Review

Roles of Chemokines in Thymopoiesis: Redundancy and Regulation

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Thymus is the primary lymphoid organ involved in the development of thymocytes. Maturation related events of thymocytes within thymus, especially the widely discussed directional migration of thymocytes, is regulated by chemokines via chemokine receptors mediated signaling pathway. Multiple types of chemokines and chemokine receptors, as components of the network-interaction within thymic microenvironment, are involved in the thymopoiesis. It appears that these chemokines are functionally redundant and such phenomenon may be explained not only by the promiscuous, non-one-to-one matching between ligands-receptors within CXC or CC chemokine subfamily, but also by the various spatio-temporal expression patterns within different cell types and developmental stages. The redundancy and regulation of thymus expressed chemokines and chemokine receptors during thymocyte development are herein discussed. *Cellular & Molecular Immunology*. 2004;1(4):266-273.

Key Words: chemokine, chemokine receptor, redundancy, regulation, thymopoiesis

Introduction

The chemokines are a family of small, structurally related cytokines, functionally involved in leukocyte activation and migration. They are divided into four subfamilies termed the CXC, CC, C and CX3C chemokine, respectively, based on the structural and genetic considerations. The CXC family is characterized by an amino acid positioned between the first and second cysteines, whereas in the CC family these two cysteines are located side by side. Two minor families have been described: the one lost the first and third cysteines is represented by lymphotactin; the other, exhibits three amino acids between the first two cysteines and is represented by fractalkine (1). Recently, chemokines are propositioned to be classified into "inflammatory" and "lymphoid" groups based on the site of production and the eliciting consequences of reactions. "Inflammatory" chemokines are produced by various types of cells, such as endothelial cells, epithelial cells, fibroblasts, as well as leukocytes, and are attractive primarily to neutrophils, and subsequently, found to attract different kinds of effector and memory lymphocytes. The common characteristic of this kind of chemokines is their high responsiveness to inflammatory stimuli. "Lymphoid" chemokines are produced within the lymphoid tissues and are involved in homeostatic lymphocyte traffic, comprising the ones participating in thymopoiesis we will discuss here. Indeed, many chemokines may play a dual role, acting in inflammatory process, and as factors triggering the migration of lymphocytes in central and peripheral immune organs, depending on the spatio-temporal expression and regulation. To date, over 50 distinct chemokines have been identified and characterized in human, whose homologous counterparts are found in mouse, as well as in other species. Chemokines mediate their biological responses by binding to targeted cell-surface receptors that belong to the large family of G protein-coupled seven transmembrane domain (7-TM) receptors (2). The receptors that bind CXC chemokines are designated CXC chemokine receptors (CXCRs), those to CC chemokines are CC chemokine receptors (CCRs), and CX3CRs to CX3C chemokines, analogically. Bevan et al. summed up the chemokines and chemokine receptors that were detected in thymus, either in thymic stroma, or in the different developmental stages of thymocytes (3).

The thymic microenvironment, composed of stromal cells, extracellular matrix and cytokines, provides a perfect-equipment residence for thymocyte growth. As they go through phenotypic and functional maturation, thymocytes are moving from the subcapsular region of the outer cortex, to the inner cortex, traverse the cortico-medullary junction and migrate to the medulla, where they camp for about 2 weeks for further maturation before devoting into the 'frontier defence barracks' (periphery

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Abbreviations: CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; DC, dendritic cell; DN, double negative; DP, double positive; SP, single positive; MMP, matrix metalloproteinase; MTEC1, mouse thymic epithelial cell line 1; MTDC, mouse thymic dendritic cell; PTX, pertussis toxin; TCR, T cell receptor.

blood and second lymphoid organs) (4). Along with the anatomical trafficking, thymocytes switch their visages, from the arrived immature $CD4^-CD8^-$ (double negative, DN) precursors, then $CD4^+CD8^+$ (double positive, DP), to the ultimately emigrants of CD4SP or CD8SP (single positive, SP). The temporally ordinal events: entry of T lymphocyte progenitors from bone marrow into the thymus, intrathymic trafficking, and emigration to the periphery are now cognized to be at least owed to the contribution of chemokines, which act via cell-surface expressed chemokine receptors. In the two concerned reviews of Bevan and Romagnani, they and colleagues summarized the up-to-date results on thymocyte migration triggered by chemokines, in terms of the consecutions of various chemokine-receptor pairs, and of the dimensional locations of chemokines and receptors within thymus during development, respectively (3, 5). Obviously, chemokines are perplexingly redundant in their actions on target cells and certain cells concomitantly produce several chemokines with an overlapping spectrum of actions. Although a great deal has been learned about chemokines and chemokine receptors in regulating the migration of mature lymphocytes, far less than satisfying information is available concerning their potential role and the regulation mechanism in thymocyte development. Recently, more functions of chemokines in thymus are discussed, such as in thymocytes proliferation, activation and cytokine production, in thymic positive and negative selection. Additionally, chemokines may have implication with the survival or apoptosis of thymocytes, which seems to be attractive, though with few reports heretofore. Some useful experimental models, such as FTOC (6), reconstitution in congenic mice, help us to pry into the more actual roles of chemokines in thymus.

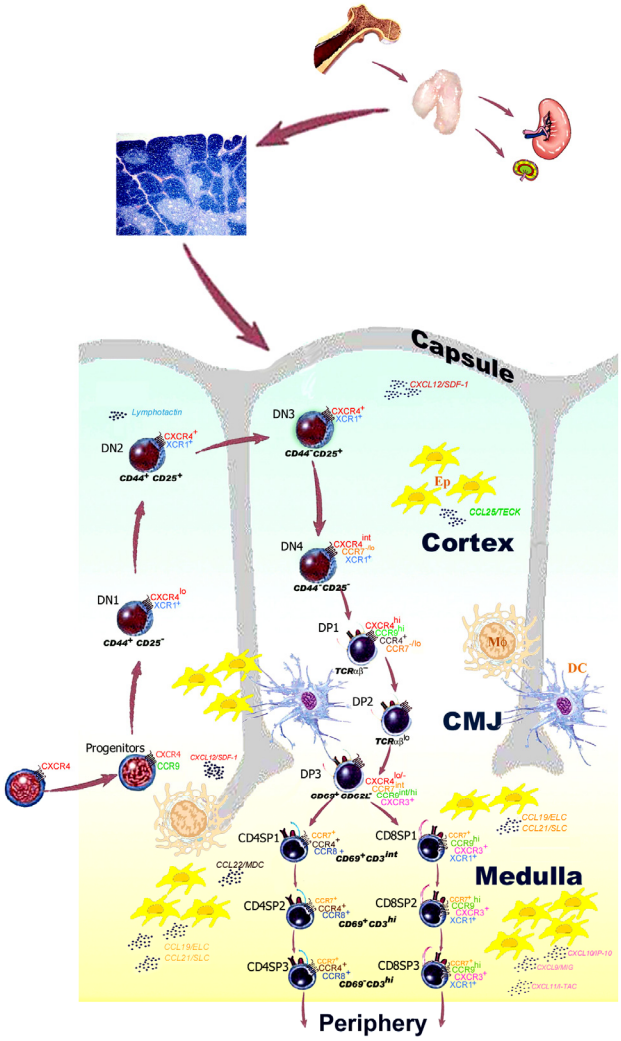

Figure 1. The schematic image shows the participation of representative chemokines and chmokine receptors involved in the schedule of thymocyte development. Bone marrow derived T progenitors maturate in the thymic microenvironment, which consisted of stromal cells, extracellular matrix and various cytokines. Chemokines drive thymocytes moving and migrating to the proper niches, where maturation related events take place. The chemokine receptors on thymocytes switch along with the developmental evolvement, implying different functions of chemokines and receptors. On the other hand, the redundant pattern of the chemokines and receptors to keep a multiple insurance of thymocyte development and to compensate when some certain chemokine or receptor is deficient. *Abbreviations:* CMJ, cortico-medullary junction; hi, high expression; int, intermediate expression; lo, low expression; +, positive; -, negative; Ep, epithelial cell; DC, dendritic cell; Mϕ, macrophage.

Dancing of chemokines in thymus

Like a player, chemokine does a splendid figure in the thymic arena, almost becoming the protagonist among the various participators that conduct the thymocytes trafficking. The routine study procedure of the previous reports is to investigate the expression profile of a thymus-expressed receptor among various thymocyte subpopulations using specific antibodies; at the same time, check the occurrence or existence of the corresponding ligand in a certain thymic position, where the cognate receptor-marked thymocyte subsets happen to pass by, thus draw the conclusion of that this pair of chemokine/receptor dominantly acts on this stage. With this strategy, multiple chemokines and receptors are exhibited to involve in the directional trafficking of thymocytes and are here summarized by the order of the topological migration of thymocytes (Figure 1).

Newly entered thymocyte progenitors are found in the subcapsular region of the outer cortex, the entrance seems to penetrate the vessels at the cortico-medullary junction observed in reconstitution assay with labeled bone marrow precursors (7). The cell surface receptor CXCR4, which has already been expressed at earlier developmental stages in the bone marrow, is remarkably contributed to thymocyte development by leading the early progenitors to a proper localization of the outer cortex and thus employing them to be ready for further differentiation (8). Here they encounter the cortical epithelial cell produced CXCL12, and this chemokine promotes the cells moving forwards, with the $CD34^+$ progenitors showing the highest responsiveness. Moreover, CXCR4 is up-regulated by c-Kit on human $CD34^+$ bone marrow progenitors (9, 10). According to the opinion of Godfrey et al., the immature DN cells are further classified into four pulsive stages defined by two surface molecules—CD44 and CD25, i.e.,

Table 1. *Chemokines and receptors mentioned in this review.*

Symbol	Alias	Definition	Receptor
CCL2	MCP-1	monocyte chemoattractant protein-1	CCR2
CCL3	MIP-1α	macrophage inflammatory protein 1 α	CCR1/5
CCL4	MIP-1β	macrophage inflammatory protein 1 β	CCR5
CCL5	RANTES	regulated on activation normal T cell expressed and secreted	CCR1/3/5
CCL19	ELC	Epstein-Barr virus-induced receptor ligand chemokine	CCR7
CCL21	SLC	secondary lymphoid tissue chemokine	CCR7
CCL22	MDC	macrophage-derived chemokine	CCR4
CCL25	TECK	thymus-expressed chemokine	CCR9
CXCL1	GRO	growth-related oncogene	CXCR1/2
CXCL4	PF-4	platelet factor 4	*ND
CXCL7	PBP	platelet basic protein	CXCR2
CXCL8	IL-8	interleukin-8	CXCR1/2
CXCL9	MIG	monokine induced by γ-interferon	CXCR3
CXCL10	IP-10	γ-interferon-inducible protein-10	CXCR3
CXCL12	SDF-1	stromal-derived factor 1	CXCR4
XCL1		lymphotactin	XCR1

* Receptor of CXCL4 is not identified so far.

$CD44^+ CD25^-$ (DN1), $CD44^+ CD25^+$ (DN2), $CD44^- CD25^+$ (DN3) and $CD44^- CD25^-$ (DN4) (11). CXCR4 was reported to be expressed lowly on $CD44^+ CD25^-$ DN, and up-regulated upon maturation to the $CD44^+ CD25^+$ stage (12). During the travel of thymocytes in the cortex, another chemokine receptor, CCR9 is up-regulated following pre-TCR signaling (13), but all of the four CD44/CD25 DNs express low levels of CCR9 and no significant migration of DN to CCL25, the ligand of CCR9, was detected. Additional chemokines and receptors that involved in the cortical niches, such as CCL3 and XCL1 were reported to be highly expressed in DN cDNA library and chemoattarctive to DN thymocytes (14, 15). But the details need further investigation.

As thymocytes move from the outer cortex to the inner cortex, they initiate the expression of CD4 and CD8, and encounter additional chemokines, which facilitate interacttions of thymocytes with peptide/MHC complexes or other factors produced by stromal cells. Positive selection occurs if the TCR engaged a peptide/MHC complex with low affinity, resulting in the cell survival and differentiation (16). In this period, CCL25/CCR9 is the star player. Remarkable expression of CCR9 is induced in the DN to DP transition and DP cells showed a strong response to CCL25 in chemotaxis assay. Down-regulated expression of CCR9 was found when the cells transmit to the CD4 SP or CD8 SP stage. The expression of CD69, a C-type lectin of very early activated antigen, hallmarks that the DP cells have been positively selected. Constitutively expression of CD69 on DP thymocytes suggests that it may be involved in regulation of developmental events in addition to activation of a variety of hematopoietic cells (17). $CD69^+$ thymocytes demonstrate enhanced CCL25-induced migration compared with $CD69^-$ thymocytes, and thymocyte migration in response to CCL25 is augmented by TCR signaling. Uehara et al. found that approximately half of all $\gamma\delta TCR^+$ thymocytes express CCR9, and migrate to CCL25 (18). The expression of CCR9 on specific γδ-T cell subsets indicates that CCR9 may also function in the development and/or trafficking of γδ-T cells (18, 19). Notably, CCR5 strikes a pose in this stage, accelerating the DN-DP transition, inducing DP cells responsive to CCL4, one of the ligands of CCR5 (20). Another important event of the DP stage is that the down-regulation of CXCR4, which has made contributions in the trafficking of early DN cells. The significance of CXCR4 decreasing may be speculated to facilitate the developing thymocytes away from the outer cortex.

Ceaselessly, thymocytes arrive the cortico-medullary junction, where and when CD69 was marked on cell surface to represent the transient thymocyte population of during or immediately after the positive selection. Thymocytes of this stage remain highly responsive to CCL25/CCR9, and also to another pair of chemokine/receptor, CCL22/CCR4. A remarkable proportion of CCL22-responsive thymocytes were $CD4^+CD8^+$ cells, whereas exhibiting reduced levels of CD8. At this moment, if TCR on thymocytes matches a peptide/MHC ligand presented by antigen-presenting cells (APCs) with high affinity, the apoptotic cell death is occurred and the self-reactive thymocytes are deleted to generate a peripheral T repertoire of self-tolerance. It seems that CCL22/CCR4 plays a key role in the migration of $CD69^+$DPs, a population of post-selection thymocytes with the phenotypic characters of $CD3^{high} CD30^+$. Chemotaxis of CCL22/CCR4 is durable on the $CD69^+ CD62L^{lo}$ stage and disappears when thymocytes evolved into more mature stage of $CD69^-CD62L^{high}$, implying a privity between CD69 and CCL22/CCR4. It is notable that CCR4 is selectively localized to the medullary $CD30^+$ cells, CCL22-producing cells are happened to be stained positive for CD30L. CD30 is a member of the TNF receptor family, thus CD30L positive CCL22-producing

cells attract $CCR4^+$ thymocytes, favor the CD30/CD30L interaction, and therefore induce apoptosis of cells by autoantigen activation (21).

Positively selected DP thymocytes mature into CD4SP and CD8SP, based on the divaricated ways with engagement of peptide/MHC I or peptide/MHC II complex, respectively. Final maturation events take place in the medulla, where the SP thymocytes appear to reside for about two weeks to undergo further development, and participation of chemokines and receptors is needed. CCL19 and CCL21, sharing the common receptor of CCR7, can selectively attract $CD4^+$ or $CD8^+$ SP. CCL19 produced by CD30L-negative medullary epithelial cells may induce the output into periphery of mature thymocytes that have survived the process of negative selection, contrast to the situation of CCL22 as described above. Suzuki et al. subdivided the SP into three subsets with hierachical maturation: $CD69^+CD3^{int}$, $CD69^+CD3^{high}$ $CD69^-CD3^{high}$. CCR7 was found up-regulated during this period with highest abundance on $CD69^-CD3^{high}$ SP. CCL25/CCR9 continues to be attractive to SP thymocytes, but presents a decline trend with one evidence of CCR9 mRNA expression in SP lower than that in DP (13). CCL22/CCR4 responsive cells of this stage are dominantly $CD3^+CD4^+$ $CD8^{low}$ SP.

In the study of chemokine production by mouse thymic stromal cells (MTSCs), we have gained more information. The MTSCs and fresh isolated thymic stromal cells produced a variety of chemokines, including CXCL12, GROα, CXCL10, CCL5, XCL1, GROβ/γ and CCL2 (22, 23).CXCL10 substantially produced by cortical type thymic epithelial cell lines was one of the potent chemokines to DP thymocytes, supporting the results of Romagnani et al.(24), whereas CCL2 produced by medullary type thymic epithelial cell line (MTEC1) and mouse thymus dendritic cell-like cell line (MTDC) was chemoattractive to both CD4SP and CD8SP thymocytes (22, 23).

The directional movement of thymocytes triggered by chemokine/receptor pairs seems to attract too much attention of investigators, which overshadowed the extended intrinsical roles of chemokines in lymphocyte development. In fact, more functions of chemokines and chemokine receptors have been made known in thymus. Recent investigations are focused on the participation of chemokines and receptors in thymocyte maturation related events, anything more than the widely discussed migration. Bevan et al. presumed that CXCL12/CXCR4 may stimulate the proliferation of DN cells, based on the knowledge that CXCL12 was originally cloned as a pre-B cell growth factor (25). This was echoed by the result that increasing number of human thymocytes was found in CXCL12 treated FTOC system, mainly affecting the most immature subpopulations, implying CXCL12 significantly increased the viability of $CD34^+$ T-cell precursors by modulating the expression of Bcl-2 and Bax genes, and stimulated the proliferation of $CD34^+$ thymic precursor cells (10).

Apoptosis and survival is a pair of conflict widely consisted in organisms, representative in immune organogenesis. As we have known, only a small portion of thymocytes survive and migrate to the periphery. Most of thymocytes are doom-destined and eliminated by neglect and negative selection. The apoptosis of thymocytes is a subtly regulated process and acts as a superior model for research. Recent findings of Flad et al. indicate that the CXCL4 (platelet factor 4, PF-4) protects monocytes from spontaneous apoptosis and induces the differentiation of monocytes into macrophages (26). It may elicit a query about the possibility of chemokines concerning in thymocytes apoptosis. Excitingly, Schabath et al. found that CXCL12 was potent to counteract dexamethasone-induced apoptosis of thymocytes (27). Recently, CCR9 was found to lead to potent resistance to cycloheximide-induced apoptosis and modest resistance to Fas-mediated apoptosis possibly *via* activation of multiple signaling components involving Akt and glycogen synthase kinase 3β (28). The fact that these two apoptotic signals involve activation of similar arrays of death execution machinery such as caspase-8, caspase-9, or caspase-3, suggests that chemokine receptor signaling may provide a wide range of anti-apoptotic activities to hematopoietic cells, including thymocytes, under certain biological conditions. Thus, dissection of signaling components involved in the CCR9-mediated anti-apoptosis could be a framework for cell survival mechanisms. Zaitseve et al. demonstrated that uptake of apoptotic thymocytes by immature dendritic cells (DCs) induced an increase in CXCR4 expression and CXCL12 mediated chemotaxis (29).

Colonization of the thymic rudiment during development is initiated before vascularization so that hematopoietic precursors must leave the pharyngeal vessels and migrate through the perithymic mesenchyme to reach the thymus, suggesting that they may be responding to a gradient of chemoattractant factors. Wilkinson et al. (30) reported that diffusible chemoattractants are produced by MHC class II^+ epithelial cells of the fetal thymus, and that the response of precursors to these factors is mediated via a G protein-coupled receptor. Indeed, a number of chemokine receptors are expressed by thymic precursors, and several chemokines are also expressed by thymic epithelial cells. CCL25 is expressed at higher levels in thymic epithelial cells and was found to show chemotactic activity for isolated thymic precursors. Whereas neutralizing Abs to CCL25, however, did not prevent thymus recolonization by T cell precursors, suggesting that other novel chemokines might be involved in this processs for redundancy.

In the review of Mebius (16), the organogenesis of lymphoid tissues, such as lymph nodes, Peyer's patches and nasal-associated lymphoid tissue, is discussed to explore several signal molecules, among which lymphotoxiņβ (LTβ) and TRANCE (TNF-related activation-induced cytokine) seem to play critical roles. LTβR signaling triggers the expression of adhesive molecules and chemokines, such as CXCL12, CCL19 and CCL21, being essential for lymphoid-organ development. Chemokines and adhesive molecules are predicted to be involved in attracting and retaining the cells that are required for the formation of the earliest anlage of a lymphoid organ. The thymus is composed of kineticly developmental thymocytes and non-lymphoid stromal components, including epithelial cells, DCs, monocytes/macrophages and fibroblasts. It has been proposed that chemokines are essential for stromal and haematopoietic cells to come together, and LTβR signaling can induce the expression of various

chemokines that might contribute to the essential accumulation of cells. Indeed, over-expression of a transgene encoding the lymphoid chemokine BLC (B-lymphocyte chemoattractant) in the pancreatic islets leads to the induction of lymphoid aggregates with distinct organization into B- and T-cell areas (31).

Complicated and confusing roles of chemokines caused by redundancy

Chemokines are double-faced players in immune system. By virtue of their target cells, chemokines have the potency to selectively induce directional migration of leukocyte subpopulations into primary or seconds lymphoid organs, or into sites of inflammation, which shows an exhibition of the specificity; on the other hand, chemokines are redundant in their actions on target cells. Thymocytes concomitantly produce several chemokines with an overlapping spectrum of actions. The fact that enlarged number of chemokines playing roles in thymus has resulted in a considerable difficulty in understanding their individual relevance and function. The redundant roles of chemokines are exhibited due to the following facts: 1. Crossed matching between chemokines and their receptors, i.e. multiple ligands for one chemokine receptor and multiple receptors for a single chemokine ligand, leading to multiple interactions to achieve similar cellular responses. 2. Most types of cells express multiple chemokines or receptors so that if one ligand or receptor is defective, an alternate set of chemokine and its receptor compensates. Targeted deletion of chemokine or receptor used to be an excellent strategy to study the function of specific chemokine–receptor interactions *in vivo*, particularly in models of inflammatory and infectious diseases. The fact that only one chemokine receptor knockout has so far proved to be embryo lethal could imply that some compensations have occurred for the loss of a given chemokine receptor since embryogenesis (32, 33), and is supportive to the opinion of chemokine redundancy. We recently found that the murine homologue of CXCL7 (platelet basic protein, PBP) was produced by thymic macrophages/monocytes, and CXCR2, the cognate receptor of CXCL7, was expressed on the four major thymocyte subsets defined by CD4 and CD8, preferentially on DN (unpublished data). The chemotactic profile of CXCL7/CXCR2 is similar to that of CXCL12/CXCR4 to a certain extent. Interestingly, T cell development is normally proceeded in $CXCL12^{-/-}$ and $CXCR4^{-/-}$ mice (33), implying that CXCL12 may not be the only chemokine that controls the trafficking of immature thymocytes in the cortex. CXCL7/CXCR2 may play a synergic function in this stage.

Consequences of the deficiency of chemokine or chemokine receptor

Due to the redundant characteristic of chemokines, targeted deletion of chemokine receptors therefore has been proved a useful tool for determining the distinct biological roles of these molecules *in vivo*. Analysis of mice in which the gene coding a single chemokine or receptor has been functionally deleted has already produced interesting information in the previous studies.

Targeted deletion of the gene for murine CXCR2 does not result in obvious changes in the development of the mouse organs, though the $CXCR2^{-/-}$ mouse is compromised with regard to its ability to resist infection, heal wounds, and maintain homeostasis when challenged with microbes and/or chemicals (34). In CXCR2 deficient mice, CCR1 expressed on neutrophils compensates the absence of CXCR2 (35). Recently, the murine sequence of CXCR1 was deposited into the GenBank database, though it is far less characterized. This may bring forward the curiosity of researchers if CXCR1 will reinforce the roles of CXCR2, as such happens in human, neutrophils can still be recruited through CXCR1 activated by CXCL8 or CXCL6 when CXCR2 was deficient (36).

Though no abnormal phenotypic change was found thymocyte development proceeds normally (33). However, in detail investigation of thymus-specific deletion of CXCR4 *in vivo* disclosed failed cortical localization of early lymphoid progenitors to the proper tissue regions of the thymus (8). These $CXCR4^{-/-}$ mice exhibited developmental defects in immune, circulatory and nervous systems and died prenatally, implicating a role for chemokines in embryonic development. These observations were similar to that made in CXCL12-knockout mice, confirming the receptor-ligand specificity, *in vivo*. (32, 33, 37).

T cell development appeared normal in adult $CCR9^{-/-}$ mice. In addition, thymocyte selection of αβ-lineage T cells was unaffected in $CCR9^{-/-}$ mice, even though most DP thymocytes expressed CCR9 at high levels and responded to CCL25. However, the results of competitive bone marrow transplantation experiments demonstrated that $CCR9^{-/-}$ bone marrow cells had a reduced capacity to repopulate the thymus compared with bone marrow cells from wild type mice. These results suggest that CCR9 may be involved in regulating the migration of progenitor cells to the thymus or the retention of T progenitor cells in the thymus (18).

CCR7 knockout and *plt* (failed to produce CCL21, one of the ligand to CCR7) mice show normal thymocyte development and export, though with a defect of T and B cells homing, indicating that CCL21/CCR7 is not essential to thymocyte emigration (38-40).

The aim of knockout is to ablate the function of the targeted gene in order to discern its role *in vivo*. However, the fact that a specific mutation has been present in the mouse from the time of its conception may lead to false conclusions, because of the inability to distinguish between phenotypic changes due to the mutation itself and changes caused by adaptation and compensation for the mutation. In this respect, knockout mice may have important differences in phenotype when compared with wild type mice treated with a specific chemokine receptor antagonist, or give unexpected results in animal models of disease. Yet in spite of these caveats, most experiments performed have yielded valuable information on the biological activity of specific chemokine receptors, and show that in spite of the redundancy observed *in vitro*, they have surprisingly specific effects *in vivo*.

To a large extent, the phenotype of the knockout animal

can usually be anticipated by previous knowledge of the gene function *in vitro*, but in many cases, the knockout has lead to an unexpected phenotype. For more extensive analysis, the reader is referred to recent reviews (41). No serious defect of thymocyte development was found in chemokine or receptor deficient mice echoed the redundancy of chemokine. It would be an alternative means to make more than one chemokine or chemokine receptor deletion to further unravel which chemokine or receptor acts as dominant effector molecule.

Complicated network-regulation for the roles of chemokines in thymocyte development

The expression of chemokines and chemokine receptors is procedurally in a temporal manner at specific stages of thymocyte differentiation to allow a programmed response of traveling thymocytes to specific chemokines. The expression levels of CXCR4 altered along with the T cell maturation. Most c-kit$^+$ hematopoietic precursors in fetal liver of the 14.5 fetal day were CXCR4$^-$, while c-kit$^+$ DN in the embryo were positive for CXCR4. The receptor expression increased along with T cell maturation up to DP cell stage. Afterwards, CXCR4 expression was down-modulated after positive selection; CD69$^+$CD3^{high} DP and CD3^{high} SP thymocytes were CXCR4 negative or low. Induction of CCR7 expression in thymocytes requires both ERK signal and Ca^{2+} signal (42). In isolated CCR7$^-$DP thymocytes, stimulation with a combination of ionomycin and PKC activator PMA induced CCR7 expression and CD8 down-regulation. These changes were inhibited by U0126, an inhibitor of the ERKK/MEK pathway. The transfectants expressing a constitutively active form of the MEK kinase Raf-1 became CD4$^+$CD8$^+$CCR7$^+$ upon stimulation with ionomycin alone. Thus, Raf-1-mediated signals and Ca^{2+}-dependent signals are essential to induce CCR7 expression in DP thymocytes as well as their differentiation. The activation of thymocytes by CCL4 appeared to be a direct, receptor-mediated event as evidenced by the rapid mobilization of intracellular calcium, increase in protein phosphorylation on tyrosine, and activation of MAPK pathway (20).

The switch from the use of one type of receptor to another is determined by the capacity of the first to undergo ligand-induced desensitization. This process is mediated by specific GRK kinases that phosphorylate the engaged receptors, resulting in their binding to β-arrestin and their localization within clathrin coated pits. Thus, it is not surprising that, as a general rule, leukocytes express several types of chemokine receptors.

Chemokine receptor is coupled with pertussis toxin (PTX)-sensitive G protein (43, 44). Therefore, PTX can block the chemokine-mediated migration of cells irrespective of the redundancy of chemokines and chemokine receptor systems. In mice treated with PTX for 2.5 days, the thymus volume decreased and the number of thymocytes reduced significantly. Suzuki et al. (12) found that PTX treatment decreased the cellularity of thymus, especially in the cortex. Moreover, it perturbed the normal distribution of SP cells and resulted in the accumulation of SP cells in the cortex. After PTX treatment, CD4$^+$ SP cells bore a decreased level of CXCR4 and a high level of CCR7 gene expression. Positively selected SP cells (or their precursors) were unable to go through the cortico-medullary junction and remained in the cortex. Thus, it suggests that a PTX sensitive process probably induced by chemokines is indispensable for trafficking across the cortico-medullary junction.

Expression of chemokines and chemokine receptors is regulated in three aspects, i.e. chemokine/receptor coding gene transcription, mRNA stability and post- translational modification; all of which are affected by inflammatory and stimulatory cytokines. Elevation of NF-κB can be detected during the transcription of chemokines, for example, expression of CCL2, CCL5 and CXCL8 have been investigated to be regulated through the NF-κB pathway (36). Chemokine and chemokine receptor mRNA stability is regulated by different, even opposing mechanisms. A pro-inflammatory stimulus on the one hand increases the mRNA stability of chemokines such as CXCL1 or CXCL8 (45); on the other hand it induces the rapid degradation of the corresponding receptors. CCR9 was found to have two alternative mRNA splicing, resulted in two distinct proteins (CCR9A and CCR9B) of different activities (46).

The thymic microenvironment is a complicated, subtly organized residence for T lymphocyte development, where showing a film of thymopoiesis: the lineage decisions taking place during this development, the selection processes responsible for shaping the T cell antigen-receptor repertoire, the interactions with the stromal components and the signal transduction pathways which transform the interactions with the thymic microenvironment into cellular responses of survival, proliferation, differentiation, and importantly, of cell death. Multiplicate adhesive molecules and cytokines, and various types of interactions make it an immune network characterized by pleiotropy, redundancy, synergy and antagonism. CXCL12 plus fibronectin or laminin induced thymocyte migration in transwell chambers is greater than that elicited by the chemokine alone (47); Savino et al. found that the chemotactic activity of CXCL12 for cortical thymocytes was enhanced more than 10-fold by laminin or fibronectin, compared with that for the medullary thymocytes, implying a relationship of laminin and fibronectin with the migration of cortical thymocytes. Moreover, the interplay of chemokine and adhesive molecules also takes place under the regulation through matrix metalloproteinase (MMP). MMP-9, for example, downregulated the expression and activity of CXCL12 (48). Eotaxin stimulates the secretion of CCL4 from human thymocytes, and expression of CCR3 in SP cells, suggesting a complex interplay within the thymus-expressed chemokines (49). Stimulated DPs release cytokines, such as IFN-γ and thereby induce the expression of the CXCR3 ligand CXCL9 in stromal cells (50).

Another consideration is the competition of chemo-attractant signals with antigen-receptor signals, with the evidence that CCL19, CCL21 and CXCL10 overcome an antigen-receptor signal and attract cells, whereas CCL22 and CXCL12 don't. This may be hypothesized that CCL22 attracts thymocytes into contact with thymic antigen-presenting cells whereas CCL19 functions to move the

cells away after a period of antigen-receptor engagement (51).

After the exerting of their appropriate functions, the chemokines/receptors appear to be cleared through proteolysis or by adsorption to binding proteins present on erythrocytes. But the question is who will act as the "wire-puller" behind the curtain to control the metabolism of a certain chemokine or receptor. Now a complicated network system appears to participate the regulation of the expression and functions of chemokines/receptors. In mice, recent thymic emigrants (RTEs) begin to seed the peripheral T cell pool late in the embryogenesis and continue to be exported from thymus at a rate of 1%-2% of thymocytes per day, and this export persists through whole life. With the knowledge that the newly input progenitors are constantly reinforced from the bone marrow, we may be more curious about the panoramic sketch of chemokines from stages of embryogenesis to adult, till the senescent.

Chemokines exhibit high similarity between sub-families and homology across species, indicating an evolutionary conservation. Hughes et al. (52) investigated the genetic relation among chemokines and chemokine receptors and found co-evolution of chemokines and the corresponding receptors by clustering and calculating the genetic distances among various species of mammalian chemokines and chemokine receptors.

Seemingly, the roles of chemokines and receptors in thymopoiesis are redundant and promiscuous. But in fact, the serial checkpoint events of thymocyte development appear to be severally guarded by certain specific switches of chemokine/receptor pairs. Loss of CXCR4 in DP, for example, facilitates the movement of maturing thymocytes among the outer cortex. However, CCR7/CCL21 plays a pivotal role in the homing of naive T cells to the peripheral lymphoid organs.

In this review, we mainly discussed the observation of chemokines and chemokine receptors within the thymus-windowed development process. In fact, prior to the intra-thymic stages and posterior to the migration out of the thymus, there are also roles of chemokines to ensure the supply of progenitors into thymus and the homeostatic output of mature SP thymocytes.

Perspectives

Thymus is an intriguing inche with subtle regulation on thymocyte maturation, going through positive and negative selection, and the most functional thymocytes with self-tolerance and non-self recognition potency survive and migrate to the periphery.

In many contexts, chemokines and their receptors are veiled with enigma and confusion, whereas, this happens to be the charm of these miscellaneous small molecules to retain "attractive", which has dual meanings—attractive to the "spellbound" targeted cells (inducing the cells to expressing cognate receptors), just as their mutual name-"chemokine" implies, which is denominated from the phrase of "chemotactic cytokine"; at the same time, having another meaning of being attractive to the investigators by showing new members and flaunting undiscovered actions, compelling the questers to exert more advanced techniques to uncover their cryptic veils, so as to dissect them in study of lymphoid organogenesis, and in disease treatment.

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