Molecular mechanisms governing thymocyte migration: combined role of chemokines and extracellular matrix

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Abstract: Cell migration is crucial for thymocyte differentiation, and the cellular interactions involved now begin to be unraveled, with chemokines, extracellular matrix (ECM) proteins, and their corresponding receptors being relevant in such oriented movement of thymocytes. This notion derives from in vitro, ex vivo, and in vivo experimental data, including those obtained in genetically engineered and spontaneous mutant mice. Thymic microenvironmental cells produce both groups of molecules, whereas developing thymocytes express chemokine and ECM receptors. It is important that although chemokines and ECM proteins can drive thymocyte migration per se, a combined role of these molecules likely concurs for the resulting migration patterns of thymocytes in their various differentiation stages. In this respect, among ECM moieties, there are proteins with opposing functions, such as laminin or fibronectin versus galectin-3, which promote, respectively, adhesion and de-adhesion of thymocytes to the thymic microenvironment. How chemokines and ECM are produced and degraded remains to be more clearly defined. Nevertheless, matrix metalloproteinases (MMPs) likely play a role in the intrathymic ECM breakdown. It is interesting that these molecules also degrade chemokines. Thus, the physiological migration of thymocytes should be conceived as a resulting vector of multiple, simultaneous, or sequential stimuli, involving chemokines, adhesive, and de-adhesive ECM proteins. Moreover, these interactions may be physiologically regulated in situ by matrix MMPs and are influenced by hormones. Accordingly, one can predict that pathological changes in any of these loops may result in abnormal thymocyte migration. This actually occurs in the murine infection by the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease. In this model, the abnormal release of immature thymocytes to peripheral lymphoid organs is correlated with the higher migratory response to ECM and chemokines. Lastly, the fine dissection of the mechanisms governing thymocyte migration will provide new clues for designing therapeutic strategies targeting developing T cells. The most important function of the thymus is to generate T lymphocytes, which once leaving the organ, are able to colonize specific regions of peripheral lymphoid organs, the T cell zones, where they can mount and regulate cell-mediated, immune responses. This intrathymic T cell differentiation is a complex sequence of biological events, comprising cell proliferation, differential membrane protein expression, gene rearrangements, massive programmed cell death, and cell migration. In this review, we will focus on the mechanisms involved in controlling the migration of thymocytes, from the entrance of cell precursors into the organ to the exit of mature T cells toward peripheral lymphoid organs. Nevertheless, to better comprehend this issue, it appeared worthwhile to briefly comment on some key aspects of thymocyte differentiation and the tissue context in which it takes place, the thymic microenvironment. *J. Leukoc. Biol.* 75: 951–961; 2004.

Key Words: *thymocyte migration · integrins · galectins · thymic epithelial cells · thymic nurse cells*

INTRATHYMIC T CELL DIFFERENTIATION AND THE THYMIC MICROENVIRONMENT

As thymocyte development comprises sequential gene expression and rearrangements of the T cell receptor (TCR) genes, we can trace the evolution of the process by evaluating the appearance and disappearance of a variety of membrane proteins. For example, the most immature thymocytes do not express the TCR complex nor the accessory molecules CD4 or CD8, being called double-negative thymocytes and corresponding to 5% of total thymic lymphocytes. This stage can be further subdivided by the differential expression of the CD25 and CD44 markers. As maturation progresses, developing cells acquire the CD4

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and CD8 markers, generating the $CD4^+CD8^+$ double-positive cells, roughly comprising 80% of the whole population. In this stage, TCR genes are fully rearranged, and productive rearrangements yield the membrane expression of the TCR in low densities (TCR^{low}). Thymocytes that do not achieve such a productive TCR gene rearrangement die by apoptosis, whereas those expressing productive TCR will be exposed to endogenous peptides presented by molecules of the major histocompatibility complex (MHC), expressed on microenvironmental cells. This interaction will determine the positive and negative selection events, crucial for normal thymopoiesis. Negative selection determines cell death by apoptosis, whereas the relatively few positively selected thymocytes down-regulate CD4 or CD8 and progress to the mature $TCR^{high}CD4^+CD8^-$ (10%) or $TCR^{high}CD4^-CD8^+$ (5%) single-positive stage. These cells will ultimately leave the organ to form the large majority of the peripheral T cell repertoire (reviewed in ref. [1]).

As mentioned above, one key interaction between developing thymocytes and microenvironmental cells involves the TCR/peptide–MHC in the context of CD4 or CD8 molecules. The thymic microenvironment comprises distinct cell types, including thymic epithelial cells (TEC), dendritic cells (DC), macrophages, and fibroblasts. Moreover, such tridimensional, cellular organization is intermingled with an extracellular matrix (ECM)-containing network [1, 2].

It has been shown that the positive selection is driven by TEC, whereas negative selection is mainly secondary to interactions involving DC with thymocytes. Although the major function of thymic macrophages seems to be the phagocytosis of apoptotic thymocytes, it has been suggested that fibroblasts play a role in the $TCR^-CD4^-CD8^- \rightarrow TCR^+CD4^+CD8^+$ progression [1].

Microenvironmental cells also modulate thymocyte differentiation by soluble polypeptides. They express cytokines, such as interleukin-1 (IL-1), IL-3, IL-6, IL-7, IL-8, and stem cell factor; chemokines, as for example, CXC chemokine ligand (CXCL)12 and CXCL10, CCL25, previously named stromal cell-derived factor-1α (SDF-1α), interferon (IFN)-inducible protein-10 (IP-10), and thymus-expressed chemokine (TECK); and thymic hormones, including thymulin, thymopoietin, and thymosin-α1 [2, 3]. Moreover, thymocytes can be influenced by the thymic microenvironment via ECM-mediated interactions [4, 5].

Among microenvironmental cell types, TEC correspond to the major component. They are seen throughout the thymic lobules in the cortex and medulla (see **Fig. 1**), where they likely form special niches, which may be involved in specific interactions with developing thymocytes. One of these niches, located in the outer cortex of the thymic lobules, is the thymic nurse cell (TNC). This is a lymphoepithelial complex in which one single epithelial cell can harbor a variable number of thymocytes [6]. Most intra-TNC lymphocytes bear the $CD4^+CD8^+$ double-positive phenotype, although immature, double-negative as well as mature, single-positive cells can be found. Once settled in culture, TNC spontaneously release thymocytes, and TNC-derived epithelial cells, once cocultured with fetal thymocytes, can reconstitute lymphoepithelial complexes [7]. As further developed below, TNC can be placed as an in vitro model of thymocyte migration within the TEC context.

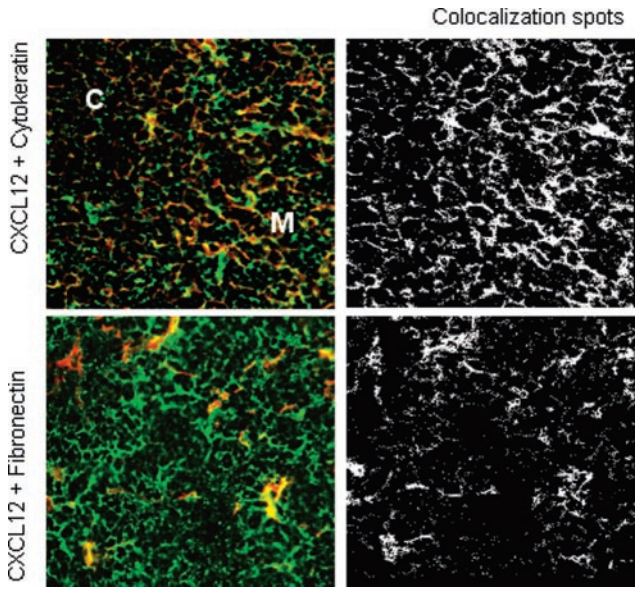


Fig. 1. Distribution of CXCL12 in the mouse thymus. In the left upper panel, CXCL12 (in green) is seen in the cortex (C) and medulla (M) of the thymic lobule. This section was double-stained with an anti-cytokeratin antibody and shows that CXCL12 is largely colocalized with TEC (stained in red). Colocalization is seen in yellow in this panel, and the computer-generated colocalization spots are seen in the upper right panel. The bottom left panel reveals a double-labeling for detection of CXCL12 (green) and fibronectin (red). We can also see in the bottom right panel numerous spots of colocalization. Original magnification, ×400.

An important issue in thymopoiesis is that maturation of thymocytes occurs as they migrate and encounter the thymic microenvironment. Accordingly, $TCR^-CD4^-CD8^-$ and $TCR^+CD4^+CD8^+$ are cortically located, whereas mature $TCR^+CD4^+CD8^-$ and $TCR^+CD4^-CD8^+$ cells are found in the medulla. As discussed in this review, chemokines and ECM are involved in driving the thymocyte journey throughout the organ.

STUDYING CELL MIGRATION IN THE THYMUS

As summarized above, thymocyte migration can be subdivided in the following segments: entrance of bone marrow-derived precursors into the organ, traffic of immature thymocytes within the cortex and from the cortex to the medulla, and exit of the thymus. A variety of in vitro, ex vivo, and in vivo strategies is now available to approach migration in these three segments.

The entrance of T cell precursors into the organ can be evaluated in vitro by means of deoxyguanosine-treated fetal thymus organ cultures (FTOC) [8]. These are cultures of the thymic microenvironment devoid of hematopoietic-derived cells, where we can seed T cell precursors to study their entrance into the microenvironmental compartment. Yet, it should be remembered that fetal thymus development differs from adult thymopoiesis. As such, the molecular mechanisms directing the process may differ between the fetus and the

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postnatal animal, thus restricting the concepts issued from the FTOC model.

T cell precursors can also be trapped in vivo after injection of hematopoietic stem cells into irradiated mice and tracing their engraftment into a thymus that has been depleted of autologous lymphoid cells by irradiation. Similar data can be obtained after injecting cells into severe combined immunodeficiency or recombination activating gene knockout (KO) mice, which do not develop thymocytes. In any of these models, we can use specific blockers that interfere with a given interaction. Using one of these protocols, the amount of cells that penetrates the organ can be traced by cytofluorometry, whereas their localization within the thymic lobules can be ascertained by immunohistochemistry. For instance, such a strategy recently allowed the demonstration that the earliest T cell precursors enter the mouse thymus intermittently with receptive and refractory periods [9]. Once gaining the organ, they remain in a particular microenvironmental niche localized in the cortex [10].

Migration of immature thymocytes can be evaluated in vitro by studying TNC. These unique, lymphoepithelial structures correspond to a unique niche that can be isolated ex vivo. Most of the intra-TNC thymocytes bear the immature $CD4^+CD8^+$, and intra-TNC lymphocyte proliferation and death have been reported (reviewed in ref. [11]). When settled in culture, these lymphoepithelial complexes spontaneously release thymocytes, so that after a shot-term culture, we can count the percentages of lymphocyte-free epithelial cells versus lymphocyte-containing structures. In a second vein, TNC-derived epithelial cells can reconstitute lymphoepithelial complexes after being cocultured with fetal thymocytes. Thus, the manipulation of TNC corresponds to an in vitro model of thymocyte migration within the TEC context.

As cell migration within a tridimensional cellular network implies sequential and tandem events of adhesion and deadhesion on the substrate, thymocyte adhesion onto TEC or any other microenvironmental component (including cells and ECM) is another tool to approach cell migration-related events of thymocyte daily life. Once adhered, thymocytes can be recovered, counted, and phenotyped [12].

Ex vivo migration of immature as well as mature thymocytes can be studied in the so-called *transwell* chambers. In these largely used devices, we can put cells in an upper chamber to migrate, whereas the migration stimuli (molecules or cells) are placed in the bottom chambers or in an intermediate filter [13]. Again, the total numbers of migrating cells that reach the bottom chamber can be counted and then phenotyped by flow cytometry.

Exit of mature thymocytes can also be evaluated ex vivo by organ chemotaxis. This system is similar to that described above, but instead of thymocytes, we place the entire thymic lobule in the upper chamber. Lastly, we can study in vivo the recent thymic emigrants by the original technique of intrathymic injection of fluorescein isothiocyanate (FITC) followed by tracing $FITC^+$ cells in peripheral lymphoid organs [14] or by the detection of TCR excision circles, which remain in the cells after the rearrangement of the TCR genes [15].

CHEMOKINE-MEDIATED INTERACTIONS IN THE THYMUS

Chemokines have been described as essential chemoattractant molecules involved in thymocyte migration and development, being expressed in defined areas of the organ and attracting distinct subpopulations of thymocytes in various maturation stages [3]. In accordance with the expression of chemokines by the thymic microenvironment, thymocytes differentially express the corresponding chemokine receptors. The main chemokine expressed in the thymus, CXCL12 (previously named SDF-1α), is secreted by TEC, particularly in the subcapsular and medullary regions (see Fig. 1), and preferentially attracts immature $CD4^-CD8^-$ and $CD4^+CD8^+$ cells [16]. Accordingly, the corresponding specific receptor CXCR4 is mainly expressed in these stages of thymocyte development (**Fig. 2**) and seems to interact with the CXCL12, even guiding immature cells, first to the subcapsular and then to the cortical-medullary regions as well as the entry of single-positive cells to the medulla [13].

Despite data showing the expression of CXCL12 and CXCR4 in the thymus, together with the chemotactic activity exerted by this chemokine on thymocytes, previous data indicated that $CXCL12^{-/-}$ as well as $CXCR4^{-/-}$ mice do not exhibit major changes in thymocyte development, and migration of $CXCR4^{-/-}$ mouse-derived fetal liver precursors into the thymus from lethally irradiated mice occurs normally [17, 18]. Nonetheless, more recent data showed impaired expansion of fetal T cell precursors in the thymus from $CXCL12^{-/-}$ and $CXCR4^{-/-}$ mice, and CXCR4 seems to affect the expansion of T cell precursors in the thymus of irradiated adult mice, leading to the notion that CXCL12/CXCR4-mediated interaction is involved in regulating fetal and adult T cell development [19]. Moreover, recent findings by Plotkin and co-workers [20] revealed that CXCL12/CXCR4-driven signaling is required for proper localization of early progenitors within the cortex: Thymus-specific deletion of CXCR4 in vivo resulted in failed cortical localization of these progenitors, together with arrest of developmental process.

The chemokine CCL25 (TECK) was first described as being produced by DC [21], but it is also expressed by TEC [22]. This chemokine attracts mainly immature thymocytes, although all thymocyte subsets are responsive, and its CCR9 receptor is expressed at all stages of murine thymocyte differentiation, with maximum expression in $CD4^+CD8^+$ cells [23, 24]. As CCR9 is down-regulated in mature thymocytes, and loss of responsiveness to CCL25 occurs just before thymocyte emigration, CCL25/CCR9 interactions seem to be important as a thymocyte retention factor into the thymus [16].

TEC produce other chemokines such as CCL4 (macrophage inflammatory protein-1β), CCL11 (eotaxin), CCL19 (Epstein-Barr-induced 1 ligand chemokine), CCL21 (secondary lymphoid tissue chemokine), CCL22 (macrophage-derived chemokine), CXCL9 (monokine induced by IFN-γ), CXCL10 (IP-10), and CXCL11 (IFN-γ-inducible T cell-α chemokine), involved in the migration and localization of mature human $TCR\alpha\beta^+$ thymocytes, $TCR\gamma\delta^+$, or natural killer cells through their respective receptors [3].

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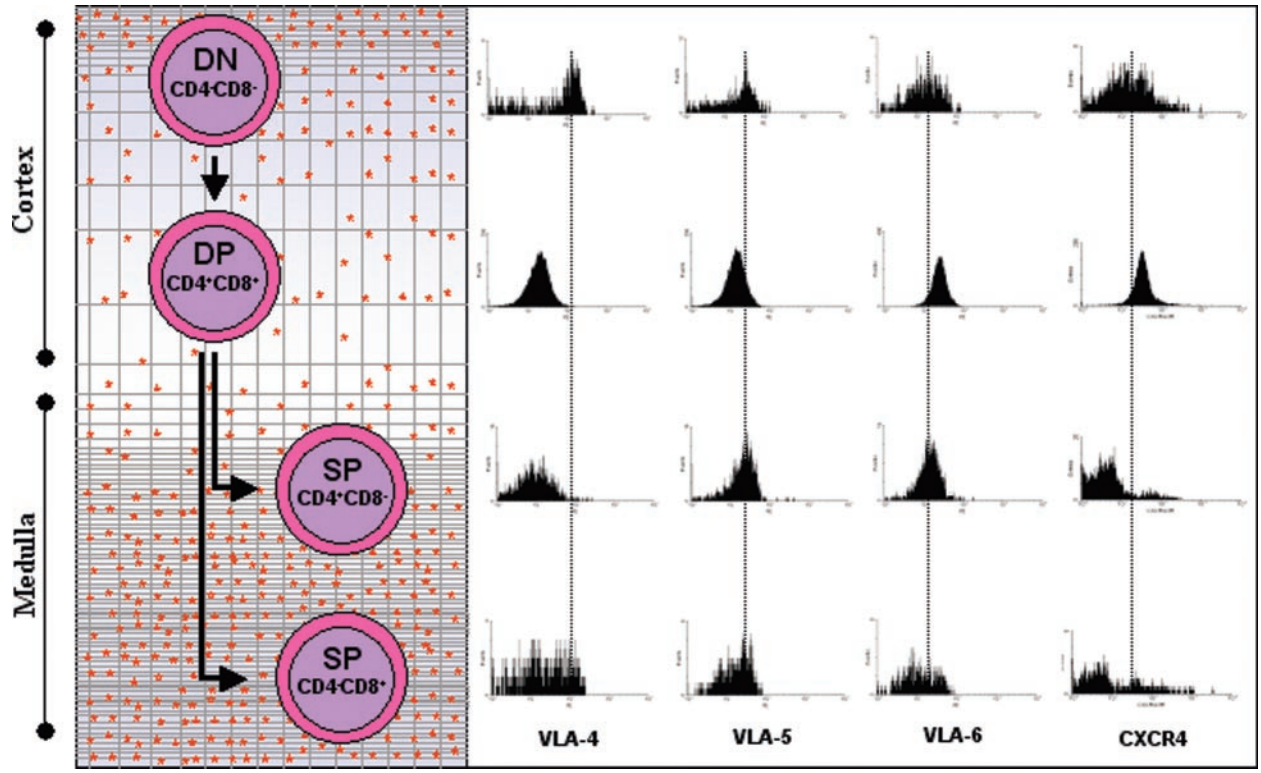


Fig. 2. Thymocyte differentiation in the context of ECM and chemokines. In this composite figure, a simplified CD4/CD8-based scheme of thymocyte differentiation is shown, placing immature cells in the cortex and CD4 and CD8 single-positive (SP) cells in the medulla of thymic lobules. In the right part of the figure, several cytofluorometric profiles reveal the density patterns for the expression of receptors for fibronectin [very late antigen (VLA)-4 and VLA-5], laminin (VLA-6), and the CXC chemokine receptor (CXCR)4 for CXCL12. The dashed lines in each marker were included to facilitate the notion that there is a fluctuation in the density of receptors, along with the differentiation stages. Bearing such ECM and chemokine receptor profiles, thymocytes migrate within an ECM network (herein, represented by the gray lines), denser in the subcapsular cortex, thinner in the inner cortex, and quite dense in the medulla. This coincides with the density of CXCL12 (red stars), which may be trapped in the fibronectin/laminin-containing ECM network, and this will favor its presentation to developing thymocytes. Simultaneously, cells would be directly interacting with the ECM molecules via their corresponding receptors. DN, Double-negative; DP, double-positive.

Another interesting concept recently raised in the literature based on ex vivo transmigration assays is that depending on the dose applied, a given chemokine may behave as a chemoattractant or chemorepulsive molecule. Thymocyte migration toward CXCL12 is observed when low concentrations of the chemokine are used, but in high concentrations, CXCL12 promotes thymocyte migration away from it, suggesting the involvement of this chemokine also in emigration of thymocytes [24], thus similar to what has been reported for neuron guidance [25].

It is interesting that CCL19/CCR7 interactions also participate in thymocyte exit [26], suggesting that thymocytes may switch their responses to a given chemokine, and this is not a characteristic restricted to one single ligand.

In $CCR9^{-/-}$ mice, thymocyte development and selection occur normally, despite abrogation of CCL25-induced chemotaxis and the fact that $CCR9^{-/-}$ bone marrow cells present a reduced capacity of repopulating the thymus of irradiated mice when compared to $CCR9^{+/+}$ cells [27]. In the same vein, CCR7-deficient mice, as well CCL19-neutralized mice show normal thymocyte development, although higher numbers of thymocytes can be observed, together with reduced numbers of circulating peripheral cells, thus suggesting a role for CCL19/CCR7 interactions in thymocyte emigration [26]. In the same study, no differences were seen in CCL21-neutralized mice. Unfortunately, although other chemokine or chemokine receptor KO mice have been reported [28], the experiments concerning thymocyte migration and differentiation remain to be performed.

Conjointly, the data raised so far in distinct KO animals point to redundant as well as nonredundant functions of different chemokines upon thymus physiology and/or a compensatory enhancement in other cell migration-related circuits, as for example, those mediated by ECM/VLA interactions.

THE INTRATHYMIC WORLD OF ECM

Adhesive proteins

The description of ECM in the thymus was first based on histological methods and dates back to the 1960s, when Henry [29] reported reticulin fibers in the organ, and the 1980s, when we [30] described the intrathymic distribution of elastic fibers. A few years later, we applied immunohistochemistry to define the presence and distribution of type I, type III, and type IV collagen, as well as laminin and fibronectin in the human thymus [31]. We showed that these three ECM proteins are

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more concentrated in the medullary region of the thymic lobules. In fact, in addition to the septum/capsule and blood vessel-associated basement membranes, these proteins form intraparenchymal fibrils, thinner in the cortex and thicker and more frequent in the medulla [31]. This pattern is also seen in the mouse thymus [32, 33], being actually phylogenetically conserved in mammals, implying their importance for thymus physiology [34].

Microenvironmental cells essentially produced the various ECM components so far described in the thymus. We have shown that cultured TEC produce laminin, fibronectin, and type IV collagen, as revealed in various TEC preparations, including TNC. Nevertheless, fibroblasts and MHC class II^+ phagocytic cells of the thymic reticulum also produce these glycoproteins [35].

Isoforms of ECM components have been reported in the thymus. The fibronectin isoform recognized by receptor VLA-5 through an arginine-glycine-aspartic acid (RGD) motif, is located throughout the thymic parenchyma, whereas the isoform derived from alternative splicing of fibronectin mRNA (and recognized by VLA-4 through the arginine-glutamic acid-aspartic acid-valine (REDV) motif) appears to be restricted to the medulla [36].

Laminin isoforms are $\alpha\beta\gamma$ heterotrimers generated by the transcription of distinct genes for each polypeptide chain. Some members of this protein family have also been detected in the thymus. Laminin-2 (the $\alpha2\beta1\gamma1$ heterotrimer) has been found in the thymus, and it has been demonstrated that thymocytes do bind to this laminin isoform. It is important that aberrant thymocyte development has been seen in the *dy/dy* mutant mouse, which lacks laminin-2. These animals exhibit precocious thymic atrophy with decreased relative numbers of $CD4^-CD8^-$ thymocytes and an increase in the number of apoptotic cells bearing this phenotype, suggesting that the $CD4^-CD8^- \rightarrow CD4^+CD8^+$ progression is partially mediated by laminin [37].

Laminin-5, which corresponds to the $\alpha3\beta3\gamma2$ heterotrimer, was detected in the human thymus: It is produced by medullary TEC and is able to trigger outside-in signals to thymocytes [38]. In the same vein, it has been shown that antilaminin-5 antibodies block thymocyte expansion, as well as $CD4^-CD8^- \rightarrow CD4^+CD8^+$ differentiation [39]. We observed that laminin-1 and laminin-2 modulate thymocyte migration within TNC complexes (Jurandy S. Ocampo et al., University of Rio de Janeiro, unpublished results). As most intra-TNC thymocytes are $CD4^+CD8^+$ cells, it is conceivable that in addition to the double-negative $\rightarrow$ double-positive progression, the migration of $CD4^+CD8^+$ thymocytes is also influenced by laminin(s).

Very recently, we characterized nidogen as a further TEC-derived ECM ligand of the thymic microenvironment. Nidogen is in general associated with typical basement membrane proteins including laminin and type IV collagen [40]. In the thymus, it is colocalized with these proteins in the basement membranes and in the intraparenchymal fibrils seen in the cortex and medulla. Functionally, others and we showed that nidogen is a further player in TEC/thymocyte interactions (ref. [41], Sandra Neves-dos-Santos et al., Federal University of Juiz de Fora, unpublished results.)

Galectins: a further ECM family expressed in the thymus

Galectins correspond to a family of lectins that bind to β-galactoside sugar residues present in a variety of glycoproteins, such as ECM molecules, β1-integrins, and CD45 [42]. Many functions were attributed to galectins as regulatory molecules of cell survival and death with adhesive or de-adhesive properties depending on their localization [43, 44]. Three galectins have been described in the thymus, produced by thymic microenvironmental cells: galectin-1 [45], galectin-3 [46], and galectin-9 [47], modulating thymocyte–ECM interactions, migration, and apoptosis [46–48]. Galectin-1 is produced by TEC [45], binding to CD7, CD43, and CD45 in thymocytes [44].

Galectin-1 and galectin-9, but not galectin-3, when exogenously added to thymocytes and lymphoblastoid T cells, interact with specific oligosaccharide ligands on the cell surface, triggering apoptosis [47, 48]. By contrast, the expression of galectin-3 has been shown to prevent T cell death induced by Fas ligation and staurosporine [49]. We showed that TEC and thymic phagocytic cells secrete galectin-3, which is found throughout the thymic parenchyma, being prevalent in the medulla, colocalizing with laminin, a polylactosamine-rich ECM glycoprotein. Moreover, galectin-3 purified from a murine TEC line-conditioned medium was able to decrease thymocyte adhesion to TEC in a dose-dependent manner, thus acting as a de-adhesion molecule [46]. Similar results were obtained when murine thymocytes were treated with human recombinant galectin-3 (**Fig. 3**). This effect could be blocked in the presence of lactose, the cognate sugar, or by the addition of antigalectin-3 monoclonal antibody (mAb). In the model of TNC complexes, galectin-3 increased thymocyte release from complexes and decreased TNC reconstitution [46].

Secretion of galectin-3 by TEC seems to be finely regulated by the interaction with thymocytes. Freshly isolated TNC complexes produce more galectin-3 in vitro than cells that bear no attached thymocytes. Moreover, we observed that galectin-3 involves thymocytes at the points of contact with the epithelial cell, as if TEC/thymocyte interactions were able to regulate galectin-3 production by the epithelial cell.

Several authors reported that antagonizing functions between galectin-1 and -3 may be related to the maintenance of a homeostatic equilibrium [44]. This is an attractive issue deserving careful investigation in terms of thymus physiology.

Conjointly, our data indicate that galectin-3 is a further modulator of thymocyte migration, by interfering with cell adhesion and subsequent de-adhesion. This occurs after oligossacharide binding on cell-surface molecules, such as β1 integrins, and to their corresponding ECM ligands [46].

In addition to this effect, it is possible that galectin-3 modulates thymocyte migration by acting as a chemoattractant moiety. Sano and co-workers [52] showed that in high concentrations, galectin-3 is a chemoattractant for monocytes and macrophages. In keeping with this, we observed that TEC-derived galectin-3 has chemoattractant effects on thymocytes,

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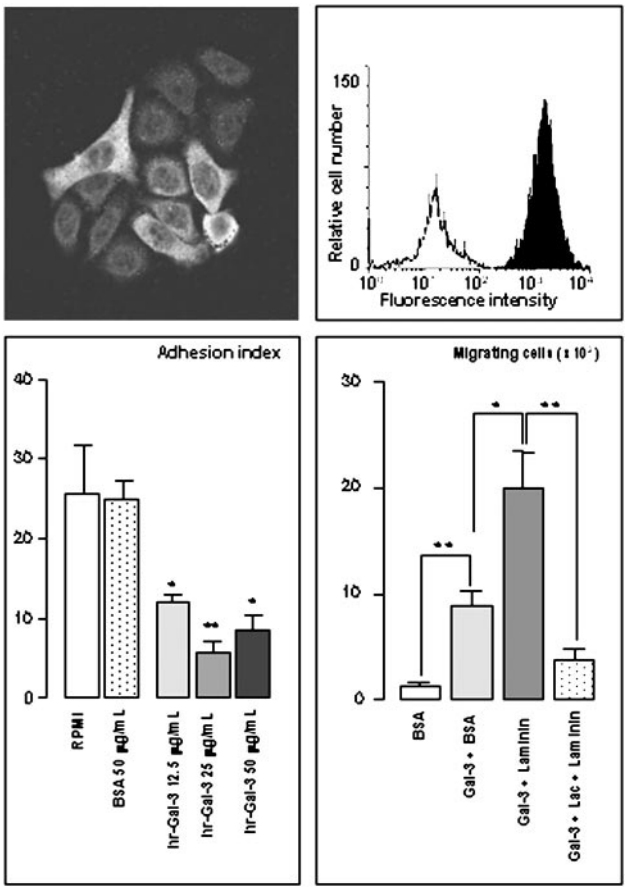


Fig. 3. TEC express galectin-3 (Gal-3): role on interactions with thymocytes. The upper panels of the figure show galectin-3 in TEC line, as revealed by immunohistochemistry (left panel) and flow cytometry (solid curve in the right panel; the open curve corresponds to the isotype-matched, negative-control immunoglobulin). Original magnification, ×1000. In the left bottom panel, it is shown that different doses of human recombinant (hr) galectin-3 significantly inhibit adhesion of thymocytes to epithelial cells (for calculation of the adhesion index, see refs. [55, 58, 59]), as compared with controls, culture medium alone, or bovine serum albumin (BSA). *, $P < 0.05$; **, $P < 0.01$. The right bottom panel illustrates the chemotactic activity of galectin-3 upon thymocyte migration in transwell chambers. This is seen when TEC-derived, purified mouse galectin-3 is applied alone (100 μg/mL) or in combination with laminin (used at 10 μg/mL). Moreover, the effect depends on the recognition by the carbohydrate-rich domain, as it is prevented by lactose (Lac; used at the concentration of 3.5 mg/mL). *, $P < 0.05$; **, $P < 0.01$. In each well, 2 million cells were led to migrate. Upper panels are derived from ref. [46].

and among those migrating cells, $CD4^+CD8^+$ (double-positive) thymocytes were the most numerous. Galectin-3 acts in synergism with laminin-potentializing migration of thymocytes (Fig. 3), and this effect can be blocked by antigalectin-3 antibody as well as lactose, its cognate sugar, but not meliobiose, suggesting that the chemoattractant effect of galectin-3 on thymocytes involves the carbohydrate-recognition domain. The mechanisms and cell-surface molecules involved in these phenomena as well as possible interactions with known chemokines are presently under investigation.

Last, it should be noticed that TEC also produce and secrete glycosaminoglycans, including heparan sulfate, and hyaluronic acid, which can also modulate TEC/thymocyte adhesion [53, 54].

ECM receptors in the thymus: the VLA family

In addition to producing ECM ligands, thymic microenvironmental cells express ECM receptors, as for example, the fibronectin receptor VLA-5 (the integrin α5β1), as well as laminin receptors, VLA-3 and VLA-6, respectively, α3β1 and α6β1 integrins. In this vein, it has been shown that TEC are sensitive to fluctuations in laminin: TEC grow faster onto a laminin substrate [55], which also enhances the expression of focal adhesion kinases by these cells [50, 51] and augment IL-6 production through a p38 mitogen-activated protein kinase-mediated signaling cascade [56].

Integrin-type ECM receptors are also expressed by differentiating thymocytes, in a pattern that is illustrated in Figure 2.

Functionally, in vivo experiments showed that anti-β1 integrin antibodies impaired migration of human T cell precursors toward the thymus of immunodeficient mice [57]. We have shown that thymocyte adhesion to ECM-producing, cultured TEC is abrogated in the presence of anti-VLA-5 or anti-VLA-6 antibodies [58, 59]. In conjunction with these data, it has been shown that the density of laminin receptors is enhanced at TEC boundaries bearing adherent thymocytes [50], suggesting that ECM molecules form bridges between thymocytes and microenvironmental cells.

In a second vein, the adhesion of enriched populations of immature $CD4^-CD8^-$ thymocytes to TEC monolayers is significantly blocked by pretreatment with anti-VLA-4 mAb and by VLA-4 (α4β1) antagonists but not by anti-VLA-5 or the corresponding RGD-serine-containing peptide, antagonist for VLA-5 [60], although α5β1/VLA-5 is expressed by these cells. Such data lead to the notion that the expression of a given integrin does not necessarily means that it is activated. This notion was further reinforced by the data showing that membrane levels of fibronectin receptor in thymocytes, after being allowed to adhere onto fibronectin, were similar in adherent and nonadherent cells [61]. In this context, a further relevant concept to have in mind was derived from experiments performed with human lymphocytes, showing that moderate rather than firm adhesion to fibronectin promotes migration [62].

Moreover, thymocyte release from TNC not only is enhanced by fibronectin and laminin but is also impaired by treatment with corresponding anti-ECM or anti-ECM receptor antibodies. Similar blocking effects were seen when TNC complexes were reconstituted after coculturing TNC-derived epithelial cells with fetal thymocytes [58]. These data tell us that ECM-mediated epithelial/thymocyte interactions affect the entrance and exit of lymphocytes in this particular microenvironmental niche. In keeping with these data, fibronectin and laminin exert a haptotatic effect on thymocytes, as they induce migration of these cells ex vivo (see **Fig. 4** for laminin-triggered migration).

Last, thymocyte exit to the periphery may also be influenced by VLA-mediated interactions; intrathymic injection of RGD-containing peptide (recognized by the α5β1/VLA-5 fibronectin receptor) results in a decreased homing of recent thymic emigrants [51].

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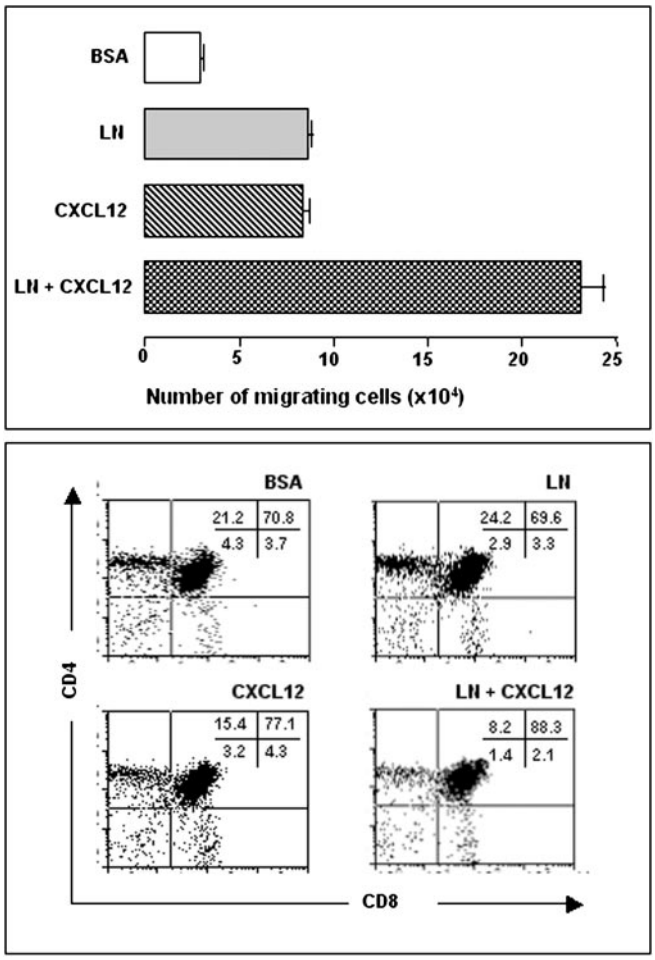


Fig. 4. Combined effects of laminin and CXCL12 upon thymocyte migration. The upper panel shows the total numbers of migrating cells in transwell chambers after stimulation with BSA (100 μg/mL), laminin (LN), or CXCL12 alone (respectively, 10 μg/mL and 100 ng/mL) or in combination. When CXCL12 and laminin were applied together, the resulting migration was higher than the sum of each stimulus alone. The bottom panel reveals CD4/CD8-defined phenotypes of a migrating cell in each experimental condition. In each well, 2 million cells were led to migrate.

DO MATRIX METALLOPROTEINASES (MMPs) PLAY A ROLE IN INTRATHYMIC, ECM-MEDIATED INTERACTIONS?

As seen in a variety of tissues in the organisms, we could predict that the presence and biological activity of ECM proteins in the thymus are dynamically controlled in matrix MMPs, as well as their natural tissue inhibitors (TIMPs). Rather surprisingly, however, very few data are available so far on this issue. Recent data indicate that some MMPs, such as MMP-9 are expressed in the human thymus, being produced at least by microenvironmental cells [51, 63].

A potential regulatory function of MMPs upon intrathymic, ECM-mediated interactions has been suggested, as the colonization of fetal thymus by T cell precursors is abrogated significantly in vitro in the presence of a MMP inhibitor [64], whereas a strong colonization of adult mouse thymuses was seen with lymphoma cell lines expressing high levels of MMP-9 [63].

Although we found by reverse transcriptase-polymerase chain reaction mRNAs for TIMP-1, TIMP-2, and TIMP-3 in mouse thymic cell preparations (our unpublished data), no functional assay has been done to place these molecules in the general context of the dynamics of intrathymic turnover of ECM proteins.

IS THERE AN ECM–CHEMOKINE INTERPLAY IN THE THYMUS?

The existence of an intrathymic ECM–chemokine interplay derives from a variety of data. CXCL12 enhances α4β1/VLA-4 expression on myeloma cells and hematopoietic progenitors, resulting in a higher degree of binding to fibronectin [65], an effect that is prevented with anti-VLA-5 antibodies [66]. Moreover, CXCL12 binds to and is presented by fibronectin to a T cell line, resulting in a greater degree of migration compared with the soluble chemokine alone [67], and the CXCL12-induced migration of $CD34^+$ precursors is enhanced by fibronectin [68]. In keeping with this concept, confocal microscopy analysis revealed a high degree of colocalization between fibronectin and CXCL12, as illustrated in Figure 1.

In the same vein, CXCL12 plus fibronectin (or laminin) induced higher thymocyte migration in transwell chambers than that elicited by the chemokine or the ECM molecule alone [69]. It is interesting that as seen as Figure 4, the CD4/CD8-defined phenotypes of migrating thymocytes were rather similar, secondary to laminin, CXCL12, or laminin plus CXCL12 stimuli, although in the latter experimental condition, the frequency of $CD4^+CD8^+$ cells was even higher. This is in keeping with the fact that most thymocytes (i.e., 95% of double-positive cells) simultaneously express α6β1/VLA-6 and CXCR-4 (**Fig. 5**). It is interesting that the notion of combined action of chemokines plus ECM upon thymocyte migration seems not to be restricted to CXCL12, as we showed that thymocyte migration induced by CCL19 plus fibronectin is higher than the migration induced by CCL19 or fibronectin alone [51].

A further, potentially interesting aspect that deserves to be investigated in the thymus is that chemokines appear to enhance T cell adhesion to ECM and activate ECM integrin-type receptors, as well as focal adhesion kinases [70]. Such a study will certainly provide new clues for the concept of chemokine–ECM interplay in thymus physiology.

Such an ECM–chemokine connectivity in the thymus should also be considered at the level of its dynamics. It has been shown by several authors that MMPs are able to cleave chemokines. This concept was originally raised after the study of CXCL12 breakdown by MMP-9 [71]. More recently, it has been shown that this enzyme is also able to cleave CXCL8, CXCL5, and CXCL6 [65]. Moreover, other MMPs can break down chemokines as shown by MMP-2, which cleaves CXCL12, and MMP-8, which also degrades CXCL8 [72]. Conjointly, these data raise the notion that in the ECM–chemokine interplay, MMPs play a dual role, regulating ECM- and chemokine-

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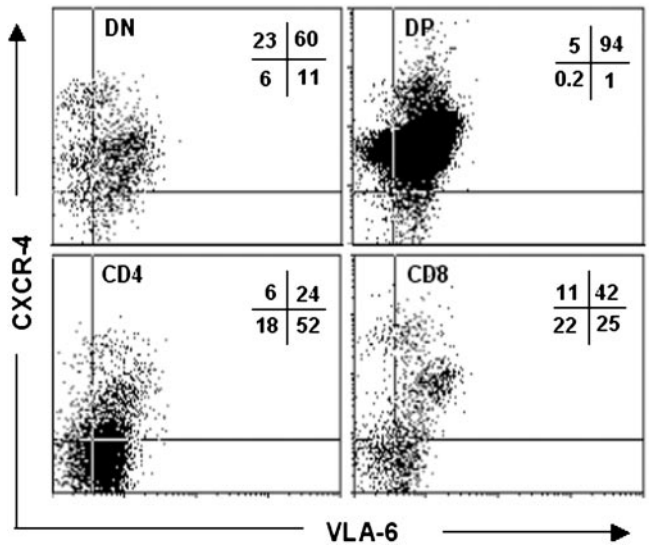


Fig. 5. Coexpression of CXCR4 and VLA-6 along with thymocyte differentiation. In these cytofluorometric profiles (derived from four-color immunolabeling), we can subdivide each CD4/CD8-defined subset into four populations (percentage values are shown in each plot). In the immature double-negative (DN) and double-positive (DP) stages, the majority of thymocytes expresses VLA-6 and CXCR4 simultaneously, whereas in the mature single-positive cells, the segregation of one receptor in relation to the other is prevalent.

mediated circuits. Although experimental data are to be obtained, we can speculate that such a scenario also occurs in the thymus.

MODULATION OF CC- AND ECM-DRIVEN THYMOCYTE MIGRATION IN HEALTH AND DISEASE

In addition to the intrinsic control likely exerted by MMPs and TIMPs, chemokine/ECM-oriented migration of thymocytes can be controlled by other physiological stimuli. In this respect, we have shown that various hormones can modulate ECM production by TEC, with corresponding effects on thymocyte adhesion and migration within TNC. For example, thyroid hormones enhance intrathymic ECM deposition in vivo and in vitro, with consequent enhancement in thymocyte adhesion to TEC and to phagocytic cells of the thymus, as well as increased thymocyte release from TNC [73, 74]. Similar effects were obtained when TNC were treated with prolactin or growth hormone, which also enhanced TNC reconstitution [75]. In fact, such an enhancement of intra-TNC thymocyte migration was seen in the context of an increase of ECM production and ECM receptor expression by TNC upon hormonal stimulation. More recently, we found that thymocytes from growth hormone transgenic mice exhibit an increased migration pattern in transwell chambers covered with laminin and/or CXCL12, as compared with the corresponding wild-type mice. It is interesting that in situ, we observed high intrathymic contents of these two molecules in the growth hormone–transgenic mouse thymus (S. Smaniotto et al., submitted). Once more, these findings indicate that distinct ligand/receptor interactions involved in thymocyte migration can be influenced by specific control mechanisms.

We have recently provided evidence that ECM/chemokine-mediated interactions can be altered in pathological conditions with consequences on thymocyte migration, which could ultimately alter the shaping of the peripheral T cell pool [5]. During experimental murine infection with *Trypanosoma cruzi*, the causative agent of Chagas disease, changes in the peripheral T cell repertoire were observed and correlated with thymocyte migration disturbances [76, 77]. We have previously shown that the TNC, when infected in vitro or isolated from *T. cruzi*-infected mice, exhibit increased ECM expression and enhanced thymocyte release [78]. Furthermore, there is an enhancement in the deposition of fibronectin by the thymic microenvironment, together with an augmentation in the membrane density of VLA-4 and VLA-5 in thymocytes from infected animals; such an increase is accompanied by augmented ex vivo thymocyte migration toward fibronectin [76]. It is interesting that in *T. cruzi*-infected BALB/c mice, in addition to a severe cortical thymocyte depletion (particularly secondary to apoptosis), we found abnormally high amounts of $CD4^+CD8^+$ T cells in lymph nodes, part of them bearing potentially autoreactive TCRs, which normally should be deleted intrathymically. In the thymus and in lymph nodes, these double-positive cells are high VLA-4 expressers [76, 77], suggesting that they may use fibronectin/VLA-4 interactions to leave the thymus. As T cells are also involved as autoimmune processes occurring in Chagas disease, abnormal fibronectin-driven thymocyte migration may play a relevant role in providing autoreactive T lymphocytes to the periphery of the immune system. Nevertheless, other driving forces are likely involved, as in infected mice, we have also noticed an increase in the intrathymic contents of galectin-3 as well CXCL12 (unpublished). Conceptually, these data tell us that the resulting vector, ultimately determining thymocyte exit in this pathological situation, involves interaction-mediated adhesive and de-adhesive ECM proteins together with chemokines.

A different scenario can be drawn in the nonobese diabetic (NOD) mouse, a largely used experimental model for the autoimmune T cell-dependent human type I diabetes. We have first demonstrated the formation of giant perivascular spaces (PVS) in the thymus, filled with mature thymocytes, which progressively accumulate in this particular region, being intermingled with a novel ECM-containing network [79, 80]. In addition, a decrease in VLA-5 expression by NOD thymocytes has been reported [81], and we found that such a defect comprises mature thymocytes and is accompanied by decreased thymocyte migration in fibronectin-containing transwell chambers [35]. In principle, these findings fit with the hypothesis that mature NOD thymocytes undergo a partial arrest within the giant PVS. Yet, short-term bromodeoxyuridine pulse-chase experiments did suggest that NOD thymocytes could actually leave the organ [80], indicating again that thymocyte exit is an affair of multiple cellular interactions. In fact, we recently found an increase of CXCL12 in the NOD thymus and an enhanced thymocyte migration upon CXCL12 stimulation (**Fig. 6**). Nevertheless, such enhanced migration is partially impaired when fibronectin is added together with the chemokine, suggesting that the rate of thymocyte exit through the perivascular spaces and blood vessel endothelium in the NOD mouse thymus depends on opposing migration pressions

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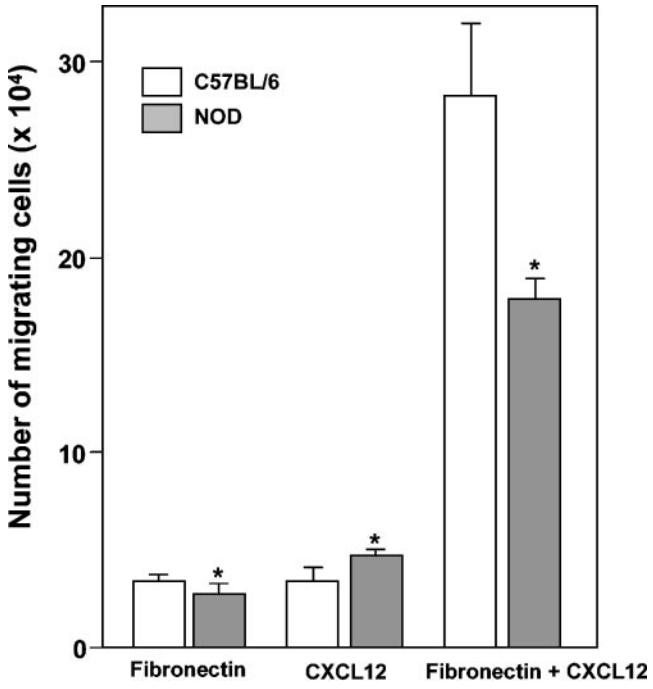


Fig. 6. Impaired fibronectin-driven thymocyte migration in NOD mice. Thymocytes (2.5 million cells) from 8-week-old C57BL/6 or NOD mice were led to migrate through transwell chambers under the stimulation of fibronectin alone, the chemokine CXCL12 alone, or both applied together. When fibronectin alone was used, NOD thymocyte migration was significantly lower than that seen in normal mice. The opposite occurred when CXCL12 was applied alone. It is interesting that when cells were subjected to fibronectin plus CXCL12, a synergic effect was observed in C56BL/6 and NOD mouse thymocytes. Yet, the migration levels seen with NOD thymocytes were largely lower. *, $P < 0.05$, as ascertained by Student's *t*-test.

provided by fibronectin/VLA-5 versus CXCL12/CXCR4 interactions.

Last, it should be noticed that the entire scenario of abnormal thymocytes exists in each of these pathological situations and is likely much more complex than indicated so far by our data. In this respect, studies on the expression and function of matrix MMPs in these animal models will inform us about the dynamics of ECM formation and degradation, an aspect that deserves to be investigated.

CONCLUDING REMARKS

The data summarized in this review clearly illustrate the notion that the molecular circuits governing thymocyte migration are complex, comprising distinct types of cellular interactions. In this respect, at least chemokines and ECM proteins appear to act in a combined way. Nevertheless, the precise cross-talk between these two ligand/receptor interactions is far from being completely unraveled and thus needs to be investigated.

The second issue urging to be dissected corresponds to the expression and role of the various MMPs in the thymus, as well as how they are regulated by TIMPs. This shall provide new clues to understand how T cell precursors enter the thymus and mature thymocytes leave the organ.

Last, the use of genetically engineered mice, bearing conditioned KO genes coding for distinct chemokines and ECM proteins and their corresponding receptors, will represent important tools to go deeper into the understanding of the relative contribution of each molecules for the entire process of intrathymic T cell migration.

In this respect, it seems apparent that a better knowledge about the mechanisms governing intrathymic T cell migration will provide new clues for understanding how thymus works in normal conditions and for designing therapeutic strategies targeting developing T cells.

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