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Review

Molecular and cellular mechanisms of T Cell development

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Abstract. The thymus is central to the establishment of a functioning immune system. Here is the place where T cells mature from hematopoietic progenitors, driven by mutual interactions of stromal cells and the developing thymocytes. As a result, different types of T cells are generated, all of which have been carefully selected for the ability to act in host defense towards non-self and against the potential to mount pathogenic self-reactive autoimmune responses. In this review we summarize our present knowlege on 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, also of cell death.

Key words. Thymopoiesis; thymocyte selection; lineage decision; rat; notch; glucocorticoids; thymic stroma.

Introduction

T cells form a major branch of the aquired immune system. Their most distinguishing feature as compared with the antibody-producing B cells is that their antigen receptors, also called T cell receptors (TCRs), are not secreted during the effector phase, thereby restricting interactions with antigen to direct cell-cell contacts. T cells recognize small peptides bound into specialized groves of polymorphic antigen-presenting molecules, encoded by the major histocompatibility complex, the MHC class I and MHC class II molecules. The major class of T cells, which bear heterodimeric TCRs consisting of an α and a β chain (αβTCR), is further subdivided into CD4 T cells which recognize MHC II-bound peptides derived from extracellular sources, and CD8 T cells which recognize MHC I-bound peptides derived from proteins sythesized within the cell such as viral antigens. Because αβTCRs recognize unique combinations of polymorphic self-MHC molecules with foreign peptides, their mode of antigen recognition is 'MHC restricted'. Accordingly, only T cells able to use the individual´s own MHC molecules for antigen recognition are useful for the immune system. While CD4 T cells regulate the immune response of B cells, other T cells and the activity of cells of the innate immune system, the main function of CD8 T cells is to lyse infected or transformed cells. Finally, a minor class of T cells expressing distinct TCR chains, termed γ and δ, also develops in the thymus, which is heterogeneous with regard to function and mode of antigen recognition.

T cells, which like all leukocytes are derived from pluripotent hematopoietic stem cells, derive their name from their maturation in the thymus. This bilobed organ, which is located just above the heart, provides a highly specialized microenvironment that guarantees proper development of T cells on their way to immunocompetent lymphocytes. Different cell types in the thymus, including epithelial and dendritic cells, contribute to this process, and consequently the integral structure of

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the thymus is indispensable for T cell development. As thymocytes mature, they move from the outer cortex to the medulla while undergoing a series of phenotypic, genetic and functional changes. In the course of this development, expression of cell surface receptors or 'markers' is altered, and new functional properties are acquired. Importantly, random rearrangements of the TCR loci take place at different steps during this process, forming the basis for the antigenic diversity of the T cell repertoire. Since these rearrangements also yield many T cells with useless or even dangerous specificities, developing thymocytes have to undergo a twofold selection process. Any cell that is unable to recognize self-MHC at least with a low affinity is unlikely to be suited for self-MHC-restricted recognition of foreign antigenic peptides and hence useless for the immune system; these cells die 'by neglect' through programmed cell death. Those expressing TCRs with high affinity for self-MHC/self-peptide complexes are potential mediators of autoimmunity and consequently eliminated by apoptosis in a process called negative selection. Only those cells which recognize self-MHC/self-peptide complexes with moderate affinity are potentially useful in self-restricted recognition of foreign antigens and therefore allowed to undergo 'positive selection' and functional maturation.

During intrathymic T cell development, immature precursors can adopt various cell fates leading to the generation of the different T cell subsets mentioned, but also of some other, minor cell types. Once such a lineage decision is made, the developing thymocyte is 'commited'. While lineage decision, selection and commitment are important cellular hallmarks of T cell development which are traced by marker acquisition and functional assays, the underlying molecular mechanisms are more difficult to resolve. They have, however, gained increasing attention during the last decade, leading to an emerging but as yet incomplete model of cell fate regulation in the thymus. Signal transduction pathways, including those driven by the Notch family of transmembrane receptors, the intracellular glucocorticoid receptor and the mitogen-activated protein (MAP)-kinase casades, determine the fate of the developing thymocyte in response to external stimuli such as cell surface molecules, most prominently the TCR itself, cytokines and hormones. In this review we summarize some of the present knowledge on the differentiation of thymocytes from hematopoietic stem cells into mature T cells, the mechanism of selection and lineage commitment as well as the architecture of the thymus. We have tried to provide a broad picture on how the thymus contributes to the generation of the enormous amount of T cells with a repertoire ready to battle inflammation and infections without attacking the organism itself, thereby doing their best to protect the body from every harm imaginable.

Hematopoiesis

Hematopoietic stem cells and lymphoid progenitors

Thymic stem cells originate in different hematopoietic sites, depending on the stage of embryonic development. Whereas in the fetus the yolk sac and liver are major sites of hematopoiesis, in the adult it primarily occurs in the bone marrow (BM). Here hematopoietic stem cells (HSCs) reside which can give rise to all lymphocyte populations and other blood cell types [1]. In mice HSCs which make up ~0.05% of all BM cells are characterized as Thy-1.1^{lo}Lin$^{-/lo}$Sca-1^{+} based on cell surface expression [2]. These cells have been further subdivided into long-term and short-term HSCs, relating to their different capacities for self-renewal, and a third group classified as multipotent progenitors, which do not self-renew.

To date it is still unclear whether the three lymphoid lineages, T, B and NK cells, develop from common lymphoid progenitors (CLPs) or whether they are derived from either lymphoid-restricted stem cells or multipotent progenitors. However, at least in the adult murine bone marrow, a population of Lin^{-}IL -7R^{+}Thy-1^{-}Sca-1^{lo}c-Kit^{lo} cells was shown to contain CLPs on the basis of their capacity to develop into lymphoid cells while being unable to support myeloid differentiation [1]. Further support for the existence of CLPs came from the finding that mutation in genes such as *Ikaros* lead to the absence of all three lymphoid lineages [3–4]. Finally, the earliest T-lineage precursors identified in the adult murine thymus, a minute population of Thy-1^{lo} CD4^{lo} CD44^{+}CD25^{-} cells, still displays the capacity to develop into lymphoid, but not myeloid cells [5–6]. However, in the context of a normal thymic microenvironment these progenitors mainly give rise to the development of T cells. Taken together, these data led to the model that HSCs in the BM differentiate into CLPs and subsequently become restricted to the lymphoid lineage only upon expression of IL-7R. After succesfully reaching the thymic microenvironment, these progenitors then differentiate into T cells, although the earliest ones still have the capacity to become B cells, NK cells and dendritic cells (DCs).

CLP-independent thymopoiesis

As outlined above, the present model suggests that early T-lineage progenitors (ETPs) in the thymus develop from BM-derived CLPs [1]. However, a recent report challenged this view by showing that thymopoiesis can proceed independently of CLPs, suggesting that ETPs are derived from HSCs as a separate lineage [7]. In particular, BM-derived CLPs are Sca-1^{lo}c-Kit^{lo} IL-7R^{+}, whereas ETPs were found to be Sca-1^{+}c-Kit^{hi} IL-7R^{-}. Consequently, both cell types differ in their responsiveness to IL-7. In further support of this model, ETPs purified from the thymus possess a superior capacity to produce

cells of the T cell lineage as compared with BM-derived CLPs, while the generation of B cells by ETPs was greatly reduced. Furthermore, adult *Ikaros*-deficient mice that are devoid of CLPs still possess normal numbers of ETPs in the thymus, allowing some aberrant thymopoiesis to proceed. From these results it was concluded that the pathways leading to T and B cells diverge from HSCs at a rather early stage. According to this model, CLPs would be the most efficient B-lineage progenitors, although retaining the capacity to generate T cells when placed in a thymic microenvironment. In contrast, ETPs would constitute a separate type of progenitor that gives rise to the majority of T cells in the thymus. In the light of these new findings, the influence of signaling pathways on lineage decisions in thymopoiesis such as the one being directed by Notch receptors (see below) also needs to be revisited.

T cell development in the thymus

Development of T cell lineages

Thymocytes can be subdivided into four main subsets based on the CD4 and CD8 coreceptor expression (fig. 1). The $CD4^-CD8^-$ double-negative (DN) cells which represent the most immature cell type among all thymocytes are located in the outer cortical areas of the thymus [8]. When differentiation of DN thymocytes further proceeds, these cells start to rearrange the TCRβ (as well as the γ and δ) gene loci, which is the first step towards the expression of a functional TCR. Importantly, successful rearrangement of the TCRβ chain is a prerequisite for survival and subsequent proliferation of thymocytes of the αβ-lineage. Thus, this step is considered the first checkpoint of T cell maturation. Mainstream development then further proceeds predominantly through an $\alpha\beta TCR^{low}$ immature single-positive stage (iCD8/iCD4) to the major $CD4^+CD8^+$ double-positive (DP) subset of thymocytes [9]. DP cells expressing intermediate levels of αβTCR on the surface are subject to a selection process which results in the induction of apoptosis in more than 90% of the newly generated thymocytes. The complex mechanisms operative at this late DP stage which guarantee the generation of an optimal T cell repertoire represent the second important checkpoint of thymocyte development (fig. 1). Finally, either CD4 or CD8 coreceptors are downregulated, and the mature MHC-restricted single-positive (SP) T cells localize to the thymic medulla where they undergo

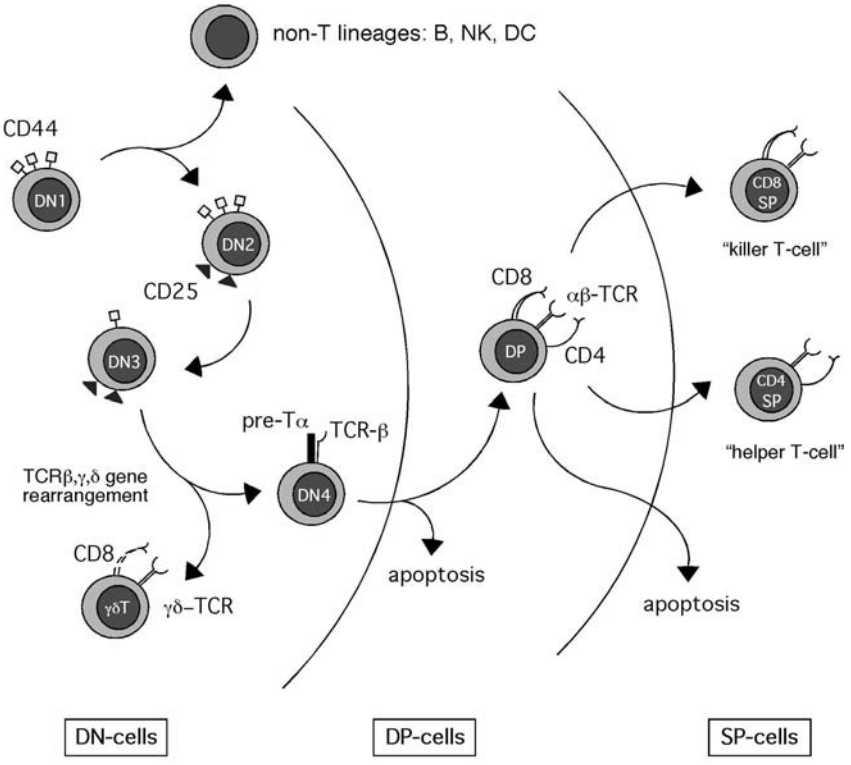

Figure 1. Model of thymocyte development. DN cells differentiate through a series of well-defined developmental stages classified through the expression of CD44 and CD25 cell surface molecules. In addition, other lymphoid lineages such as B cells, NK cells and dendritic cells (DCs) as well as T cells of the minor class expressing γδTCRs may be generated within the thymus. At the two checkpoints of thymocyte development, one in the late DN stage and one at the end of the DP stage, 'useless' T cell precursors are blocked from further progression and undergo apoptosis. Finally, positively selected and lineage-committed immunocompetent $CD4^+$ and $CD8^+$ SP cells are generated which migrate to the periphery.

several rounds of cell divisions [10–11] before they finally migrate to peripheral lymphoid organs (fig. 1).

The DN cell compartment

The earliest progenitor cells entering the thymus from the BM or other sites of hematopoiesis are to be found within the $CD4^{-}CD8^{-}$ DN (or rather the $CD8^{-}CD4^{lo}$) population of thymocytes. In mice the DN compartment can be subdivided into four stages by the consecutive expression and disappearance of CD44 and CD25 (IL2-R α chain) [12] (fig. 1). Accordingly, the earliest subtype of DN cells in mice is classified as $CD44^{+}CD25^{-}$ (DN1). Indeed, it is a subset of these cells which is considered to contain the CLPs and/or ETPs. Differentiation then further proceeds via a $CD44^{+}CD25^{+}$ stage (DN2), while the TCR genes still largely remain in germ line configuration. Afterwards, CD44 is markedly downregulated, and the $CD44^{-}CD25^{+}$ cells (DN3) start to rearrange their TCRβ, γ and δ genes. In mainstream development of αβ T cells, the TCRβ chain is subsequently expressed together with the surrogate pre-Tα chain and CD3 components on the cell surface forming the pre-TCR complex. At the same time, expression of both CD44 and CD25, is extinguished from these cells (DN4). Although pre-TCR expression is about 50–100-fold lower than that of TCR on mature cells, it represents a critical checkpoint in T cell differentiation, as failure to develop a functional pre-TCR results in apoptosis [13]. Signaling from the pre-TCR, known as β selection, confers survival, inhibition of further DNA recombination at the TCRB gene locus (allelic exclusion), and signals for significant cell division and differentiation of DN3 and DN4 cells. One feature of the pre-TCR, in contrast to α/β or γ/δ TCRs, is that expression in immature cells results in its spontaneous inclusion into membrane glycolipid-enriched microdomains, so-called lipid rafts, and constitutive downstream signaling [14] without apparent requirement for putative ligand interactions on thymic stroma cells [15].

Whereas this subdivision of DN cells in the mouse is well established, a similar classification of this compartment has failed both in rats and humans [16–17]. This not only questions the relevance of the expression of low-affinity IL2-Rα on early thymocytes (in the absence of significant levels of IL-2Rβ), but also made it necessary to find alternative subdivisions for DN cells in other species. In the rat this was achieved on the basis of the sequential expression of the cell surface molecules CD45RC and CD2 [16]. Here, the development of DN cells proceeds via $CD45RC^{+}CD2^{low}$ and $CD45RC^{+}CD2^{high}$ cells to a $CD45RC^{-}CD2^{high}$ stage. These cells then start to rearrange their TCR gene loci, and during mainstream differentiation they finally present the TCRβ chain on the cell surface as described above for mice. Similar to mice the earliest thymic progenitor cells are found in the first $CD45RC^{+}$ subsets of DN cells. Classified based on the epitope recognition by the Ox22 antibody, these $Ox22^{+}$ cells where shown to contain the progenitor cells according to their ability to repopulate the thymus of sublethally irradiated rats [16]. Finally, DN cells in the human are subdivided based on the expression of yet another set of markers, namely CD34 and CD1A. In particular, upregulation of CD1A is strongly associated with T cell commitment, suggesting that this represents a hallmark in early human T cell development [17].

γ/δ T cells and other hematopoietic lineages

Besides αβ T cells, cells expressing a γδ TCR also develop in the thymus. This thymocyte population is particularly prominent during fetal development, while its frequency strongly declines until birth. Commitment to this second T cell lineage, which accounts for less than 5% of thymocytes in adults, appears to depend on a productive rearrangement of both the γ and δ chain genes [18]. It was found that αβ T cells which also possess rearranged γ and δ TCR genes are selectively depleted of in-frame rearrangements in these loci. This suggests that completion of a productive γ and δ chain gene rearrangement diverts the two T cell lineages and favors a γδ T cell fate. In addition, evidence has been presented which involves Notch signaling in the lineage decision between the two T cell populations [19]. In agreement with the model described above, it was suggested that the presence of either in-frame β or γδ TCR gene rearrangements determines whether a precursor cell receives a Notch signal. This, in turn, would then drive development into either of the two T cell lineages.

Although the thymus is primarily the site of T cell maturation, other hematopoietic cells can develop there. In particular, it is known that early progenitors in the thymus possess the potential to become not only T but also B cells, a lineage decision which is predominantly under the control of Notch transmembrane receptors (see below). In addition, data have also been presented suggesting the existence of common intrathymic NK/T and T/DC precursor cells downstream of the T/B cell decision [20–21]. Although it was shown that NK cell development is thymus independent, evidence has accumulated that T and NK cells share a common precursor. Using clonal analysis it was demonstrated that bipotent NK/T precursor cells exist in the mouse fetal thymus which can give rise not only to cells of the T but also the NK lineage [22]. Although similar findings in rats and humans suggest that NK cells generated within the thymus may play a physiological role under certain conditions, the signals directing the choice between both lineages have not yet been delineated [23].

DCs originate from BM cells, but it is unclear whether they mature within the BM itself or differentiate in other

organs. Two subsets of DCs can be distinguished, one of myeloid and the other of lymphoid origin. Thymic DCs which mainly function to mediate negative selection of autoreactive thymocytes (see below) are exclusively constituted of cells of lymphoid origin. The development of these DCs and T cells has been linked by a common intrathymic precursor cell [21]. Several findings favor the existence of such a common T/DC precursor cell downstream of the common T/B precursor. However, experiments using Notch1-deficient BM cells have rather favored a model where T cells and DCs develop from independent intrathymic precursor cells [24]. Thus, although it appears to be clear that precursor cells exist in the developing thymus which have the potential to become B cells, NK cells and lymphoid DCs, their exact lineage relationship remains elusive.

Lymphostromal interactions in thymus development

Thymus organogenesis

The adult mammalian thymus is a bilobed structure located just above the heart. Each lobe is surrounded by a capsule formed by dense connective tissue of mesenchymal origin with septae pushing into the organ. The main structural component is formed by a network of thymic epithelial cells representing the stroma. This network is completely filled with large numbers of lymphoid cells, as well as macrophages and DCs which are scattered throughout the organ.

The thymic stroma is predominantly formed by thymic epithelial cells which originate from the endoderm of the pharyngeal pouch. During ontogeny, mesenchymal cells from the neural crest invaginate the epithelial bud, which subsequently begins to differentiate into cortical and medullary areas. Among the few known molecular regulators of thymic epithelial development is the nude gene locus [25]. Nude mice which are mutant for the transcription factor FoxN1 initially display a rather normal thymus 'anlage', but upon onset of lymphocyte colonization, subsequent development and differentiation into the typical thymic architecture is severely perturbed. Whereas transcriptional control of this important gene remained an unsolved issue for a long time, recent work has identified Wnt proteins as crucial regulators of FoxN1 expression, and consequently, thymic function and morphogenesis [26]. Wnt proteins constitute a group of secreted glycoproteins which bind to receptors of the Frizzled family of cell surface molecules. Downstream signaling of Frizzled receptors leads to the dephosphorylation of β-catenin and subsequent activation of the transcription factors TCF-1 (T cell factor 1) and Lef1 (lymphoid enhancing factor 1) [27]. In the thymus, activation of Wnt signaling induces FoxN1 expression in TECs independent of de novo protein synthesis. This argues that FoxN1 is a direct target of this pathway and that signaling via Wnt proteins is critical for thymic development. In a different approach, transgenic expression of constitutively active β-catenin in thymocytes demonstrated that besides its role in the thymic stroma, the Wnt signaling pathway is also required for proper thymocyte development [28]. In particular, enforced β-catenin signaling in this setup led to the generation of DP and some SP T cells lacking αβTCR expression even in the absence of pre-TCR signaling. Taken together, these and other experiments assign the Wnt-signaling pathway a central role in both the development of the thymic stroma and thymocyte differentiation. In addition to Wnt proteins, two other major signaling pathways located at the interface of TECs and thymocytes have been found to control T cell development. Whereas signaling via Notch transmembrane receptors will be discussed later in this review, the one involving Sonic hedgehog (Shh) will be shortly outlined. Hedgehog proteins expressed by TECs bind to their transmembrane receptors Patched and Smoothened, primarily found on immature thymocytes. When using a neutralizing antibody against Shh, differentiation of DN thymocytes into DP cells was increased. Similarily, recombinant Shh proteins arrest thymocyte development at the DN stage just after initiation of TCRβ rearragment has occurred [29]. These data clearly implicte Shh signaling in the control of thymocyte development exerted by the stroma and highlight again the importance of TECs for proper T cell differentiation in the thymus.

Homeostasis of epithelial tissues is often maintained by constant self-renewal of epithelial stem cells. Although it is still under debate whether a common stem cell giving rise to cortical as well as medullary epithelial lineages resides in the thymus, convincing evidence in favor of the existence of such a thymic progenitor comes from identification of the cell surface glycoprotein MTS24 [30–31]. Using monoclonal antibodies, it could be demonstrated that differential expression of this 'marker' defines a subpopulation of embryonic TECs that upon grafting give rise to a functional epithelial stromal compartment able to support normal thymopoiesis. This clearly argues for a common origin of both subsets of TECs. Besides the epithelium, mesenchymal cells represent the second important stromal cell type which influences thymocyte development [32]. This heterogenous cell population is derived from the neural crest and predominantly locates to the capsule and the septae. Based on the current knowledge, a two-stage model for the role of mesenchyme in thymopoiesis was proposed. First, these cells appear to be required for the initial stage of thymic formation when the surrounding mesenchyme invaginates into the epithelial primordium. Later in thymopoiesis they also become involved in thymocyte development. In particular, mesenchymal fibroblasts were found to be essential in reaggregate thymus organ cultures (RTOCs) for normal thy-

mocyte maturation [32]. It was hypothesized that growth factors and cytokines as well as direct cell-cell contact may play a role in this process, similar to the requirement of BM stromal cells for the development of hematopoietic stem cells.

Functional aspects of the cortical compartment

The thymic cortex represents a three-dimensional network of MHCII-positive epithelial cells which serves as a microenvironment for the development of immature thymocytes. This environment appears highly important for supporting the development of mature T cells from precursor cells (fig. 2). However, thymocytes are also required for normal development of the thymic stroma, as highlighted by the analysis of CD3ε transgenic mice [33]. These animals, which encounter a profound block at the earliest stage of T cell development, also lack the three-dimensional epithelial structure of the thymus. This finding strengthens the notion that lymphocyte and stromal development in the thymus support each other, establishing a process known today as 'thymic cross-talk' [34].

During embryonic development thymus colonization occurs prior to vascularization, indicating that the lymphoid progenitor cells have to enter the thymus primordium through the mesenchymal capsule. In contrast, in adults the lymphoid progenitor cells are blood-born, leaving the vessels at the corticomedullary junction. Once the progenitor cells have entered the thymus, they localize to the outer cortical regions and subsequently pass through a series of developmental steps (fig. 2). It appears that it is the thymic microenvironment bearing cell-surface molecules and secreting soluble factors which then drives the development of these precursor cells into the T cell lineage. In particular, members of the Notch family and their ligands are good candidates for determining the fate of the precursor cells in the thymus [35]. This will be discussed in more detail later in this review.

A particularly important feature of cortical epithelial cells is their unique ability to drive positive selection of the T cell receptor repertoire [36]. On the one hand this is achieved by providing peptide-bound MHCI and MHCII ligands for the αβ T cell receptors of the developing thymocytes (fig. 2); on the other hand, these cells also secrete survival factors which enhance viability. In addition, expression of accessory and costimulatory molecules on thymic epithelial cells is hypothesized to play an important role in positive selection. However, the exact contribution of these molecules is so far unknown.

The medullary microenvironment

As mentioned above, positive selection of maturing T cells takes place in the cortex, with the epithelial cells

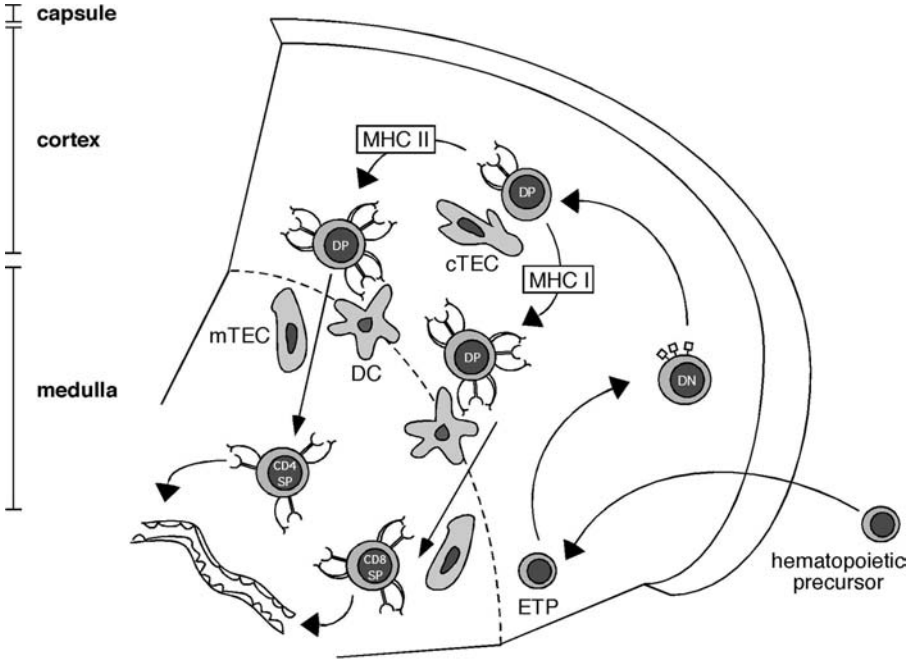

Figure 2. Lymphostromal interactions taking place during thymocyte development. Derived from hematopoietic precursors, early T lineage progenitors (ETPs) give rise to DN thymocytes developing in the thymic cortex. Positive selection mainly takes place through interaction of DP cells with cortical thymic epithelial cells (cTECs) bearing MHC class I and MHC class II molecules on their surface. In contrast, negative selection predominantly is achieved through interaction of DP cells with dendritic cells (DCs) as well as medullary thymic epithelial cells (mTECs) located at the corticomedullary junction. Subsequently, SP cells locate to the thymic medulla from where they finally leave the organ via the circulation.

located here being the most efficient mediators of this process. However, cortical epithelial cells presumably lack the ability to mediate negative selection. This appears to occur at the corticomedullary junction, mainly mediated by BM-derived MHCII$^+$ DCs, which pick up and present circulating antigens (fig. 2). Thus, the thymic medulla is the place where newly generated SP CD4$^+$ and CD8$^+$ thymocytes locate after having gone through both selection processes. In addition, medullary epithelial cells also seem to play an important role in achieving self-tolerance (fig. 2). This model is supported by the finding that they express many proteins previously thought to be tissue specific [37–38]. Although the ability to induce self-tolerance is a feature shared between thymic DCs and medullary epithelial cells, the induction of self-tolerance by the latter appears also to involve nondeletional mechanisms such as induction of anergy [39].

After having gone through selection, SP thymocytes usually reside in the thymic medulla for several days. This is consistent with the medullary epithelial cells playing a role beyond inducing self-tolerance. However, whether changes in the expression of cell-surface molecules observed in SP thymocytes while residing in the medulla are causally linked to interactions with stromal cells or whether they occur cell autonomously is presently unknown. Another possible function of the medullary stroma might be located in the TCR-independent expansion of intrathymic T cells, which comprises at least six rounds of cell division in an IL-7-dependent manner [10–11].

Is the thymic stroma indispensable for T cell development?

For many years it was thought that the presence of thymic stroma is strictly required for normal thymocyte development. In the absence of the thymic microenvironment, normal development of T cells could not be supported. Therefore, fetal thymic organ culture (FTOC) was established, delivering exactly the environment required to provide the necessary cellular interactions and soluble factors for proper thymocyte development [40]. Furthermore, this system was also used to repopulate thymus rudiments which had been depleted of lymphocytes by 2-deoxyguanosine treatment [41]. Finally, by reaggregating thymic epithelial cells, mesenchymal fibroblasts and defined subsets of lymphocytes, T cell development could also be reconstituted [42]. However, the notion that the thymic stroma is indispensable was recently challenged by showing that expression of the Notch ligand Delta-like 1 on the OP9 BM stromal cell line supports T cell development from hematopoietic progenitors into mature T cells [43]. This is another important step towards elucidating the molecules and signals which are relevant for proper thymocyte development, leading the way to new cell culture scenarios which allow T cell development in the absence of the classical thymic microenvironment.

Thymocyte selection and lineage commitment

The mouse model

As outlined before, the second checkpoint of α/β T cell development occurs at the CD4$^+$CD8$^+$ DP stage. Interaction of $\alpha\beta$TCRs with MHC/self-peptides on thymic epithelial and stromal cells initiates selection processes that lead either to death by neglect, positive or negative selection, and lineage commitment [44]. Although the bias of the unselected TCR repertoire for MHC is quite high due to the intrinsic MHC reactivity of germ line-encoded V-segments [45–47], the majority of DP thymocytes (around 90%) will die by death by neglect due to lacking or inefficient interaction of their TCR with self-MHC. According to the quantitative/avidity model of selection [48–49], DP cells with TCRs interacting with moderate affinity to self-ligands will be positively selected and further differentiate into mature functional T cells. High-affinity interaction with self-MHC, which would be equivalent to autoreactivity in the periphery, destines DP cells to apoptosis (negative selection) [50]. Lineage commitment entails the shutdown of one of the coreceptors and the activation or maintenance of genetic programmes that link the specificity of the TCR for either MHC class II or MHC class I molecules with the functional properties of CD4$^+$ T-helper or CD8$^+$ cytotoxic T cells [8].

In recent years the transcriptional control elements of the mouse CD4 and CD8 loci, the only indicators of lineage commitment presently known, have been extensively studied, and the crucial cis-regulatory elements for the expression of CD4 and CD8 in DP and mature T cells have been identified. Trans-acting factors that are involved in the transcriptional regulation of the coreceptor genes include the basic helix-loop-helix proteins HEB and E2A, Ikaros and the runt-related transcription factors Runx1 and Runx3 [51–52]. Future studies now have to establish other key players and how their expression and activity are controlled by intracellular and extracellular signals such as provided by TCR-MHC-ligand interaction.

TCR recognition of self-ligands on DP cells leads to the generation of intracellular messengers that control selection and lineage commitment. One of the earliest events triggered by TCR ligation is the activation of Src-family kinases, most prominently the tyrosine kinase p56^{lck} (Lck), which phosphorylates the immunoreceptor tyrosine-based activating motifs (ITAMs) of the TCR/CD3ζ chains, thus providing docking sites for the Syk-family kinase ZAP70. Phosphorylation of ZAP70 by Lck in turn enables ZAP70 to phosphorylate the important transmembrane adapter molecule LAT (linker of activation of

T cells). This initiates the cascade of recruitment and activation of further signaling intermediates, including protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K), intracellular calcium mobilization and activation of one or several mitogen-activated protein kinase (MAPK) cascades [53–54]. Many of the molecules employed by αβTCR signaling are also used by the pre-TCR, including Lck-ZAP70-MAPKinase signaling, and elimination of these molecules by gene targeting leads to complete or partial arrest at the pre-TCR checkpoint (see above) [55].

Lck in lineage commitment

A role for Lck in lineage commitment first became obvious when it was shown that Lck associates with CD4 and CD8 coreceptor molecules [56–58], whereby in mouse thymocytes CD4 cytoplasmic domains bind more Lck than CD8α cytoplasmic tails [59]. Coligation of TCR and CD4 or CD8 molecules on MHC ligands likely facilitates optimal signaling by additional recruitment of Lck and LAT, which also associates with both coreceptors [60], thereby changing quantity and/or quality of TCR interaction.

Experiments from mice expressing additional transgenic coreceptor molecules, particularly chimeric CD8-CD4 coreceptors in which the intracellular tail of CD8α was replaced with the cytoplasmic domain of CD4, led to the proposition of the 'strength of signal' model of lineage commitment (fig. 3) [8]. In CD8-CD4 coreceptor transgenic mice DP cells with TCRs specific for MHCI efficiently developed into the CD4 lineage instead of differentiating to CD8 T cells, i.e. they adopted the incorrect cell fate-generating CD4 T cells bearing 'mismatched' TCRs [61]. The notion that 'strong' intrathymic Lck signals as provided by CD4 coligation would instruct DP cells to the CD4 lineage and 'weak' signals as provided by CD8 into the CD8 lineage was further supported by experiments from mice bearing MHCII-restricted TCRs in the absence of CD4 molecules. Rather than failing to mature at all, DP cells from these mice developed into the CD8 lineage [62]. Furthermore, disruption of CD4-MHCII interaction by mutating the CD4 binding site in MHCII molecules blocked the development of $CD4^+$ T cells [63]. Experiments from mice with inducible Lck expression [64] and expression of Lck only at the DP stage [65] confirm that changes in Lck activity influences CD4/CD8 lineage choice. The importance of signal strength, including differential Lck activation, was before supported by studies using in vitro culture systems. Higher and longer exposure of unselected DP cells to the reagents phorbol 12-myristate-13-acetate (PMA) and ionomycin, which activate PKC and induce calcium flux, induced $CD4^+$ T cell maturation but lower concentrations and short-exposure $CD8^+$ T cells [66]. Likewise, in thymic organ culture using CD3/CD4 $F(ab')_2$ bispecific Abs that coligate TCR/CD3 molecules with the CD4 coreceptor, DP cells with specificity for MHCI could efficiently differentiate into mature and functional $CD4^+$ T cells [67].

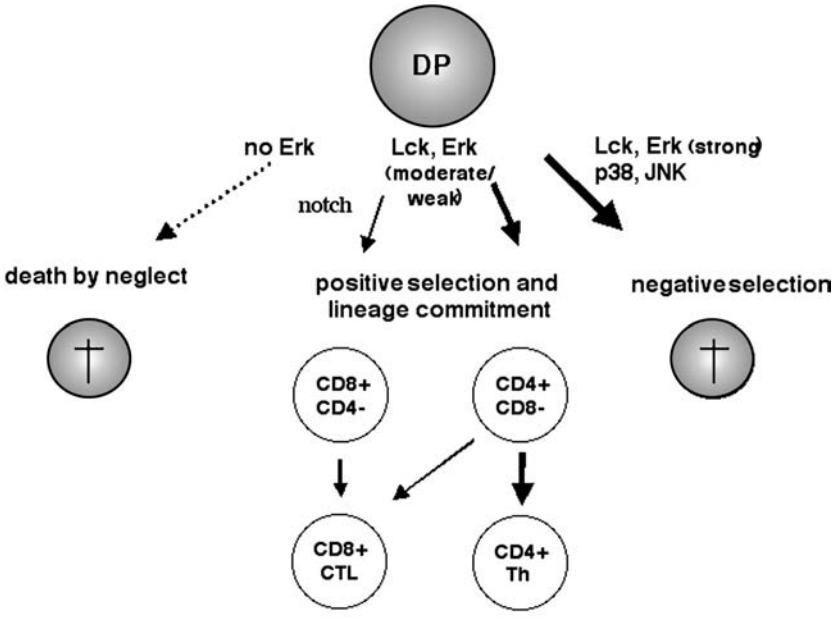

Figure 3. Model of selection and lineage commitment of mouse DP cells. DP cells die by death of neglect in absence of appropriate TCR/MHC interactions and efficient signaling, e.g. via Erk. TCR/self-MHC interactions inducing signaling above a certain threshold initiate selection processes that depending on the strength and duration of signals, lead to positive or negative selection. Negative selection results from 'very strong' signaling via Lck, Erk and activation of the MAKinases p38 and JNK. 'Moderate' signals induce positive selection whereby continued 'stronger' signaling is required for CD4 than for CD8 T cell commitment. Signals provided via Notch receptors may also favor CD8 maturation.

This study also showed that coligation of TCR with CD8 molecules by CD3/CD8α $F(ab')_2$ Abs could induce CD4 maturation, although with less efficiency, presumably because less Lck was recruited into the TCR complex. In the presence of low concentrations of CD3/CD4 or CD3/CD8 $F(ab')_2$ Abs that could not induce CD4 maturation, additional ligation of either CD4 or CD8 molecules strongly promoted CD4 development. Indeed, the latter setup might reflect the in vivo situation more closely and indicates that lineage commitment may depend on the relative level of TCR/CD3 signaling compared with coreceptor Lck signaling [68]. The data also suggest that low TCR-Lck signaling can be strengthened by 'noncognate' interactions of either coreceptor with MHCI or MHCII, which induces additional activation of Lck. Such interactions indeed may contribute to lineage commitment considering data showing that unsialylated CD8 molecules of DP cells can have an adhesion function in noncognate CD8-MHCI interactions [69–70]. In addition, a number of reports have demonstrated that CD4 maturation can be mimicked by antibodies ligating a variety of surface molecules, including targeting thymocytes to thymic cortical epithelium [71], extensive engagement of TCRβ chains [72] or coligation with CD2 [73], CD5, CD28 (which also associate with or recruit Lck) and other molecules [74]. Thus, it seems that in the mouse, CD4 maturation can be fostered by various costimulatory interactions within the 'broader' window of CD4 lineage decision, simultaneously implying that the window for CD8 maturation, either in quality and/or quantity, is relatively narrow. It should be noted that in the rat system, lineage commitment seems to follow different rules [75], as discussed in more detail in one of the following chapters.

Selection of murine $CD8^+$ T cells in thymic organ culture was first demonstrated using altered peptide ligands with antagonist or partial agonist activity [48, 76, 77], which in contrast to agonist peptides cannot fully activate mature T cells to proliferate and secrete significant amounts of cytokines [78]. Monospecific CD3/CD3 $F(ab')_2$ Abs, in contrast to the bispecific Abs mentioned earlier, cannot recruit Lck to the TCR-CD3 complex and signal in mature T cells similar to altered peptide ligands [79]. In thymic organ culture these antibodies can efficiently select mature CD8 T cells even in settings where DP cells express MHCII-restricted TCRs [80]. Under these conditions, addition of CD3/CD3 $F(ab')_2$ Abs simultaneously blocked normal selection of $CD4^+$ T cells, similar to inhibition of CD4 maturation observed using MHCII-binding antagonist peptide [81]. The data with CD3/CD8 and CD3/CD3 $F(ab')_2$ Abs raised the question how TCR-MHCI-CD8 interaction is regulated to prevent efficient recruitment of Lck into the TCR complex. One possibility of differential Lck activation is via alternatively spliced forms of CD8α, so-called CD8α′, that cannot interact with Lck. About half the CD8 molecules on mouse DP thymocytes, but not mature T cells, consist of CD8α′β heterodimers, naturally occurring 'dominant-negative' forms of CD8 that lead to inefficient Lck recruitment during MHCI interaction [82]. In contrast to CD8α transgenes, expression of a CD8 minigene that encodes both α and α′ forms of CD8 is inefficient in promoting the development of mismatched $CD4^+$ T cells with MHCI TCR specificity and even blocks development of such cells on a CD8α transgene background. These data suggest that CD8α′ serves to dampen Lck activation upon MHCI recognition and to favor CD8 cell fate [83]. Although the CD8β chain does not bind Lck, elimination of CD8β by gene targeting [84–85] or overexpression of a tailless CD8β chain [86] strongly reduces selection of CD8 lineage cells, similar to CD8α-deficient mice where MHCI-restricted cytotoxic T cells seem to be completely absent [87]. While the previous data show a role for CD8α and β chains, 'CD8 T cell' maturation can also occur in the absence of CD8 molecules by increasing the affinity of the TCR for the positively selecting ligand, although higher concentrations or higher affinity of peptides was insufficient for switching CD8 to CD4 lineage commitment [88].

The model of lineage instruction by signaling strength of Lck was recently challenged by data from mice expressing CD8-CD4 chimeric coreceptor molecules in the absence of endogenous CD8α coreceptors. On this background CD8-CD4 coreceptors quantitatively promoted the differentiation of MHCI-specific T cells into both CD4 and CD8 lineages. The majority of MHCI-restricted DP cells still differentiated into $CD8^+$ T cells, which apparently seems to contradict instruction into the CD4 lineage by the CD4 tail [89]. Further clues for lineage commitment come from elegant experiments using two-step thymic organ culture systems, which allow discrimination between initial lineage commitment and subsequent signals that could either sustain or alter lineage commitment or might induce cell death. Of particular interest are experiments where DP cells from HY TCR transgenic mice, which bear TCRs recognizing a male-specific antigen presented by MHC class I molecules [90], were exposed in primary culture to male antigen, which induces negative selection in vivo. Cells were then recovered and reaggregated in secondary culture with female stromal cells that lack the deleting antigen and foster maturation into the CD8 lineage in accordance with MHC restriction. When the exposure to male antigen was limited to a few hours, the cells subsequently developed into $CD8^+$ T cells, whereas if DP cells were exposed to HY antigen for more than 14 h, they developed into $CD4^+$ T cells [91]. Thus, the initial temporal encounter with MHC would bias for lineage commitment, and subsequent encounters would confirm the cell fate with long duration signals instructing DP cells into the CD4 lineage and short duration signals into the CD8 lineage. One way to control duration

of signaling seems to be via availability of antigen and quantity of TCR interactions, as stimulation of DP thymocytes with low levels of MHCI ligand can induce CD8 maturation, whereas high ligand concentrations promote CD4 cell fate [92]. In another version of the duration of signal model, known as the 'kinetic signaling' model, DP thymocytes first convert to $CD4^+CD8^-$ intermediate thymocytes after TCR/coreceptor signaling irrespective of the specificity of the stimulating signal. Lineage commitment then occurs at the $CD4^+CD8^-$ intermediate stage whereby persistence of the initiating TCR/coreceptor signals induces CD4 maturation, and cessation of signals CD8 commitment [93].

MAPkinase signaling in selection and lineage commitment

Positive selection of DP thymocytes requires activation of the Ras-Raf–MEK-Erk signaling cascade (fig. 3) as shown by lack of T cell maturation in mice expressing dominant-negative versions of Raf, Ras and/or MEK molecules or deficient for ERK1 or ras-Grp [94]. Gene ablation of the CD3δ chain of the TCR/CD3 complex [95] or mutation of a special TCRα domain also results in lack of Erk activation and positive selection [96]. Furthermore, Ras-Erk signaling is necessary for expansion and differentiation of DN to DP cells [97–98].

Initial studies indicated that negative selection is regulated by the JNK and p38 pathways only (fig. 3). JNK2-deficient mice [99] or mice expressing a dominant-negative JNK1 transgene [100] showed reduced apoptosis of thymocytes after treatment with anti-CD3 Abs or deleting peptides, and blocking p38 activation in fetal thymus organ cultures by pharmacological reagents interfered with negative but not positive selection [101]. Likewise, in mice expressing dominant-negative Ras and MEK transgenes, positive selection was ablated, but negative selection was normal [94]. On the other hand, other data clearly demonstrate that Erk signaling is involved in positive as well as negative selection (fig. 3). Deletion of DP cells in in vitro suspension and in thymic organ cultures was strongly reduced when MEK activity was attenuated by pharmacological inhibitors. Interestingly, by diminishing Erk signaling, agonist signals could be modified to signals that allowed or even enhanced positive selection of CD8 lineage cells [102–103].

Further studies show that a critical parameter in discriminating positive versus negative selection is the kinetics of MAPkinase activation (fig. 3). Mice hemizygous for the adapter molecule Grb2 show normal Erk activation and positive selection but inefficient negative selection connected with reduced JNK and p38 activation. The observed difference in MAPKinase activation was linked to lower thresholds required for ERK activation than that for JNK or p38 [104]. Furthermore, whereas positive-selecting (low-affinity) TCR ligands induce low-level long lasting Erk activation, negative-selecting (high-affinity) ligands induce intense but transient Erk signals [96, 105].

A role for Erk signaling in lineage commitment was first demonstrated in mice expressing a hypersensitive mutant of Erk2 in the thymus which enhanced the development of CD4 lineage cells at the expense of $CD8^+$ T cells [106]. This was confirmed in newborn thymic organ cultures from a variety of transgenic/knockout mouse strain combinations that fail to mature either $CD4^+$ or $CD8^+$ T cells. In the presence of the MEK inhibitor PD98059 CD4 lineage cells, either induced by endogenous ligands or by bispecific Abs were redirected into the CD8 lineage [107–108]. Thus continued/strong ERK signaling is essential for CD4 lineage cells but less so for CD8 commitment (fig. 3). In this context, an interesting aspect concerning duration/strength of signaling is the possible feedback mechanism of MAPKinase activation on further upstream signaling molecules. Phosphorylation of Serine 59 of Lck by active Erk abrogates the binding of the tyrosine phosphatase SHP1 and thus inactivation of Lck. This would lead to prolonged TCR-Lck signaling and CD4 cell fate, whereas inhibition of or low Erk signaling would lead to a faster shutdown of TCR signaling by SHP1 and induce the CD8 cell fate [109].

Since the Erk cascade is a strong candidate for directing lineage commitment, future studies have to establish the critical target molecules of Erk activation and how the Erk cascade is interconnected with other signaling molecules that are induced after TCR ligand interaction such as PI3-K [110] or Notch [111] (fig. 3, and see below). The central unresolved question, namely by what mechanisms strength/duration or accumulation of signaling mediators during a certain time frame are translated into alterations of genetic programmes, awaits further experimentation.

Lineage commitment in the rat

An interesting aspect of quantitative signaling in lineage commitment is the comparison of mouse and rat thymocytes [75]. Unselected 'virgin' DP rat thymocytes that mature in overnight culture from isolated immature CD8 precursor cells [112–113] develop into the CD8 lineage when treated with the phorbol ester PMA and ionomycin (see fig. 4A) or TCR plus anti-CD4 or anti-CD8 Abs in the presence of IL-2 [114]. As discussed before, this is exactly the opposite lineage decision as observed in mouse DP thymocytes, indicating that the same or similar signals promote different fates in the two systems. Since rat CD8α molecules cannot form CD8α′ splice variants as observed in the mouse, this may be one parameter underlying the difference in lineage commitment [114]. As shown in fig. 4B, treatment of rat DP cells with anti-CD3 Ab or the lectin concanavalin A (ConA), which leads to CD4 maturation of mouse DP thymocytes [115] as well as

short-term stimulation (1 h) with anti-CD3 plus anti-CD4 or anti-CD8 Abs also induced CD8, but not CD4 cell fate. Inhibition of Src kinases by PP1 or inhibition of PKC by Gö6983 (fig. 4C) blocked differentiation of rat DP cells, confirming a role for their necessity in positive selection and lineage commitment [116]. Remarkably, in the presence of two different MEK inhibitors (PD98059 and UO126) DP cells still developed into the CD8 lineage, although CD8 levels were markedly lower (fig. 4C). A role for ERK in final CD8 maturation has been observed in mouse thymic organ culture where the MEK inhibitor had to be withdrawn for full upregulation of TCR levels on maturing CD8 cells [107]. The continuous presence of MEK inhibitor in the rat culture system thus failed to inhibit CD8 commitment but still may affect final maturation of CD8-committed cells. Likewise, inhibition of the calcium-regulated phosphatase calcineurin by the immunosuppressant FK506 [117] did not affect CD8 T cell maturation (fig. 4C). In contrast, in the mouse inhibition of calcineurin activity blocks selection of DP cells [118], although CD8 maturation seems to be less dependent on calcineurin activity than CD4 selection [119]. In no case

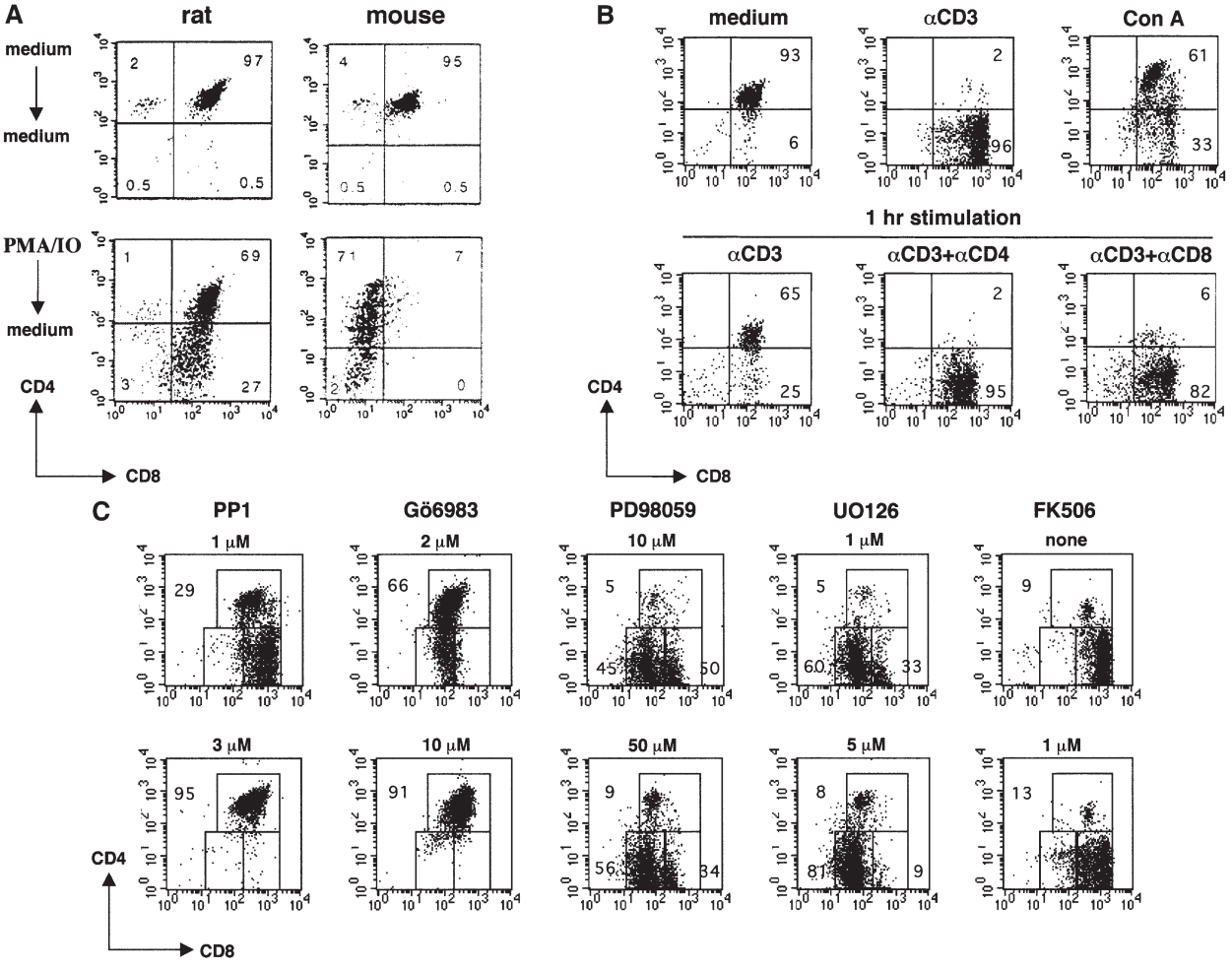

Figure 4. Lineage decision in the rat. (*A*) Stimulation of DP cells with PMA and ionomycin (IO) induces opposite lineage decision in rat and mouse cells. Isolated DP thymocytes from Lewis rats and BALB/c mice were stimulated with PMA (0.4 ng/ml) and IO (0.2 μg/ml) for 20 h or left unstimulated. After another 20 h of rest in medium, CD4 and CD8 expression was analyzed by flow cytometry (reproduced with permission from [114]; Copyright 1998. The American Association of Immunologists, Inc.). (*B*) CD8 lineage decision of rat DP cells after anti-CD3, ConA and short-term anti-CD3 plus coreceptor activation. In the upper panel DP cells from Lewis rats, differentiated overnight from purified immature CD8 precursors (iCD8), were cultured in medium alone, with plate-bound anti-CD3 mAb (G4.18, 1 μg/ml) or with the lectin ConA (2 μg/ml) for 2 days in the presence of IL-2 (150 U/ml), rested in medium for 1 day and analyzed on day 4 for CD4/CD8 expression. In the lower panel DP cells were activated in suspension for 1 h with anti-CD3 Ab (3 μg/ml) or anti-CD3 plus anti-CD4 (W3/25/1, hybridoma supernatant) or anti-CD8 Abs (OX8, 2.5 μg/ml) and cross-linking anti-Ig (immunoglobulin) G $F(ab')_2$ Abs. Cells were washed, cultured with IL-2 alone and analyzed as above. (*C*) Inhibition of various signaling molecules fails to alter lineage decision of rat DP cells. 'Virgin' DP cells obtained by overnight culture of iCD8 cells were cultured for 2 days on plates coated with anti-TCR Ab (R73, 1 μg/ml) in the presence of IL-2 and various inhibitors of signaling pathways at the concentration indicated. Cells were rested and analyzed on day 4 as described above.

did attenuation of the investigated signaling molecules induce a switch of rat DP cells to the CD4 lineage. This suggests that CD4 and CD8 lineage commitment in the rat either requires qualitatively different signals or that the rat culture system is missing some essential components that are provided by thymic stromal cells as given in mouse thymic organ or reaggregate cultures.

$CD4^+CD25^+$ regulatory T cells

Another unique lineage of $\alpha\beta TCR^+$ $CD4^+$ T cells that arises in the thymus comprises immunoregulatory $CD4^+$ T cells which are found in rat, mice and humans controlling autoimmunity, allograft rejection and inflammation [120]. Reduction or functional alteration of regulatory cells is generally associated with the spontaneous development of various autoimmune diseases, including gastritis, thyroiditis, type 1 or inflammatory bowel disease. Phenotypically, a major subset of regulatory T cells, which make up about 10% of peripheral $CD4^+$ T cells and 2–5% of $CD4^+$ thymocytes, can be characterized by the constitutive expression of the IL-2 receptor α chain (CD25), cytotoxic T lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) molecules. Constitutive expression of CD25 on these cells likely relates to the observations that IL-2 is required for thymic differentiation and/or survival of the $CD25^+$ T cells. Indeed, many, but not all, mouse knockout strains deficient for essential components of the IL-2/IL-2R system show a generalized autoimmune inflammatory syndrome [121].

The majority of $CD25^+CD4^+$ regulatory T cells are constantly produced from birth, appearing around day 3, to adult life and arise during 'altered' negative selection of DP cells. It seems that the required TCR avidity for self-peptide/MHC or MHC class II itself has to be higher for the induction of regulatory $CD4^+$ T cells than that necessary for the generation of normal $CD4^+$ T cells, but still below the level for negative selection [122]. Their TCR repertoire, however, is polyclonal, similar to that of $CD25^-CD4^+$ T cells. Recently, several groups have identified the forkhead-winged helix transcription factor Foxp3 as a key regulatory gene for the thymic development and function of $CD25^+CD4^+$ regulatory T cells [123–125]. Thus, transduction of T cells with Foxp3 or other yet to be identified genes should provide a useful tool for the therapeutic treatment of autoimmune and inflammatory diseases and in transplantation tolerance.

Notch and lymphoid cell development

The molecular basis of Notch signaling

Notch proteins are transmembrane receptors which were first identified in *Drosophila* and *Caenorhabditis elegans* by their ability to direct cell fate decisions as well as to control survival and proliferation in developmental scenarios [126]. So far, four members of the Notch family have been identified in mammals which are widely expressed in different tissues, including all hematopoietic ones [127]. Notch proteins undergo ligand-dependent activation involving enzymatic processing by a γ-secretase that cleaves off the intracellular domain of Notch (NIC) (fig. 5). This part of the protein, which harbors a RAM domain, cdc10/ankyrin repeats, a transcriptional activation and a PEST domain, subsequently translocates to the nucleus where it binds to DNA in a complex with the CSL transcription factor and other cofactors [126] (fig. 5). This finally leads to the induction of gene expression, including major effectors of the Notch signaling pathway such as Hairy/Enhancer of Split 1 (HES-1).

In mammals, five Notch ligands have been identified, including Delta-like 1, 3 and 4 and the two Serrate-like ligands Jagged-1 and -2. Whereas Notch proteins in the thymus are predominantly located on lymphoid cells, their ligands are found on thymic epithelial cells (TECs) [128] (fig. 5). The observation that extrathymic sites of hematopoiesis such as the fetal liver hardly express any Notch ligand has led to the conclusion that it is presumably the presence of these ligands on TECs which generates a 'productive' thymic microenvironment [35]. This in turn would allow induction of Notch signaling and thereby initiate and sustain T cell lineage commitment.

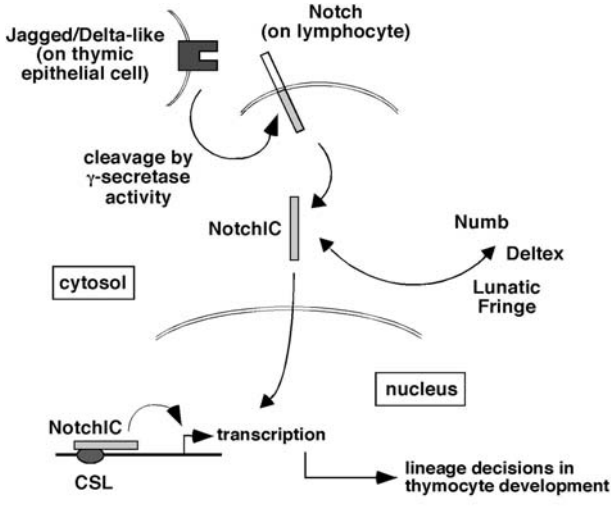

Figure 5. Signaling pathway of Notch transmembrane receptors. Notch proteins located on thymocytes become activated by their cognate ligands of the Jagged/Delta-like family expressed on the surface of thymic epithelial cells. Activation occurs though cleavage of the intracellular domain of Notch (NotchIC) by an enzymytic γ-secretase activity. Consequently, NotchIC may either interact with other proteins such as Numb, Deltex or Lunatic Fringe, or bind to specific response elements on the DNA together with the transcription factor CSL. This finally leads to activation of gene transcription, presumably directing lineage decisions in thymocyte development.

Insights from molecular genetics

Initial evidence that Notch proteins may participate in thymic development came from the discovery of the first mammalian Notch homologue (Notch-1) being a partner of the T cell receptor β-chain gene in a chromosomal translocation characterizing a subset of human T cell leukemias [129]. Extensive additional evidence that Notch proteins affect different aspects in intrathymic development has been reported since, including many transgenic and knockout models.

The first and probably most important lineage decision in lymphoid development involving Notch1 appears to take place in BM CLPs or ETPs. As discussed above, progenitor cells in the thymus can give rise to T and B cells as well as other hematopoietic lineages. In particular, there are several lines of evidence that Notch1 is involved in the decision between T and B cells. First, inactivation of Notch1 in BM precursor cells leads to an early block in T cell development accompanied by an aberrant development of immature B cells [130]. Second, overexpression of either Deltex1 or Lunatic Fringe redirects lymphoid progenitor cell development to the B cell lineage by antagonizing Notch1 function [131–132]. Later, in cells committed to the T cell lineage, reduced Notch1 activity has been shown to favor the development of γ/δ versus α/β T cells [19]. Finally, α/β T cells are subjected to the decision of becoming either MHC I-restricted CD8 SP cells or MHC II-restricted CD4 SP cells. Whereas most data point towards a role of Notch1 in this process, controversial models concerning the underlying mechanism have been put forward. Some authors provided evidence that Notch1 selectively promotes a CD8 fate, whereas others have suggested that Notch1 favors survival of both CD4 and CD8 SP cells [133–135]. Yet another model implies that Notch1 exerts its effect indirectly by reducing signal strength through the TCR [91,136]. In contrast to these studies, two strains of conditional Notch1 knockout mice deleting the gene at different stages in the DN compartment failed to demonstrate any effect on the CD4/CD8 lineage decision [137–138]. Although the aforementioned data argue against a nonredundant role of Notch1 in the lineage decision between CD4 and CD8 SP cells, the possibility that other familiy members in these experiments have compensated for the lack of Notch1 formally remains.

Whereas the role of Notch1 in the T/B-cell lineage decision is rather clear, only few studies so far have addressed its role in the fate determination of the proposed NK/T and T/DC precursor cells in thymus. We have recently obtained evidence showing that at least in the rat, Notch proteins are involved in the lineage decision between T and NK cells, with Notch signaling being a prerequisite for promoting the T cell fate and preventing the development of NK cells [H. M. Reichardt, unpublished data]. With regard to the role of Notch in the development of thymic DC, experiments have been reported which demonstrate that DCs develop completely normally from Notch1-deficient BM precursors [24]. Although these results argue against the existence of a common DC/T precursor cell, the approach did not exclude the possibility that other Notch familiy members may participate in this lineage commitment. Thus, it remains open to what extent Notch transmembrane receptors control the lineage decisions between T cells on the one hand and NK cells or DC on the other hand.

Glucocorticoids and thymocyte development

The molecular basis of glucocorticoid signaling

Glucocorticoids belong to a class of lipophilic hormones which are synthesized by the adrenal gland and released in response to stimuli such as stress and inflammation [139]. Their pleiotropic effects range from regulation of energy metabolism and control of cognitive function to the modulation of the immune system. Their activity is mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor which translocates to the nucleus following hormone binding. Subsequently, GR either activates transcription by binding to specific response elements (designated GREs) or it represses the activity of other transcription factors such as activating protein-1 (AP-1) and nuclear factor-κB (NF-κB) [140]. Using genetically manipulated mice, it could be demonstrated that these two modes of transcriptional regulation can be separated and thus represent distinct ways by which glucocorticoid hormones can modulate gene expression [141].

Whereas the antiinflammatory and immunosuppressive activity of glucocorticoid hormones is well established [142], their role in the thymus is still highly controversial. It is unquestionable that glucocorticoids induce apoptosis in thymocytes and peripheral lymphocytes with the double-positive thymocytes being the most sensitive cell type. However, whether glucocortiocids are involved in thymocyte development is still debatable. Furthermore, it is yet an unresolved issue whether the thymus is able to synthesize glucocorticoids autonomously and if yes, whether these hormones are involved in thymopoiesis.

Insights from molecular genetics

Since thymopoiesis to a large extent takes place during fetal life and at the same time circulating levels of glucocorticoids are low during this phase, it is an important issue whether the thymus or in particular thymic epithelial cells are able to autonomously produce glucocorticoid hormones. Jenkinson et al. were unable to detect P450scc [143], the key enzyme of the steroidogenic pathway leading to the production of corticosterone, in $MHCII^+$ TECs.

In contrast, Ashwell et al. have presented indirect evidence for steroid production as well as for a functional role of intrathymically synthesized glucocorticoids [144]. Thus, the question remains unsolved whether glucocorticoids are produced at extraadrenal sites.

Several animal models have been generated and analyzed to investigate the potential role of glucocorticoids in thymus development. The first transgenic mouse specifically developed for this purpose expressed antisense RNA to the GR in immature thymocytes [145]. Cells recovered from these mice display reduced levels of GR protein and are hyporesponsive to hormone treatment. Furthermore, these transgenic mice have reduced thymocyte numbers as well as impaired progression from the DN to the DP stage. When crossed into MRL-*lpr/lpr* mice, greatly diminished autoimmunity and an altered T cell repertoire were found [146–147]. These findings strongly suggest that glucocorticoids are important for thymocyte development and selection. In contrast to these studies, gene-targeting experiments failed to demonstrate any influence of GR on thymus development. First, GR^{dim} mice, which carry a point mutation that prevents DNA-binding-dependent transcriptional regulation, have normal thymocyte numbers and no impairment of T cell lineage progression [141]. Second, GR knockout mice carrying a hypomorphic allele neither show any impairment in thymocyte development nor in positive and negative selection [148]. However, although these mice, which are designated $GR^{-/-}$ or GR^{hypo} (both refering to the same strain of mice!) don't express full-length GR, they still make a truncated 39-kDa form of GR due to an unnaturally occurring splice event [149–150]. Thus, residual transcriptional activity of GR cannot be excluded in these mice. Taken together, in the case of GR^{dim} mice the repressive activity of GR is still intact, and in the case of GR^{hypo} mice a truncated protein of unknown characteristics is present. Consequently, both models formally do not exclude the possiblility that glucocorticoids are involved in thymus development. Finally, two strains of transgenic mice overexpressing GR either ubiquitously or specifically in immature thymocytes have been described [151–152]. These animals show an increase in sensitivity towards GC-induced apoptosis, a reduction in cell recovery and an altered $CD4^+$:$CD8^+$ ratio. It is noteworthy that a lower cell recovery was found both after overexpressing GR [151] as well as after reducing it by an antisense strategy [145]. How these diverging findings may be explained remains unclear. Taken together, despite the generation and analysis of several animal models where GR was genetically manipulated, the answer to which role GR plays in the developing thymus still awaits analysis of a complete knockout of GR. Since this mutation is lethal (in contrast to the hypomorphic allele described above) [149], transfer experiments have to be conducted in order to solve this question.

Conclusion

Since the first descriptions of the thymus as a site of lymphocyte differentiation, we have come a long way in understanding the process of T cell development in the exquisitely specialized microenvironment of this organ. Thus, a coherent picture is emerging of the early progenitors that enter the thymus, their crosstalk with thymic stroma cells in building up the thymic network, and of the multiple cell lineages of both the adaptive, and innate immune system that arise from early thymocyte precursor cells. Transgenic, knockout and in vitro culture technologies have made intrathymic T cell development one of the best-studied examples for mammalian cell lineage decisions, and it has become clear that signaling molecules that govern cell growth and differentiation in primitive organisms such as worms and flies are also an integral part of cell fate decisions in the thymus. Many of the thymocyte differentiation processes are regulated by cell-extrinsic soluble factors and/or receptor-ligand interactions in direct cell-cell contact, which control proliferation and direct alternate differentiation programmes of the developing thymocytes. Positive and negative selection based on self-MHC recognition and CD4/CD8 lineage commitment of immature α/β T cells are hallmarks of the thymus, building a repertoire of mature T cells that can successfully recognize and eliminate foreign antigens while simultaneously avoiding autoimmunity. The acquisition of self-nonself discrimination is supported by ectopic expression of peripheral organ-specific antigens by thymic epithelial cells, and by differentiation of regulatory or suppressive T cells that can be produced in the thymus and which are potent guardians against inflammatory and autoimmune responses. Comparison of T cell development in different species shows that there are alternate ways to achieve the same goal and that similar signaling mechanisms can lead to different cell decisions. Although we have learned much about the differentiation pathways and the signals guiding them, in molecular terms we are far from a complete understanding, and some essential regulators or concepts might still await discovery. Therefore, the complex machinery of the thymus with all its implications for the proper functioning of the immune system and its failure in immunodeficiency and autoimmune pathology will continue to be a fascinating field of research.

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