligands, we demonstrate a selective reduction in thymic pre-cDC and DC1 in CCL21Ser-deficient (Ccl19ser−/−) but not CCL19-deficient (Ccl19+/−) mice. Collectively, our study demonstrates a mechanism in which CCR7 regulates thymic DC1 development by controlling the intrathymic availability of pre-cDC via its ligand CCL21Ser.

Materials and Methods

Mice

Wild type (WT) C57BL/6 (CD45.2+), BoyJ (CD45.1+), WT C57BL/6 CD45.1−CD45.2+ plp/pol (35), and Ccr7−/− (36) mice were housed at the University of Birmingham Biomedical Services Unit. All experimental procedures were approved by the Birmingham Animal Welfare and Ethical Review Body and performed in accordance with U.K. Home Office regulations. CCL19-deficient Ccl19−/− mice (37) were housed at the University of Lausanne, Switzerland, and CCL21Ser-deficient Ccl12α−/− mice (38) were housed at The University of Tokushima, Japan. All mice were used at 8–12 wk of age.

Abs and flow cytometry

For analysis of DC and pre-cDC, thymus and spleen samples were digested using collagenase D (Roche) and DNase I (Roche). Analysis of pre-cDC was also performed on bone marrow preparations flushed from isolated femurs and tibias. Cell suspensions were stained with Abs to the following: CD11c (N418), PDCA-1 (129C1), Sirpα (P84), CD45.1 (A20), CD45.2 (30-F11), CCR7 (4B12), MHC II (M5/114.15.2), and Flt3 (A2F10). Analysis of DC and pre-cDC was performed after electronic gating on lineage− (Lin−) cells using FITC-conjugated Abs to the following: CD3 (145-2C11), CD19 (eBioID3), NK1.1 (PK136), TER119 (TER119), and B220 (RA3-6B2).

Mixed bone marrow chimera generation

Bone marrow samples from the femurs and tibias of CD45.1+ WT, CD45.2+ WT, or CD45.2−Ccr7−/− mice were T-depleted using an anti-CD3 PE Ab and Anti-PE MicroBeads (Miltenyi Biotec). WT WT and WT Ccr7−/− cells were then mixed at a 50:50 ratio, and a total of 5 × 106 T-depleted cells was i.v. injected into CD45.1+CD45.2+ WT host mice that had previously been lethally irradiated (two split doses of 500 rad). Mice were sacrificed after 8 wk, and tissues were harvested for flow cytometry.

Tracking DC migration in vivo using fluorescent microbeads

Short-term tracking of DC migration in vivo was performed exactly as described (25). In brief, 200 µl of yellow/green (YG) fluorescent (505/515) carboxylate-modified microspheres (FluoSpheres, 0.2 µm diameter; Invitrogen) were i.v. injected into adult WT or Ccr7−/− mice. Forty-eight hours postinjection, thymus and spleen tissues were isolated, and bead-labeled DC subsets and pre-cDC were analyzed by flow cytometry.

Proliferation analysis using BrdU

BrdU incorporation was used to detect proliferation of cDC. A total of 1.5 mg BrdU was injected i.p. into mice, which were sacrificed 18 h later. Thymic cell suspensions were prepared by enzymatic digestion, and cDC1 and cDC2 populations were identified as described above. To reveal BrdU incorporation, cells were permeabilized and stained using the APC BrdU Flow Kit according to the specification (BD Pharmingen).

Results

CCR7 controls intrathymic availability of Sirps−/− cDC1 and their progenitors

Although chemokine receptors are known to play important roles in the recruitment of peripheral cDC2 and pDC to the thymus (24, 25, 39), mechanisms that establish intrathymic cDC1 from immature thymus-colonizing progenitors are less clear. Given that CCR7 and its ligands play an important role in the migration of DC in peripheral lymphoid tissues (40–42), we first examined the intrathymic DC pool in Ccr7−/− mice. Thymus and spleen cell suspensions from adult WT and Ccr7−/− mice were prepared, and Lineage− (Lin−) CD11c+PDCA1− cDC1 were identified by flow cytometry (Fig. 1A). Both the proportion and absolute number of intrathymic cDC were significantly reduced in Ccr7−/− mice (Fig. 1A–C). Further subdivision of total thymic cDC using Sirpα to identify Sirps−/− cDC1 and Sirpα− cDC2 revealed that there was a significant reduction in cDC1 numbers (Fig. 1B, 1C). Importantly, splenic cDC1 proportions and numbers were comparable in WT and Ccr7−/− mice (Fig. 1D, 1E), arguing against a systemic loss of these cells in the absence of CCR7. Interestingly, cDC2 numbers were comparable in the thymus of WT and Ccr7−/− mice (Fig. 1C), indicating that the mechanisms controlling cDC2 entry to the thymus are not limited by CCR7 deficiency. In contrast, the selective cDC1 reduction in the thymus of Ccr7−/− mice suggests that CCR7 is required for the thymic entry of these cells or their progenitors. In support of this, analysis of intrathymic DC populations following in vivo BrdU administration demonstrated comparable proportions of BrdU+ cDC1 in both WT and Ccr7−/− thymus (Fig. 2A, 2B), indicating that reduced thymic cDC numbers in Ccr7−/− mice are not due to reduced cell proliferation.

Although pre-cDC have been defined in peripheral lymphoid tissues (32–34), the precise nature of corresponding DC progenitors in thymus is still not fully clear. For example, the presence of Lin−CD11c+ MHC II+Flt3−Sirpα−/low pre-cDC (32) in the thymus has not been studied, and the relationship between these cells and other thymic DC progenitors described in additional studies is not fully clear (21, 22, 43). Interestingly, we found that Lin−CD11c+MHC II+Flt3−Sirpα−/low pre-cDC were readily detectable in the thymus of adult WT mice (Fig. 3A), albeit at a lower frequency compared with both spleen and bone marrow (Fig. 3B). To see whether the reduction in intrathymic cDC1 in Ccr7−/− mice correlated with alterations in the frequency of pre-cDC, we first used flow cytometric analysis and anti-CCR7 Abs to examine CCR7 expression on thymic DC subsets. In agreement with earlier reports (21, 44, 45), we found that thymic cDC1 and cDC2 both expressed CCR7 (data not shown). Interestingly, pre-cDC in the
thymus were also CCR7+ with higher CCR7 levels detectable on thymic CCR7+ pre-cDC, suggesting a role for CCR7 in the recruitment of these cells to the thymus. Furthermore, that pre-cDC are primary to these alterations in medulla size, we generated bone marrow chimeras using mixtures of CD45.1+ WT and CD45.2+ Ccr7−/− progenitors, in which WT haematopoietic cells restore thymic medulla architecture (6). As controls, we established similar chimeras using mixtures of congenically marked CD45.1+ WT and CD45.2+ WT bone marrow, and all cells were transferred into CD45.1+CD45.2+ lethally irradiated hosts to allow identification of transferred WT and Ccr7−/− mice (Fig. 4A). Mice were harvested after 8 wk, and anti-CD45.1/anti-CD45.2 Abs were used to examine chimerism within thymic cDC and pre-cDC populations. As expected, the contribution of each donor to total thymus cellularity was comparable in both WT:WT and WT:Ccr7−/− chimeras (Fig. 4B). Moreover, WT and Ccr7−/− bone marrow showed comparable contributions to intrathymic cDC2 in WT: Ccr7−/− chimeras (Fig. 4C). In contrast, we saw a significant decrease in the proportion of cDC1 generated from Ccr7−/− bone marrow in WT:Ccr7−/− chimeras (Fig. 4D). Moreover, this reduction in intrathymic cDC1 generated from Ccr7−/− marrow was accompanied by a significant reduction in the proportion of Ccr7−/−/− derived pre-cDC (Fig. 4E). Thus, reductions in cDC1 and pre-cDC in unmanipulated Ccr7−/− mice still occur in the presence of WT counterparts, indicating these effects are not secondary to medulla disorganization in Ccr7−/− mice.

To directly examine the recruitment of pre-cDC to the thymus in the steady-state, we adopted a short-term homing assay used previously to examine pDC entry to the thymus, in which migratory DC are labeled by uptake of fluorescent microbeads (25). Thus, WT mice were i.v. injected with YG-labeled microbeads, and splenic and thymic DC populations were analyzed for YG labeling 2 d postinjection. As expected following i.v. transfer into WT mice, YG+ cells were clearly detectable within all cDC1, cDC2, and pre-cDC populations in the spleen (Fig. 5A, 5C). Interestingly, we saw differential labeling of DC populations in the thymus (Fig. 5B, 5C). Thus, YG+ cells were labeled by uptake of fluorescent microbeads (25). Thus, WT thymus were also CCR7+, with higher CCR7 levels detectable on thymic pre-cDC, as compared with bone marrow pre-cDC (Fig. 3C). In addition, although pre-cDC numbers were comparable in the bone marrow of WT and Ccr7−/− mice (Fig. 3D), we saw a significant reduction in pre-cDC in the thymus of Ccr7−/− mice (Fig. 3D). Thus, our findings indicate that the selective loss of cDC1 in the thymus of Ccr7−/− mice is accompanied by a reduction in numbers of intrathymic CCR7+ pre-cDC, suggesting a role for CCR7 in the recruitment of these cells to the thymus. Furthermore, that pre-cDC are present at normal frequency in bone marrow also indicates that their reduction in the thymus is not likely due to limited availability caused by alterations in pre-cDC development at extrathymic sites.

**Thymic recruitment of pre-cDC is impaired in Ccr7−/− mice**

CCR7 plays an important role in the migration of newly selected CD4+ and CD8+ thymocytes into the thymus medulla (6), and the absence of CCR7 or its ligands results in disrupted medulla organization and small medullary areas (5, 46). To examine whether the defects in cDC1 and pre-cDC in Ccr7−/− mice are secondary to these alterations in medulla size, we generated bone marrow chimeras using mixtures of CD45.1+ WT and CD45.2+ Ccr7−/− progenitors, in which WT haematopoietic cells restore thymic medulla architecture (6). As controls, we established similar chimeras using mixtures of congenically marked CD45.1+ WT and CD45.2+ WT bone marrow, and all cells were transferred into CD45.1+CD45.2+ lethally irradiated hosts to allow identification of transferred WT and Ccr7−/− mice (Fig. 4A). Mice were harvested after 8 wk, and anti-CD45.1/anti-CD45.2 Abs were used to examine chimerism within thymic cDC and pre-cDC populations. As expected, the contribution of each donor to total thymus cellularity was comparable in both WT:WT and WT:Ccr7−/− chimeras (Fig. 4B). Moreover, WT and Ccr7−/− bone marrow showed comparable contributions to intrathymic cDC2 in WT: Ccr7−/− chimeras (Fig. 4C). In contrast, we saw a significant decrease in the proportion of cDC1 generated from Ccr7−/− bone marrow in WT:Ccr7−/− chimeras (Fig. 4D). Moreover, this reduction in intrathymic cDC1 generated from Ccr7−/− marrow was accompanied by a significant reduction in the proportion of Ccr7−/−/− derived pre-cDC (Fig. 4E). Thus, reductions in cDC1 and pre-cDC in unmanipulated Ccr7−/− mice still occur in the presence of WT counterparts, indicating these effects are not secondary to medulla disorganization in Ccr7−/− mice.

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**FIGURE 1.** Selective reduction in intrathymic Sirpα− cDC1 in Ccr7−/− mice. (A) Representative flow cytometric plots showing the gating strategy used to identify thymic DC. Lin− refers to the exclusion of NK1.1, CD19+, CD3−, and TER119− and B220-expressing cells. Total cDC were identified as Lin−CD11c+PDCA1−, which were then subdivided further to identify Sirpα− cDC1 and Sirpα+ cDC2. Data shown are typical of at least three separate experiments. Analysis of the proportions (B) and absolute numbers (C) of DC subsets in the thymus of WT (black bars) and Ccr7−/− (open bars) mice. Data in (B)-(E) are from at least three separate experiments, with at least three mice per group. Error bars represent the SEM using an unpaired Student two-tailed t test. *p < 0.05, ***p < 0.001, ****p < 0.0001.
perhaps because of the blood–thymus barrier (47). Importantly, analysis of intrathymic pre-cDC in the same mice showed that ∼10% of these cells were YG+, indicating their migration to the thymus from peripheral sites (Fig. 5A, 5C). Next, when we compared YG-labeled DC populations in tissues from WT and Ccr7<sup>2/2</sup> mice after i.v. microbead transfer, we saw a significant reduction in the numbers of both YG+ pre-cDC and cDC1 in the thymus of Ccr7<sup>2/2</sup> mice (Fig. 5D). This was not due to differential cell labeling between strains, as no differences in the numbers of YG+ pre-cDC and cDC1 were seen in the spleens of WT and Ccr7<sup>2/2</sup> mice (Fig. 5E). Moreover, and consistent with unaltered cDC2 numbers in Ccr7<sup>2/2</sup> mice (Fig. 1), numbers of YG+ cDC2 in the thymus of WT and Ccr7<sup>2/2</sup> mice were comparable (Fig. 5D). Thus, by tracking the steady-state migration of DC subsets using short-term in vivo homing assays, our findings indicate that Lin<sup>2</sup>CD11c<sup>+</sup>MHC II<sup>2</sup>Flt3<sup>+</sup>Sirp<sub>a</sub>low pre-cDC enter the thymus from the periphery and that this process is reduced in the absence of CCR7.

CCL21, but not CCL19, controls intrathymic DC pool formation

CCL19, CCL21Ser, and CCL21Leu represent the three known functional chemokine ligands for CCR7 (48). As the genes encoding both CCL19 (Ccl19) and CCL21Ser (Ccl21a) are expressed in multiple thymic stromal cell types (49–51), we next investigated whether the requirement for CCR7 in intrathymic cDC1 development mapped to specific chemokine ligand requirements. Initially, we examined intrathymic DC in plt/plt mice, in which expression of both Ccl19 and Ccl21a is absent (35). Thus, freshly isolated thymus tissue from adult WT and plt/plt mice was enzymatically digested, and intrathymic Sirp<sub>a</sub>2 cDC1 and Sirp<sub>a</sub>+ cDC2 DC subsets were identified by flow cytometry. Consistent with the requirement for CCR7 and the phenotype of Ccr7<sup>2/2</sup> mice, plt/plt mice showed a reduction in the absolute numbers of total thymic cDC and a selective reduction in the proportion and numbers of cDC1 (Fig. 6A–C). In addition, we also saw a significant reduction in the absolute numbers of pre-cDC in plt/plt mice (Fig. 6C). Thus, for intrathymic DC populations, plt/plt mice essentially mirror the effects seen in Ccr7<sup>2/2</sup> mice. Next, to examine the requirement for individual CCR7 ligands in thymic DC development, we examined Ccl19<sup>2/2</sup> and Ccl21a<sup>2/2</sup> mice that individually lack expression of the CCR7 ligands CCL19 or CCL21Ser. Interestingly, no alterations in the numbers and proportions of cDC1, cDC2, and pre-cDC were seen in the thymus of Ccl19<sup>−/−</sup> mice (Fig. 6A, 6B, 6D). In contrast, analysis of Ccl21a<sup>−/−</sup> mice...
mice showed alterations in thymic DC populations. In particular, we saw a reduction in the number of total cDC (Fig. 6E) that was caused by a specific reduction in both the proportion and number of cDC1 (Fig. 6A, 6B, 6E). Moreover, pre-DC were also reduced in the thymuses of Ccl21a^{-/-} mice (Fig. 6E). Thus, analysis of mice that lack CCR7 ligands either individually or in combination indicates that although CCL19 is dispensable, CCL21Ser plays an essential role in controlling the intrathymic development of cDC1.

Discussion
In the thymus medulla, interactions between mTEC, DC, and newly selected thymocytes are essential for both negative selection and Foxp3^{+} Treg generation, which represent key aspects of T cell tolerance. Although thymic DC are known to be heterogeneous, the mechanisms that control formation of the intrathymic DC pool from its constituent components of peripherally derived pDC and cDC2 and intrathymically produced cDC1 are unclear. Given that chemokine receptors play important roles in the thymic recruitment of pDC and cDC2 (24, 25), we investigated their potential role in the development of cDC1 in the thymus. In particular, given the role of CCR7 in both thymocyte migration and DC migration in peripheral lymphoid tissues, we examined the role of this chemokine receptor and its ligands during development of the intrathymic DC pool.

In this article, we show that the thymus of Ccr7^{-/-} mice has a selective defect in the frequency of cDC1. Mixed bone marrow chimeras show this defect maps to CCR7 expression by haemopoietic cells and is not an indirect consequence of the medullary disorganization seen in these mice. Furthermore, we show that a pre-cDC subset, previously described only in peripheral lymphoid tissues (32), is present in the thymus and expresses CCR7. Moreover, such pre-cDC are reduced in the thymus of Ccr7^{-/-} mice, with in vivo migration assays indicating this deficiency is caused by their reduced capacity to enter the thymus. Thus, the contribution of cDC1 to the intrathymic DC pool occurs via a mechanism involving CCR7-mediated recruitment of pre-cDC. This requirement for CCR7 by cDC1 draws parallels with the respective requirements of pDC for CCR9 and cDC2 for CCR2.
maturation of their cDC1 progeny. This scenario is perhaps similar to the multiple roles played by CCR7 during conventional cδT cell development in the adult thymus that include lymphoid progenitor colonization and cortex-to-medulla migration of positively selected thymocytes (52–55). In addition, that the reduction in pre-cDC in Ccr7<sup>−/−</sup> mice does not result in increased compensatory proliferation in either these cells or their cDC1 progeny (data not shown) may also indicate a limited availability of intrathymic growth factors for DC and/or their progenitors.

Our findings are also significant in relation to the intrathymic developmental potential of pre-cDC defined by a Lin<sup>−</sup>CD11c<sup>+</sup>MHC II<sup>+</sup>Ftlt3<sup>−</sup>Sirp<sub>plt</sub>phenotype. For example, when such pre-cDC were isolated from bone marrow and transferred i.v., both cDC1 and cDC2 progeny were detectable in the spleen of recipient mice (32). Thus, our finding that the reduction in thymic pre-cDC in Ccr7<sup>−/−</sup> mice results in a selective deficiency in cDC1, but not cDC2, appears at odds with their capacity to act as common progenitors for cDC. One possible explanation is that as cDC2 can enter the thymus from the periphery as already mature cells, these cells then occupy a finite number of appropriate niches (43). This may then limit the intrathymic generation of cDC2 from colonizing pre-cDC, which may result in their intrathymic skewing toward cDC1 development. Alternatively, pre-cDC that enter the thymus may represent a particular subset of these cells that may already be biased toward cDC1 development. Further comparative analysis of DC progenitors in thymus and peripheral lymphoid tissues may help in discriminating these possibilities. It is also interesting to note that although cDC1 and cDC2 both develop independently of mTEC, these cells then occupy a finite number of appropriate niches, that of which may already be biased toward cDC1 development.

In addition, by analyzing the chemokine ligand requirements of thymic DC, we show that CCL21Ser is both essential and sufficient for CCR7-mediated control of thymic cDC1 and their progenitors. Interestingly, that thymic DC require CCL21Ser but not CCL19 may be similar to the requirements of DC in lymph nodes, where DC homeostasis and function were reported to be unaffected in Ccl19<sup>−/−</sup> mice (56). It is also worthy to note that in the thymus, CCL21Ser expression has recently been shown to map specifically to mTEC (38), which also control the intrathymic positioning of cDC1 via their expression of XCL1 (57). Taken together, such findings emphasize the importance of mTEC in the regulation of thymic DC and highlight roles for multiple chemokines in both the recruitment (CCL21Ser) and intrathymic positioning (XCL1) processes that take place during thymic cDC1 development. Interestingly, however, although mTEC expression of CCL21 is controlled by LTβR signaling (50), absence of LTβR expression by TEC does not perturb thymic DC numbers (58). Thus, additional receptors expressed by the thymic epithelium may also trigger CCL21 expression to regulate intrathymic cDC. Finally, the paucity of thymic cDC1 in Ccr7<sup>−/−</sup> and Ccl21a<sup>−/−</sup> mice described in this article may also be important in explaining the importance of CCR7 and its ligands in central tolerance. Indeed, CCL21Ser, but not CCL19, has recently been shown to be important for T cell tolerance in the thymus, where it controls medulla entry of positively selected thymocytes (38). Taken together, these findings indicate that CCR7–CCL21Ser interactions may be important for central tolerance in two separate ways: regulation of

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**FIGURE 6.** A CCR7-CCL21Ser axis regulates intrathymic cDC1 and their progenitors. (A) is a representative example of flow cytometric analysis of adult thymus preparations from the indicated mouse strains to show Sirp<sub>α</sub><sup>+</sup> expression in pre-gated Lin<sup>−</sup>CD11<sup>+</sup>PDCA1<sup>−</sup> total cDC. (B) Quantitative analysis of the proportions of Sirp<sub>α</sub><sup>−</sup>cDC1 in plt/plt, Ccl21a<sup>−/−</sup>, and Ccl21a<sup>−/−</sup> mice (black bars), compared with control mice (open bars). (C–E) show numbers of total cDC, Sirp<sub>α</sub><sup>−</sup>cDC1, Sirp<sub>α</sub><sup>−</sup>cDC2, and Lin<sup>−</sup>CD11c<sup>−</sup>MHC II<sup>+</sup>Ftlt3<sup>−</sup>Sirp<sub>α</sub>pre-cDC in adult thymus preparations from plt/plt (C), white bars], Ccl21a<sup>−/−</sup> [(D), open bars], and Ccl21a<sup>−/−</sup> mice [(E), open bars], compared with controls (black bars). All analysis was obtained from a minimum of nine mice per strain across at least three independent experiments. Error bars represent the SEM using an unpaired Student two-tailed t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
thymocyte cortex-to-medulla migration and the regulation of thymic cDC1 availability. In conclusion, our study shows that CCR7 determines cDC1 development in the thymus via a mechanism involving its ligand CCL21Ser and the recruitment of CCR7-expressing pre-cDC. These findings highlight the importance of multiple chemokine receptors in controlling the makeup of the intrathymic DC pool and demonstrate further the key influence of CCR7 on thymus function.

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Disclosures

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References

1. Takada, K., K. Kondo, and Y. Takahama. 2017. Generation of peptides that promote positive selection in the thymus. J. Immunol. 198: 2215–2222.
2. Klein, L., B. Kyewski, M. Allen, and K. A. Hergquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don’t see). Nat. Rev. Immunol. 14: 377–391.
3. Boehm, T., and J. B. Swann. 2014. Origin and evolution of adaptive immunity. Annu. Rev. Anim. Sci. 2: 259–283.
4. Hu, Z., J. N. Lancaster, C. Sasiponganan, and L. I. Ehrlich. 2015. CCR4 promotes medullary entry and thymocyte-dendritic cell interactions required for central tolerance. J. Exp. Med. 212: 1947–1965.
5. Cowan, J. E., N. I. McCarthy, S. P. Parnell, A. J. White, A. Bacon, A. Serge, M. Irela, P. J. Lane, E. J. Jenkins, W. E. Jenkinson, and G. Anderson. 2014. Differential requirement for CCR4 and CCR7 during the development of innate and adaptive oT cells in the adult thymus. J. Immunol. 193: 1204–1212.
6. Ueno, T., F. Saito, D. H. Gray, S. Kuse, K. Hishima, H. Nakano, T. Kakiuchi, M. Lipp, R. Long, and Y. Takahama. 2004. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. [Published erratum appears in 2004 J. Exp. Med. 200: following 496]. J. Exp. Med. 200: 493–505.
7. Koble, C., and B. Kyewski. 2009. The thymic medulla: a unique microenvironment for interfascicular self-antigen transfer. J. Exp. Med. 206: 1505–1513.
8. Abrahmson, J., and Y. Goldfarb. 2015. AIRE: from promiscuous molecular partnerships to promiscuous gene expression. Eur. J. Immunol. 46: 22–33.
9. Tai, X., B. Erman, A. Alag, J. Mu, M. Kimura, G. Katz, R. J. Steptoe, S. H. Naik, M. H. Lahoud, Y. Liu, P. Zheng, et al. 2008. Dendritic cells correspond to myeloid-type double-negative 1c cells. Immunity 32: 259–283.
10. Link, A., T. K. Vogt, S. Favre, M. R. Britschgi, H. Acha-Orbea, B. Hinz, and B. Hinz. 2009. Mice lacking expression of secondary lymphoid organ chemokine receptor 7-deficient mice with altered T cell migration under inflammatory and steady-state conditions. J. Exp. Med. 208: 1039–1049.
11. Proietto, A. I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D’Amico, K. Nutsch, Z. Yang, J. I. Gordon, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. J. Exp. Med. 190: 45–53.
12. Malchow, S., D. S. Leventhal, V. Lee, S. Nishii, N. D. Socci, and P. A. Savage. 2016. Aire enforces immune tolerance by directing autoreactive T cells into the regulatory T cell lineage. Immunity 44: 1102–1113.
13. Derbinski, J., and B. Kyewski. 2010. How thymic antigen presenting cells sample the body’s self-antigens. Curr. Opin. Immunol. 22: 592–600.
14. Abraham, J., and G. Anderson. 2017. Thymic epithelial cells. Annu. Rev. Immunol. 35: 85–118.
15. Gallegos, A. M., and M. J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J. Exp. Med. 200: 1039–1049.
16. Perry, J. S. A., C. L. Ilo, A. L. Kau, K. Nutsch, Z. Yang, J. I. Gordon, K. M. Murphy, and C. S. Hsieh. 2014. Distinctive compartments of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus. Immunity 41: 414–426.
17. Proietto, A. I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D’Amico, R. J. Steptoe, H. S. Naik, M. H. Lahoud, Y. Liu, P. Zheng, et al. 2008. Dendritic cells correspond to myeloid-type double-negative 1c cells. Immunity 32: 259–283.
18. Li, J., J. Park, D. Foss, and I. Goldschneider. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. J. Exp. Med. 206: 607–622.
19. Wu, L., and K. Shortman. 2005. Heterogeneity of thymic dendritic cells. Semin. Immunol. 17: 304–312.
immunogenic dendritic cell maturation in thymus and periphery. *Immunity* 45: 305–318.

46. Kurobe, H., C. Liu, T. Ueno, F. Saito, I. Ohigashi, N. Seach, R. Arakaki, Y. Hayashi, T. Kitagawa, M. Lipp, et al. 2006. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* 24: 165–177.

47. Ribatti, D. 2015. The discovery of the blood-thymus barrier. *Immunol. Lett.* 168: 325–328.

48. Förster, R., A. C. Duval-Misslitz, and A. Rot. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* 8: 362–371.

49. Ueno, T., K. Hará, M. S. Willis, M. A. Malin, U. E. Höpken, D. H. Gray, K. Matsushima, M. Lipp, T. A. Springer, R. L. Boyd, et al. 2002. Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity* 16: 205–218.

50. Lkhagvasuren, E., M. Sakata, I. Ohigashi, and Y. Takahama. 2013. Lymphotoxin β receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells. *J. Immunol.* 190: 5110–5117.

51. Lucas, B., K. D. James, E. J. Cosway, S. M. Parnell, A. V. Tumanov, C. F. Ware, W. E. Jenkinson, and G. Anderson. 2016. Lymphotoxin β receptor controls T cell progenitor entry to the thymus. *J. Immunol.* 197: 2665–2672.

52. Misslitz, A., O. Pabst, G. Hintzen, L. Ohl, E. Kremmer, H. T. Petrie, and R. Förster. 2004. Thymic T cell development and progenitor localization depend on CCR7. *J. Exp. Med.* 200: 481–491.

53. Nitta, T., S. Nitta, Y. Lei, M. Lipp, and Y. Takahama. 2009. CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proc. Natl. Acad. Sci. USA* 106: 17129–17133.

54. Zlotoff, D. A., A. Sambandam, T. D. Logan, J. J. Bell, B. A. Schwarz, and A. Bhardwaj. 2010. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* 115: 1897–1905.

55. Krueger, A., S. Willenzon, M. Lyszkwicz, E. Kremmer, and R. Förster. 2010. CC chemokine receptor 7 and 9 double-deficient hematopoietic progenitors are severely impaired in seeding the adult thymus. *Blood* 115: 1906–1912.

56. Britschgi, M. R., S. Favre, and S. A. Luther. 2010. CCL21 is sufficient to mediate DC migration, maturation and function in the absence of CCL19. *Eur. J. Immunol.* 40: 1266–1271.

57. Lei, Y., A. M. Ripen, N. Ishimaru, I. Ohigashi, T. Nagasawa, L. T. Jeker, M. R. Bisol, G. A. Holländer, Y. Hayashi, R. W. Malefyt, et al. 2011. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *J. Exp. Med.* 208: 383–394.

58. Cosway, E. J., B. Lucas, K. D. James, S. M. Parnell, M. Carvalho-Gaspar, A. J. White, A. V. Tumanov, W. E. Jenkinson, and G. Anderson. 2017. Redefining thymus medulla specialization for central tolerance. *J. Exp. Med.* 214: 3183–3195.