Trafficking on serpentines: molecular insight on how maturating T cells find their winding paths in the thymus

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Summary: Maintenance of the peripheral T-cell pool throughout the life requires uninterrupted generation of T cells. The majority of peripheral T cells are generated in the thymus. However, the thymus does not contain hematopoietic progenitors with unlimited self-renewing potential, and continuous production of T cells requires importation of such progenitors from the bone marrow into the thymus. Thymus-homing progenitors enter the thymus and subsequently migrate throughout distinct intrathymic microenvironments while differentiating into mature T cells. At each step of this scheduled journey, developing thymocytes interact intimately with the local stroma, which allow them to proceed to the next stage of their differentiation and maturation program. Undoubtedly, thymocyte/stroma interactions are instrumental for both thymocytes and stroma, because only their ongoing interplay generates and maintains a fully operational thymus, able to guarantee unimpaired T-cell supply. Therefore, proper T-cell generation intrinsically involves polarized cell migration during both adult life and embryogenesis when the thymus primordium develops into a functional thymus. The molecular mechanisms controlling cell migration during thymus development and postnatal T-cell differentiation are beginning to be defined. This review focuses on recent data regarding the role of cell migration in both colonization of the fetal thymus and T-cell development during postnatal life in mice.

Introduction

The thymus is a primary lymphoid organ crucially involved in the development of T cells (1). The unique capacity of the thymus to efficiently promote T-cell differentiation and repertoire selection is mediated by thymic epithelial cells (TECs), which are the major constituents of the thymic stroma. The thymic stroma itself is organized into two main compartments, the cortex and the medulla, each of which is composed of several distinct subsets of TECs. Recent studies indicate that different subtypes of TECs generate discrete microenvironments inside the thymus, which support the progressive differentiation of T-cell progenitors into mature T lymphocytes.
In accordance with this view, it has been shown that the orchestrated migration of developing thymocytes through distinct epithelial microenvironments is essential for proper T-cell development (3, 4). The present literature indicates that interactions between developing thymocytes and stromal cells, which occur during migration, are also required for the generation and maintenance of intact epithelial compartments (2). Together, these observations implicate an essential role for cell migration in the regulation of intrathymic T-cell homeostasis. Because intrathymic progenitors have limited self-renewing potential, the migration of hematopoietic progenitors from the bone marrow into the thymus is also an essential requirement for uninterrupted T-cell generation throughout postnatal life. Finally, newly generated T cells need to emigrate from the thymus into the peripheral circulation. Therefore, polarized cell migration into, within, and out of the thymus are crucial events regulating both intrathymic T-cell generation and peripheral T-cell homeostasis throughout adult life. Besides its essential role during postnatal lymphopoiesis, cell migration is also crucially involved in the development of the thymus itself as well as in the colonization of the thymic primordium during embryogenesis.

Despite the crucial role of cell migration in thymus development and function, the molecular mechanisms coordinating these movements are not completely understood. This review focuses on recent data regarding the role of cell migration in the colonization of the fetal thymus as well as in T-cell development during postnatal life in mice. Other aspects of thymus biology such as thymus organogenesis and thymus degeneration have been reviewed recently by others (5, 6) and are not covered here.

Colonization of the fetal thymus

The thymic primordium develops from the third pharyngeal pouch endoderm around embryonic day 11 (E11) (5). Its subsequent colonization by lymphoid progenitors occurs in two waves peaking between E11 and E13 and between E18 and E21 (7). Lymphoid commitment precedes colonization of the thymic primordium and clusters of CD45^+ c-kit^+ CD34^+ progenitor cells are already present in the aorta-gonad-mesonephros (AGM) region at E10 (8, 9). AGM-derived progenitors are subsequently found in the circulation and fetal liver at E11 to E12, coinciding with the time of thymic rudiment colonization, suggesting that the first lymphoid progenitors that seed both the fetal liver and the thymic primordium are generated in the AGM region (10). At E11, the thymic primordium consists of two to three layers of epithelial cells, which are surrounded by a mesenchymal layer. In contrast, at E12, the epithelium develops further into a three dimensionally arranged cell cluster (11). The first wave of colonization of the thymic primordium by hematopoietic progenitors precedes the onset of intrathymic vascularization and includes two distinct migratory steps. In a first step, starting at E11, basophilic cells accumulate in the jugular vein, in perithymic capillaries, and in the mesenchyme surrounding the thymic rudiment (10, 11). In a second step, starting at E12, progenitor cells enter the epithelial cluster itself and proliferate (11). Hematopoietic progenitors are still observed in the mesenchyme surrounding the thymic primordium at E13 but not at E14, suggesting that the first colonization wave finishes between E13 and E14 (12). The second wave of thymic primordium colonization occurs between E18 and E21 (7).

Progenitors colonizing the thymic rudiment during this period are probably generated in the bone marrow, because precursor cells appear in the murine femoral marrow at E17 (13). Interestingly, there is some evidence that the fetal liver might be the source of progenitors colonizing the bone marrow and that the chemokine CXCL12 (SDF-1) and its receptor CXCR4 are involved in this process (14). Despite the well-known migratory kinetics of progenitors into the thymic primordium, some of the published data were generated using E14.4 to E15.5 embryos. Unfortunately, this time interval neither correlates to the first nor to the second migration wave. However, because the first wave appears to finish between E13 and E14, we consider these data as being more relevant for the second colonization wave.

It has been suggested that expression of chemokines by stromal cells is responsible for the attraction of fetal progenitors into the thymic rudiment (15–17). According to this idea, the mRNA expression of CCL21 (SLC), CCL25 (TECK), and CXCL12 has been detected in the thymic primordium of E12.5 embryos by in situ hybridization (15) (Table 1). More recently, CCL21 expression has also been identified in the thymic primordium of E11.5 embryos by immunohistology (17) (Table 1). In accordance with the early expression of CCL21, mice of the spontaneous mutant strain plt/plt (paucity of lymph node T cells), which lack expression of both CCL19 and of the lymphoid form of CCL21, CCL21-ser, showed reduced numbers of thymocytes at E13.5 but not at day 17.5 or after birth (17). Comparable results have also been obtained for CCR7-deficient mice, which lack expression of the receptor for CCL19 and CCL21 (17). Together, the data suggest that CCL21 plays an essential role in the migration of progenitors during the first but not during the second colonization wave. Furthermore, the very early expression of CCL21 protein...
indicates the participation of this chemokine in the first migratory step occurring during the first colonization wave, which is the recruitment of progenitors into the mesenchymal layers surrounding the epithelial core. In contrast to CCL21, expression of CCL25 protein was first detected in the thymic primordium of E12.5 embryos (17). Because the CCL25 protein is not present at E11.5, it is unlikely that this chemokine is involved in the migration of progenitors into the mesenchyme surrounding the thymic primordium. Expression of CXCL12 protein has not been investigated at this early stage; nevertheless, in situ hybridization data (15) indicate its involvement in the regulation of cell migration during the first colonization wave. Similar to CCL25, CXCL12 is probably not involved in the first migratory step because of the fact that comparable numbers of hematopoietic progenitors are found in the mesenchyme surrounding the thymic rudiment of wildtype and CXCL12-deficient E11.5 embryos (14).

The functional importance of chemokine expression during the first colonization wave is undoubtedly illustrated by the failure of lymphoid progenitors to seed the thymic rudiment of nude mice, which lack expression of CCL25 and CXCL12 (15, 16). The nude phenotype is caused by a recessive mutation in the gene for the transcription factor Foxn1 (forkhead box N1). Absence of functional Foxn1 protein results in hairlessness and, more importantly, the development of a rudimentary thymus with lack of T-cell differentiation (18). In these mice, the initial thymic development (up to E11) proceeds normally (19). Its subsequent development is impaired, however, coinciding with the time when seeding of the thymic primordium by lymphoid progenitors occurs (16). Even though hematopoietic progenitors arrive in the mesenchymal layer surrounding the thymic rudiment of nude mice, they fail to invade subsequently the epithelial cluster (15, 16). These observations indicate that the first step of progenitor colonization occurs normally. In contrast, the second step involving migration of progenitors into the epithelial cluster seems to be disturbed. The incapacity of progenitors to migrate into the epithelial cluster leads to an accumulation of these cells in regions located caudally to the parathyroid primordium and cranially in the parathyroid primordium itself (11, 15). Correlating with the misplacing of progenitors in the thymic and parathyroid primordia, expression for CCL25 and CXCL12 is absent in the thymus of E12.5 nude mice embryos. In contrast, expression of CCL21 mRNA is not impaired (15). Together, the results indicate that CCL25 or CXCL12 (or both) but not CCL21 is essentially involved in the migration of progenitors from the mesenchyme into the epithelial cluster of the thymic primordium. It is of note that, although the generation of TECs is not disturbed in nude mice, their subsequent differentiation is strongly impaired, resulting in the formation of an aberrant rudimentary thymus (5). It is conceivable that the lack of interactions between developing thymocytes and TECs make an important contribution to the thymic phenotype observed in nude mice. Therefore, unimpaired migration of progenitors into the thymic primordium is essential for the development of a fully functional thymus.

Besides chemokines, there is also evidence of the involvement of diverse adhesion molecules in regulating the migration during the first colonization wave. At the time of entry into the epithelial cluster (E12), immigrating progenitors express α4 integrin (12). Additionally, the expression of the integrins α4, α5, α6, and of CD44 has been detected in fetal liver progenitors isolated from C57BL/6 E12 embryos (20). Kawakami and colleagues (20) also demonstrated that α4 integrin is necessary for the adhesion of fetal liver progenitors to the surface of fetal thymic lobes in culture and that interactions between CD44 and its major ligand, hyaluronate, are essential for the migration of the adhered progenitors into the lobes. In agreement with these data, antibodies against α4 integrin have been shown to inhibit the development of thymocytes from E13 liver-derived progenitors in fetal thymic organ cultures (FTOC) (21).
Little is known about the mechanisms coordinating migration during the second colonization wave. Nevertheless, mRNA expression for the chemokines CCL21, CCL25, and CXCL12 has been detected in the thymic primordium of E15.5 embryos by in situ hybridization (15) (Table 1). Consistent with the chemokine expression pattern found in the E15.5 thymic primordium, fetal prothymocytes (Thy1+ c-kitlow) and uncommitted (Thy1− c-kit+) progenitors isolated from the blood of embryos at the same developmental stage migrate in response to CCL21 and CXCL12 in in vitro chemotaxis assays. Under the same experimental conditions, both kinds of blood progenitors respond with much less efficacy to CCL21 (15, 16). More recently, the mRNA expression profiles of all 36 chemokines identified so far in the mouse genome were analyzed in thymus lobes of E14.5 embryos, as well as in isolated E14.5 EPCAM+/I-A+ TECs (17). This analysis confirmed earlier data obtained by in situ hybridization (15) and revealed mRNA expression of additional nine chemokines including CCL19 (Table 1), which had only been detected in the thymus of newborn mice in prior studies. Using an in vitro time lapsed visualization approach, Liu and colleagues (17) also demonstrated that fetal thymus lobes attract progenitors isolated from the blood and liver of E14.5 embryos. Interestingly, this attraction can be inhibited by the addition of pertussis toxin to the culture, indicating the participation of G-protein-coupled receptors, presumably chemokine receptors, in this process. In support of this idea is the finding that addition of neutralizing antibodies against CCL21 and CCL25 inhibited the immigration of fetal liver progenitors into the cultivated thymic lobes. The effect of CXCL12 inhibition on the migration of liver-derived progenitors has not been addressed in this work, but because the immigration of E14.5 intrathymic progenitors into fetal thymic lobes in culture was not affected by the addition of anti-CXCL12 antibodies, the authors of this study suggested that CXCL12 is not involved in fetal thymus colonization (17). This conclusion requires further experimental confirmation, because the chemotactic behavior of E14.5 intrathymic progenitors might not necessarily reflect that of progenitors derived from E18 blood or bone marrow, which will actually seed the thymus during embryogenesis.

The aforementioned studies have identified chemokines and adhesion molecules that are likely to be involved in the process of thymus colonization. So far, however, it is difficult to discern the relevance of each of these molecules in the colonization process, because they are at least in part simultaneously expressed in the thymic primordium. Therefore, it seems possible that the lack of a given chemokine might be compensated for by the expression of others. A further obstacle in understanding the importance of the investigated molecules is because of the variety of fetal progenitor populations used in the different studies, hampering useful comparisons among the published data. In general, the use of fetal intrathymic progenitors is probably less adequate for this kind of study, because these progenitors would express chemokines and adhesion molecules necessary for intrathymic migration rather than for thymic colonization.

**Cell migration during postnatal thymopoiesis**

T-cell development in the postnatal thymus is characterized by periodic recruitment of bone marrow-derived progenitors into the thymus (22). Progenitors enter the thymus at the corticomedullary junction (CMJ) and undergo progressive differentiation through several distinct developmental stages. Interestingly, differentiation occurs concomitantly with the directional migration of developing thymocytes through distinct microenvironments within the thymus, indicating that differentiation-inducing signals are provided by distinct intrathymic microenvironments (23). Recently immigrated progenitors lack CD4 and CD8 expression and are referred to as double-negative (DN) cells. Based on the differential expression of the surface molecules CD25 and CD44, the DN subset is further subdivided in four differentiation stages (DN1–4). Differentiation to the DN1 stage (CD25CD44high) occurs in proximity to the site of thymic entry, whereas the consecutive differentiation of stages DN2 (CD25CD44high) and DN3 (CD25+CD44low) occur while cells migrate from this region into the mid and outer cortex, respectively. DN3 cells accumulate in the subcapsular zone (SCZ), where they differentiate to DN4 (CD25−CD44+). In the same thymic region, DN4 cells differentiate subsequently into double-positive (DP) cells (CD4+CD8+). Transition to the DP stage is accompanied by a reversion of the migration polarity, which finally guides the DP thymocytes across the cortex toward the medulla, while only positively selected cells will actually enter the medulla. After successfully passing through negative selection in the medulla, fully mature CD4+ or CD8+ single-positive (SP) cells egress from the thymus into the peripheral circulation (Fig. 1). Although proper T-cell development is highly dependent on migratory events like the recruitment of bone marrow-derived precursors into the thymus and the orchestrated migration of developing thymocytes throughout discrete intrathymic microenvironments, the mechanisms guiding these migratory events remain largely unknown. Only recent advances made in the field provide
important insights into the mechanisms controlling cell migration during postnatal thymopoiesis. These new findings are summarized in the next sections.

Recruitment of blood progenitors into the thymus

Intrathymic progenitors have a very limited self-renewing capacity. Therefore, the continuous generation of mature T cells in the postnatal thymus requires recruitment of blood-circulating, bone marrow-derived progenitors into the thymus (22). Progenitors enter the thymus through postcapillary venules located in the vicinity of the CMJ (23, 24). It has been shown recently that their recruitment is a gated event characterized by brief periods of receptivity and longer periods of refractivity (25). Therefore, it is assumed that the periodic influx of progenitors is temporally regulated and depends on interactions of circulating blood cells with the endothelium of the postcapillary venules. Although only little is known about the molecular mechanisms regulating migration of blood-circulating progenitors into the thymus, some prerequisites necessary for this process can be deduced based on knowledge about extravasation of leukocytes in other tissues. Leukocyte extravasation involves at least three distinct steps: rolling, firm adhesion, and transendothelial migration. Rolling involves loose attachment of leukocytes to endothelial cells, mainly through interactions between selectins expressed on the surface of circulating leukocytes and their ligands expressed on the endothelium. Firm adhesion involves the attachment of rolling lymphocytes to ligands expressed by endothelial cells. This step requires mainly interactions between integrins expressed on the surface of the rolling leukocytes and their counter-receptors, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), expressed by the endothelium. Interestingly, cytokine and chemokine-mediated signals trigger a high-affinity binding stage of integrins thereby strengthening adhesion to the endothelium (26). Transendothelial migration involves the directed movement of the adhered leukocytes through the endothelium. This intricate process consists of various coordinated cellular actions involving repeated adhesion and detachment from endothelial surfaces, cytoskeletal rearrangements, proteolysis of the basement membranes, and re-adhesion of the extravasating leukocytes to the extracellular matrix (27).

Fig. 1. Cell migration during postnatal T-cell differentiation. Bone marrow-derived progenitors enter the thymus through postcapillary venules at the corticomedullary junction (CMJ) and differentiate in this region to DN1 cells. DN1 cells migrate outwards of the perimedullary cortex and differentiate into DN2 cells. A further differentiation stage intermediate between DN1 and DN2, called DN1-2 stage, has been recently identified. Yet it is not clear whether part of the pluripotent DN1 population is able to differentiate directly to DN2 cells. DN3 cells differentiate from DN2 cells in the mid and outer cortex and accumulate in the SCZ, where they undergo proliferation and differentiate to DN4 cells. Transition to the DP stage correlates with reversion of migration direction back into the cortex toward the medulla. During their migration throughout the cortex, double-positive (DP) cells are subjected to positive selection and differentiate to SP cells. Positively selected single-positive (SP) cells migrate across the CMJ and enter the medulla, where they undergo functional maturation. Finally, mature SP cells leave the thymus probably through postcapillary venules and enter the peripheral circulation.

An earlier study identified CD44 as a molecule involved in the recruitment of circulating progenitors into the thymus, because blocking of this molecule with specific antibodies reduces homing of progenitors into the thymus (28) (Table 2). Another molecule implicated in the migration of progenitors into the thymus is Vanin-1, a glycosylphosphatidylinositol-linked molecule. Antibodies against this protein inhibit the re-population of the thymus from sublethally irradiated mice (29) (Table 2). Expression of Vanin-1 has been detected in radially aligned capillaries throughout the cortex and, more importantly, in large vessels located in the medulla and in the vicinity of the CMJ. However, Vanin-1 is not expressed on the luminal side of
<table>
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<td>CD44</td>
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<td>Reduction of thymic homing</td>
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<td>Vanin-1</td>
<td>In vivo blocking of Vanin-1 by i.v. injection of specific antibodies</td>
<td>Decrease of thymic re-population after sublethal irradiation</td>
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<td>P-selectin</td>
<td>Analysis of P-selectin and C2-deficient mice as well as analysis of thymus chimerism in parabiotic and competitively reconstituted mice</td>
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<td>CXCR4</td>
<td>Analysis of wildtype mice competitively reconstituted with adult bone marrow of lck(Cre)/CXCR4loxPloxP mice</td>
<td>Developmental arrest at the DN1 stage associated to lack of migration of DN1 cells into the middle cortex</td>
<td>(3)</td>
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<td>CCR7</td>
<td>Analysis of CCR7-deficient and plt/plt mice as well as from wildtype mice reconstituted with adult bone marrow of CCR7-deficient animals</td>
<td>Decreased numbers of thymocytes, altered thymic morphology, partial developmental arrest at the DN1-2 stage associated with accumulation of DN1-2 cells in areas adjacent to the CMJ, accumulation of mature SP cells in the cortex and reduced egress of mature thymocytes in the periphery of newborn mice</td>
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<td>CCR9</td>
<td>Analysis of CCR9-deficient mice</td>
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<td>Analysis of laminin 2-deficient mice (dy/dy mice)</td>
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<td>S1P</td>
<td>In vivo blocking of S1P by injection of S1P receptors agonist</td>
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<td>S1P1</td>
<td>Analysis of T-cell-specific S1P1-deficient mice(lck(Cre)/S1P1loxPloxP mice) and from mice reconstituted with E12.5 fetal liver progenitors of S1P1-deficient embryos</td>
<td>Reduction of thymocyte egress from the thymus in the periphery</td>
<td>(65, 66)</td>
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CMJ, corticomedullary junction; DN, double-negative; DP, double-positive; SP, single-positive.
the vessels but rather in vascular and perivascular zones (29). Vanin-1 belongs to a family of proteins that are thought to be involved in transendothelial migration (27). Together, these observations indicate that Vanin-1 is involved in the transmigration of recruited progenitors through the endothelium. In search of vascular ligands which could regulate the recruitment of circulating progenitors into the thymus, Lepique and colleagues (30) have shown that the molecules CD34, MEC79, VCAM-1, and VAP-1 (vascular adhesion protein 1) are all expressed in thymic blood vessels. However, the expression of these molecules is not restricted to the endothelium of the postcapillary venules. Furthermore, none of these endothelial ligands is differentially expressed in periods of receptivity and refractivity to circulating progenitors (30, 31). A recent study identified P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) as molecules involved in the regulation of progenitor recruitment in the adult thymus (32). While P-selectin is expressed on thymic vessels, P-selectin ligands are expressed in circulating thymic progenitors. Accordingly, homing efficiency of progenitors is strongly reduced in the absence of either P-selectin or PSGL-1 expression (32) (Table 2). As mentioned, the immigration of progenitors into the thymus is a gated event that occurs periodically. This engraftment correlates with the availability of unoccupied stromal niches within the thymus (25, 33). An important insight provided by Rossi and colleagues (32) was the observation that stromal niche occupancy modulates the expression of P-selectin. Therefore, P-selectin is the first molecule identified that fulfills the aforementioned requirements for recruitment of circulating cells into the thymus. It is expressed on postcapillary venules, allowing interactions between endothelium and circulating progenitors, and, more importantly, its expression is temporally regulated according to the availability of the thymus to engraft new progenitors from the bloodstream.

Cell migration inside the thymus

T-cell development in the adult thymus occurs in three distinct phases: lymphopoiesis, T-cell receptor (TCR)-mediated selection, and functional maturation. Lymphopoiesis refers to the early phases of T-cell development including lineage commitment and proliferation (23). TCR-mediated selection refers to positive selection in the cortex as well as negative selection in the medulla, while functional maturation refers to the maturation of thymocytes that were not negatively selected in the medulla before their export from the thymus. Each of these developmental events occurs while differentiating cells migrate throughout the thymus (23, 34) (Fig. 1). This highly coordinated migration allows immature thymocytes to receive differentiation signals transmitted by different sets of epithelial cells that are located in distinct thymic microenvironmental niches. Generally, such polarized migration requires the presence of a stable adhesive matrix, which provides a substrate for migration, as well as directional signals, which polarization the direction of movement. A stable adhesive matrix for migration can be provided by secreted extracellular matrix proteins such as fibronectin and laminin or by cells expressing counter-receptors for adhesion molecules on their surface (35). Directional signals may be provided by chemokines, which are the canonical molecules involved in polarization of cell migration. The expression of chemokines as well as several molecules involved in cell matrix or cell–cell interactions has been detected in the murine thymus (Table 3), and developing thymocytes were shown to express several chemokine receptors and integrins.

Table 3. Expression and localization of cell migration related molecules within the postnatal thymus

<table>
<thead>
<tr>
<th>Chemokine/adhesion molecule</th>
<th>Thymic localization</th>
<th>Receptor/integrin expressed by developing thymocytes</th>
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<td>CCL17 (TARC)</td>
<td>Medulla</td>
<td>CCR4</td>
<td>(15, 37)</td>
</tr>
<tr>
<td>CCL19 (ELC)</td>
<td>Blood vessels, medulla, CMJ and adjacent areas and cells scattered throughout the cortex</td>
<td>CCR7</td>
<td>(4, 61)</td>
</tr>
<tr>
<td>CCL21 (SLC)</td>
<td>Medulla, CMJ and adjacent areas and cells scattered throughout the cortex</td>
<td>CCR7</td>
<td>(4, 61)</td>
</tr>
<tr>
<td>CCL22 (MDC)</td>
<td>Medulla</td>
<td>CCR4</td>
<td>(15)</td>
</tr>
<tr>
<td>CCL25 (TECK)</td>
<td>Abundantly expressed in the cortex and medulla</td>
<td>CCR9</td>
<td>(4, 46)</td>
</tr>
<tr>
<td>CXCL12 (SDF-1)</td>
<td>Medulla, CMJ, few cortical cells and capsule</td>
<td>CCR4</td>
<td>(4)</td>
</tr>
<tr>
<td>Collagen type I and IV</td>
<td>Cortex, medulla, CMJ, perivascular spaces, and capsule</td>
<td>α5β1</td>
<td>(40)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Cortex, medulla, CMJ, perivascular spaces, and capsule</td>
<td>α4β1, α4β7, α5β1</td>
<td>(40–42)</td>
</tr>
<tr>
<td>Laminin (not specified)</td>
<td>Cortex</td>
<td>α6β1, α6β4</td>
<td>(40, 42)</td>
</tr>
<tr>
<td>Laminin 5</td>
<td>SCZ</td>
<td>α6β4</td>
<td>(44)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Cortex</td>
<td>α4β1, α4β7</td>
<td>(35)</td>
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CMJ, corticomedullary junction; SCZ, subcapsular zone.
(Table 3). By modulating the expression of such molecules and depending on their developmental stage thymocytes are capable of redefining their migratory pattern on demand (see below).

Although the contributions of some molecules in coordinating migratory events have started to be elucidated, the real dimension of the network consisting of adhesion molecules, chemokines, and their receptors are not known. Because different chemokines are expressed in identical or overlapping regions of the thymus and because the same chemokine can be expressed in distinct cortical and medullary areas (Fig. 2), it is hard to predict which molecules may be involved in the distinct migratory steps based solely on their histological detection. It is therefore more appropriate to assess the expression pattern of integrins and chemokine receptors on thymocytes of different developmental stages to identify those players that coordinate the migration during the corresponding phase of differentiation. The differential and temporal expression of integrins and chemokine receptors at distinct stages of differentiating T cells and their possible role in cell migration are discussed in detail in the following sections.

**Cell migration during lymphopoiesis**

Thymic homing progenitors enter the thymus through post-capillary venules at the CMJ. The earliest intrathyphic progenitors (DN1 cells) include cells with multilineage lymphoid potential, which can give rise to T, B, and natural killer (NK) cells as well as dendritic cells (DCs). DN1 cells move very little and remain in the vicinity of the site of thymic entry for the first 9–11 days of intrathyphic residence. During this period, they undergo proliferation and lineage commitment, losing their potential to give rise to B and NK cells. Having

![Fig. 2. Localization of chemokine expression in the thymus of adult C57BL/6 mice.](image-url)
completed this program, differentiated cells move asynchronously outwards of the CMJ. This movement correlates with the appearance of DN2 cells, which are still able to give rise to T cells and DCs, in the middle cortex. Two days later, DN3 cells, which are committed to the T-cell lineage (including α/β and γ/δ T cells), appear in the outer cortex. The minimum time to reach the capsule, where the transition to the DP stage occurs, is approximately 13 days (34). Differentiation past the DN1 stage requires active migration across the cortex to the capsule. DN1 cells express low to intermediate levels of α4 integrin, intermediate to high levels of α5 and α6 integrins, and low levels of β1 integrin (35, 36). This pattern indicates the potential expression of α4β1, α5β1, and α6β1 heterodimers, which would bind to VCAM-1, fibronectin, and laminin 1, respectively. DN1 cells express the chemokine receptor CXCR4, which binds CXCL12. In addition, a small part of this population expresses CCR7 and CCR9 (3, 4, 37, 38). Both laminin and fibronectin are present in areas adjacent to the CMJ (Table 3). However, it remains speculative whether these molecules support the migration of those cells leaving the CMJ region. In contrast, an elegant study by Plotkin and colleagues (3) demonstrated that CXCR4 signaling is essentially required for the migration of developing cells into the middle cortex, where they receive signals necessary for their further differentiation (Table 2). Furthermore, cells lacking CXCR4 are unable to differentiate past the DN1 stage, illustrating the importance of intrathymic migration for the continuous generation of T cells during postnatal life (3). We have recently shown that lack of CCR7 also affects the migration of early progenitors toward the outer thymic cortex (4) (Table 2). We found that a CD25intCD44high DN cell population, which most likely represents a transitional stage of differentiation between DN1 and DN2, expresses CCR7. We termed this stage DN1-2. About 50% of the DN1-2 cells express high levels of CCR7. In the absence of CCR7 signaling, an accumulation of DN1-2 cells is observed at the CMJ (Fig. 3). According to this observation, part of the DN1-2 population undergoes developmental arrest at this transitional stage. These results indicate that at least part of the CCR7+ DN1-2 population migrates into the cortex in response to CCL19 or CCL21, and for this particular population, these signals can not be compensated for by other chemokines/chemokine receptors.

Since at this early developmental stage, intrathymic precursors are not committed to the T-cell lineage, it is unclear to what extent DN1-2 cells contribute to the pool of developing T cells. Although the lineage potential of various progenitor populations is discussed controversially in the literature (39), it is largely accepted that T-cell progenitors are c-kit+. Because more than two-thirds of the CCR7+ DN1-2 thymocytes express c-kit (unpublished data), it is likely that at least part of this population gives rise to T cells. Although the developmental block found in CCR7-deficient animals is not
complete, it has a drastic influence on the size and morphology of the adult thymus (Fig. 4).

In contrast to DN1 cells, DN2 cells migrate more actively throughout the cortex. DN2 cells express the integrins α4, α5, α6, β1, β4, and β7 (35). This expression pattern could indicate the expression of α4β1, α4β7, α5β1, α6β1, and α6β4 heterodimers, which would bind to fibronectin, laminin 1 and 5, collagen I and IV, and VCAM-1. All these molecules, with the exception of laminin 5, have been identified throughout the thymic cortex (35, 40–42) (Table 3). Although all molecules found in the middle cortex could provide a matrix for migrating DN2 cells, there is currently only evidence for the participation of VCAM-1 in this process (35). The DN2 population uniformly expresses CXCR4 and low levels of CCR9 (3, 4, 37, 38) and migrates in vitro in response to CXCL12 (3). As previously mentioned, CXCR4 plays a critical role in the migration of DN1 cells, which is essential for their further differentiation. Therefore, CXCR4 signaling is essential for the development of DN2 cells. In contrast, it is not clear whether CXCR4 is guiding the migration of DN2 cells throughout the cortex. Unfortunately, it is not possible to evaluate the in vivo importance of CXCR4/CXCL12 signaling in the migratory behavior of thymocytes beyond the DN1 stage, because lack of CXCR4 leads to a complete developmental block at this very early stage. A role for CCL25/CCR9 signaling in the migration of CD25+ cells, which include the DN2 and DN3 developmental stages, into the SCZ has been demonstrated recently (43). However, because only the expression of CD25 but not that of CD44 has been investigated, the requirement of CCR9 for migration of DN2 cells (CD25+CD44hi) into the middle cortex needs experimental confirmation.

DN3 cells migrate from the middle and outer cortex into the SCZ. Similar to DN2 cells, DN3 cells express the integrins α4, α5, α6, β1, and β4 but not β7 (35). Therefore, DN3 cells are not able to interact with VCAM-1-expressing stromal cells and need another kind of adhesion matrix for their migration. Interestingly, the DN3 population expresses the highest levels of the integrins α6 and β4, suggesting that these cells could express α6β4 heterodimers. The α6β4 heterodimers are capable of interacting with laminin 5, which is known to be expressed in the SCZ. Furthermore, anti-laminin 5 antibodies block T-cell development at the DN2 and DN3 stage in FTOC (44). Another potential heterodimer expressed by DN3 cells is α6β1, which binds to laminin 2. A significant reduction in the size of the thymus and in the total number of thymocytes has been observed in mice deficient for laminin 2, dy/dy mice (Table 2). Analysis of the different cell populations found in the thymus revealed that dy/dy mice have strongly reduced numbers of DP cells. This reduction is due to increased apoptosis of their predecessors, DN3 and DN4 cells, which locate to the SCZ (45). Although it is not clear whether the interactions between these extracellular matrix proteins and developing thymocytes are relevant for the migration process, it is evident that both laminin 2 and 5 are required for normal T-cell development. Like DN2, DN3 cells express the chemokine receptors CXCR4 and CCR9 (3, 4, 37, 38) and migrate in vitro in response to CXCL12 (3). Because expression of this chemokine has also been detected in the thymus capsule (Fig. 2), it seems plausible that CXCL12 directs the movement of DN3 cells toward this region. In addition, CCR9 is involved in the migration of DN3 thymocytes to the SCZ, because CD25+ cells of CCR9-deficient animals are unable to accumulate in this thymic region (43). Interestingly, in accordance with early studies (46), no abnormal T-cell development was observed in CCR9-deficient mice, despite the aberrant location of DN3 cells in the thymic cortex (43) (Table 2).

The DN4 population differentiates to DP cells in the SCZ. DN4 cells express the same α-integrin repertoire as DN3 cells (36). Although the expression of β-integrins on these cells has not been investigated, it is likely that DN4 cells express the same (or at least very similar) pattern of integrins and

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**Fig. 4. Altered thymus architecture in CCR7-deficient mice.** H&E staining from representative sections of adult thymus of wildtype (left) and CCR7-deficient (right) animals. Compared to wildtype thymi, those of CCR7-deficient mice shown numerous small medullary areas distributed through the entire cortical area. Note that medulla is even found in the SCZ (indicated by arrows). Reproduced from The Journal of Experimental Medicine 2004;200:481–491 by copyright permission of The Rockefeller University Press.
chemokine receptors as DN3 cells, because both populations reside in the same thymic region (23). With respect to the expression of chemokine receptors, DN4 cells express homogenously CCR9 and CXCR4, whereas CCR7 expression could be detected only in a minor part of this population (4). DN4 cells also migrate in vitro in response to CXCL12 (3).

Cell migration during TCR-mediated selection and functional maturation

The differentiation of DN cells into DP cells occurs in the SCZ of the cortex. Transition to the DP stage is accompanied by a reversion of the migration polarity, which guides the DP thymocytes across the cortex toward the medulla. During their migration throughout the cortex, DP thymocytes undergo positive selection, and only positively selected thymocytes are able to migrate across the CMJ into the medulla, where functional maturation proceeds. Positive selection requires interactions between DP thymocytes and cortical stromal cells, which expose self-peptide bound to major histocompatibility complex (self-MHC) on their surface (24). Only DP cells, whose TCR binds with low avidity to self-MHC molecules, receive signals for survival and continue maturation. Although cell migration is required to guarantee encounters between stromal cells and DP thymocytes, the mechanisms involved in this process have not yet been elucidated. Consistent with the reversion of migration direction observed after DN to DP transition, DP cells downregulate the expression of the integrin α6β4 (47) whose ligand, laminin 5, is expressed in the SCZ. Therefore, the downregulation of α6β4 integrin by immature DP cells could be necessary to allow their escape from this thymic area. DP cells express uniformly the chemokine receptors CCR9 and CXCR4 and migrate in vitro in response to CCL25 and CXCL12 (4, 44, 46, 48–51). Together, these observations suggest an involvement of CCR9/CCL25 and CXCR4/CXCL12 in the migration of DP cells across the cortex, where they interact with stromal cells and undergo positive selection.

The next migration step encompasses crossing of the CMJ and relocation of positively selected cells in the medulla, where negative selection and functional maturation occurs (31, 52). The relocation of positively selected cells to the medulla seems to be a tightly regulated process, because only already selected cells, not immature DP cells, are able to enter this area. Migration of positively selected cells across the CMJ can be inhibited by pertussis toxin, indicating that this migration step might involve G-protein-coupled receptor-mediated signals (37). In contrast to immature DP cells, mature DP cells that have undergone positive selection downregulate the expression of CXCR4. Additionally, positively selected cells express CCR4 and CCR7 as well as increased levels of CCR9 (48, 53–55). Consistent with the chemokine receptor expression pattern found in this population, the ligands for CCR4 (CCL17 and CCL12) are expressed exclusively in the medulla (15, 37) (Table 3). The ligands for CCR7 (CCL19 and CCL21) and CCR9 (CCL25) are expressed in both cortex and medulla, although the expression of CCL19 and CCL21 seems to be more pronounced in medullary areas (Fig. 2). Additionally, positively selected cells (mature DP and immature CD4+ cells) migrate in vitro very successfully toward CCL19, CCL21, CCL22, and CCL25 but do not respond to CXCL12 (49), supporting the idea that down-regulation of CXCR4 as well as upregulation of CCR4, CCR7, and CCR9 expression direct the migration of positively selected cells from the cortex into the medulla. At least for CCR7, this supposition seems to be correct, because premature expression of CCR7 leads to an aberrant positioning of immature DP cells in the medulla (56). Furthermore, an accumulation of SP cells has been observed in the cortex of mice deficient for CCR7 or its ligands (55).

An intriguing model for cell migration during positive selection and medullar relocation has been recently suggested by H. T. Petrie (31). This model predicts that directional movements of immature DP cells through the cortex occurs passively, as a consequence of the strong proliferation of cells occurring in the SCZ, whereas the migration of positively selected cells across the CMJ into the medulla occurs actively and would be directed by an increased responsiveness of these cells to chemokines and integrin ligands expressed in the medulla. This model is compatible to the observation that the expression of chemokine receptors, whose ligands are expressed in the medulla, is upregulated on positively selected cells. Interestingly, recent data obtained by two photon-imaging analysis of either intact thymic lobes (57) or 400 μm thick thymus slices loaded with DP thymocytes (58) provided further evidence to support this model. Both studies have shown that the migration of immature DP thymocytes from the SCZ toward the medulla occurs as a net result of many random movements rather than in a straightforward fashion. Although this observation does not preclude the participation of chemokines directing this apparently uncoordinated migration, it correlates very well with a passive migration of immature DP thymocytes driven by the displacement of proliferating cells from the SCZ. Remarkably, this migratory pattern changes dramatically during and after positive selection. The motility of DP thymocytes is strongly
reduced during antigen recognition either as a consequence of prolonged productive interactions between DP thymocytes and stromal cells or as a prerequisite for it (58). In contrast, after antigen recognition, positively selected cells migrate directly and rapidly toward the medulla (57), consistent with the coordination of migration by guidance cues such as chemokines and integrins. Although experimental data support the aforementioned model, other kinds of signals, including repulsive signals produced by medullary microenvironments, could prevent the migration of immature DP cells into the medulla (31).

Upon arrival in the medulla, positively selected cells remain in this thymic region for an additional period of 3–7 days (59). During this time, they finish selection and undergo functional maturation. It is very likely that functional development of different T-cell subsets present in the periphery of adult mice (T-helper cells, regulatory T cells, cytotoxic T cells, etc.) occurs in distinct yet undiscovered medullary microenvironments. In this case, migration of maturing T cells within the medulla itself would be important for their proper maturation. Although chemokines and integrins could be involved in positioning of maturing T cells within the medulla, this possibility has not been addressed experimentally.

Egress of mature T cells from the thymus

Emigration of newly differentiated T cells from the thymus into the circulation is essential for the maintenance of the peripheral T-cell pool. The first molecular insight into the mechanisms involved in thymic export came from the observation that thymocytes expressing the catalytic subunit of pertussis toxin are unable to leave the thymus (60), implicating the involvement of G-protein-coupled receptors in the export of newly generated T cells to the periphery. On the basis of this observation, it was originally suggested that chemokine-mediated signals might regulate this process. In accordance with this idea, expression of CCL19 has been identified on endothelial vessels within the medulla (Fig. 2), and a role for CCL19 in the export of newly generated T cells from the neonatal thymus has been suggested (61). However, as discussed by H. T. Petrie (31), chemokine signals can only guide migration up to the chemokine sources themselves, indicating that the migration of mature thymocytes from the thymus into the bloodstream requires further signals in addition to CCL19 (31). In pioneering work, Yagi and colleagues (62) demonstrated that long-term administration of the sphingosin-1-phosphate (S1P) receptor agonist FTY720 inhibits the emigration of mature thymocytes from the thymus to the periphery (Table 2), indicating the participation of this G-protein-coupled receptor family in thymocyte egress. This result has been confirmed by short-term experiments showing a more than 95% inhibition of thymocyte egress within 2 h after administration of another S1P receptor agonist (63) (Table 2). Together, these studies clearly implicate the involvement of S1P receptors in the migration of mature thymocytes to the periphery. More recently, two groups demonstrated independently that the receptor S1P1 is essentially required for the egress of mature thymocytes into the peripheral blood (64, 65) (Table 2). Because S1P is present at concentrations of 100–300 nM in the plasma (66), S1P receptors are probably mediating the migration of mature thymocytes from the thymus into the bloodstream, whereas CCL19 is likely to guide the positioning of mature thymocytes near to the blood vessels where they exit the thymus as proposed earlier (31).

Concluding remarks

Generation of T cells in the thymus is essential for the maintenance of the peripheral T-cell pool and unimpaired T-cell-mediated immune functions. A plethora of evidence suggests that orchestrated cell migration is crucially required for proper thymic development and postnatal T-cell differentiation. The molecular mechanisms governing directed cell migration in thymus development and function, however, are complex and comprise distinct kinds of interactions, like those between chemokines and their receptors or adhesion molecules and integrins. Although recent studies provided important insights in the field (3, 4, 15, 17, 32, 43, 55, 61, 64, 65), several aspects remain to be clarified. Only few molecules are known to be involved in the colonization of the thymic primordium. The identification of signals involved in this essential migratory process is complicated by the fact that the thymus is colonized in two migratory waves by progenitors of distinct origins (10). In general, the most frequently used approaches to investigate thymic colonization are in vitro migration to chemokines and in vitro migration of fetal progenitors into alymphatic fetal thymic lobes. However, under these experimental conditions, diverse kinds of fetal progenitors, regardless of their origin, are able to migrate in response to the same chemokines as well as into the thymic lobes. Therefore, it is necessary to define and use progenitor populations that reflect the in vivo situation. The analysis of the expression of chemokine receptors and integrins on these populations could give valuable insights in the elucidation of
molecules guiding the migration of progenitors during the two distinct colonization waves.

More is known about the molecules directing migratory events during postnatal T-cell development (Tables 2 and 3). Interestingly, mice deficient for CCR7 are still able to generate sufficient amounts of T cells despite of the essential role of CCR7 in the positioning of early progenitors within the cortex and mature thymocytes in the medulla (4, 55). This observation suggests that CCR7-mediated signals can be compensated for by other molecules. Similar results are observed in mice deficient for other chemokine receptors and migration-related molecules (Table 2), indicating that combinatorial signals of many molecules are controlling cell migration during postnatal T-cell development. Therefore, it is hard to evaluate the individual participation of single molecules in this complex process. The identification of molecules involved in the control of cell migration during adult thymopoiesis is hampered further by the plasticity of the developing T cells. Unless an early developmental block provoked by the absence of a given molecule is complete, a reduced number of cells will be able to overcome this block and pass to the next developmental stage. The reduced number of cells able to continue differentiation may be compensated for in later stages by a more vigorous proliferation replenishing the final pool of cells to an apparently normal level. This renders the analysis of molecules possibly required for cell migration during early developmental stages particularly tedious when using mice that are deficient for one gene only. Here, the study of mice deficient for two or more candidate molecules may prove to be of greater promise.

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