T CELL DEVELOPMENT IN MOUSE THYMUS
Studies on lymphostromal interactions

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T CELL DEVELOPMENT IN MOUSE THYMUS
Studies on lymphostromal interactions

DE ONTWIKKELING VAN T-CELLEN IN DE THYMUS VAN DE MUIS

PROEFSCHRIFT

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'Twijfel is een eerbetoon aan de waarheid'

Ernest Renan

Aan mijn ouders
Aan Dorien, Inge en Cleo

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CHAPTER 1

General introduction

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Preface

T lymphocytes, the effectors of *cell-mediated immunity*, are concerned with the control of intracellular infections: cytotoxic T lymphocytes recognize and destroy virally infected cells, whereas helper T lymphocytes, through lymphokines, may activate macrophages in killing intracellular parasites. The parasital/viral antigens are recognized by the T lymphocytes in association with molecules of the major histocompatibility complex (MHC). This ability, to recognize antigens in the context of self-MHC molecules, also termed MHC-restriction, is learned by the T lymphocytes during their development in the thymus.

On their pathway to development (maturation), T cells are differentially influenced by various thymic stromal cells. The stromal cells constitute microenvironments, where developing T cells, through cell-cell interactions and locally secreted cytokines, receive signals to proliferate and mature.

This thesis focuses on the specific role of thymic microenvironments in promoting sequential stages in T cell development in the murine thymus. Our understanding of these lymphostromal interactions will be introduced in this chapter, containing three major sections. The first section on "**Development and architecture of thymic microenvironments**" discusses the variety of thymic microenvironments that have presently been identified. The phenotypic characterization of thymic microenvironments, presented in this section, is more extensively reviewed in chapter 2. The second section on "**T cell differentiation**" introduces a major pathway of T cell differentiation, including the $\alpha\beta$ and the $\gamma\delta$ T cell lineages, showing the complexity of this process. The third section on "**Lymphostromal interactions**" links the different types of stromal cells to distinct stages of T cell development. The chapter ends with an "**Introduction to the experimental work**".

Development and architecture of thymic microenvironments

Thymic microenvironments are composed of stromal cell types derived from different embryonic germ layers (1-7). Three elements are critical to the normal development of the murine thymus: ectoderm of the third branchial cleft, endoderm of the third pharyngeal pouch and mesenchyme from the pharyngeal arch (1,2,8,9). If any one of these components is missing, the thymus fails to develop. This is well illustrated in the nude mouse, that lacks a thymus due to an absence of the ectodermal component (1,2). Moreover, fetal thymi of gestational day 12 (referred to as GD12) fail to support lymphocyte development when their mesenchymal capsules were removed (8). Removal of the neural crest cells, that eventually give rise to the mesenchymal capsule, leads to similar results (9).

From the embryonic germ layers a thymic primordium of simple architecture develops, with an inner epithelial mass surrounded by a capsule of mesenchyme. Initially, in the mouse around GD10-11, all thymic epithelial cells (TEC) belong to an apparently single homogeneous population and share the same morphology (10). However, from GD13, based on morphological characteristics (10) and immunostaining with TEC-specific mAb (11), the thymic primordium can be divided into a cortex and a medulla. During the next days of gestation, further developments involve: proliferation of TEC to accomodate the increasing size of the thymus; invagination of the thymus by connective tissue septae and bloodvessels, leading to thymic lobulation; the growth and extension of subcapsular TEC along the septa and around bloodvessels; and the maturation of Hassall's corpuscles (5,10). By GD18, a complete epithelial network has developed that resembles the epithelial network of the adult thymus. This network is supplemented with two bone marrow derived, non-resident stromal cell types: the interdigitating cells (IDC) and the macrophages (Mø). Morphologically, the presence of macrophages and interdigitating cells in the fetal mouse thymus is first identified around GD14 resp. GD17 (10,12).

A more detailed description of heterogeneity in thymic microenvironments has been obtained by analyzing the architecture of the adult thymus. The thymic epithelial cells, the major stromal cell component, provide a three-dimensional network within which all other cell types lie. At least four types of epithelial cell types are identified by their reactivity with TEC-specific mAb, as determined by immunohistological analysis (Fig. 1; reviewed in chapter 2; ref. 13-16) and their ultrastructural morphology, as analyzed with transmission and scanning electron microscopy (TEM resp. SEM; ref. 10,17-21):

- *Subcapsular and perivascular TEC* (sc/pvTEC) form a single layer of TEC that lines the external surface of the thymus along the capsule and septa and surrounds bloodvessels and perivasculair spaces.
- *Cortical TEC* (cTEC) are stellate cells with long cytoplasmic extensions forming a

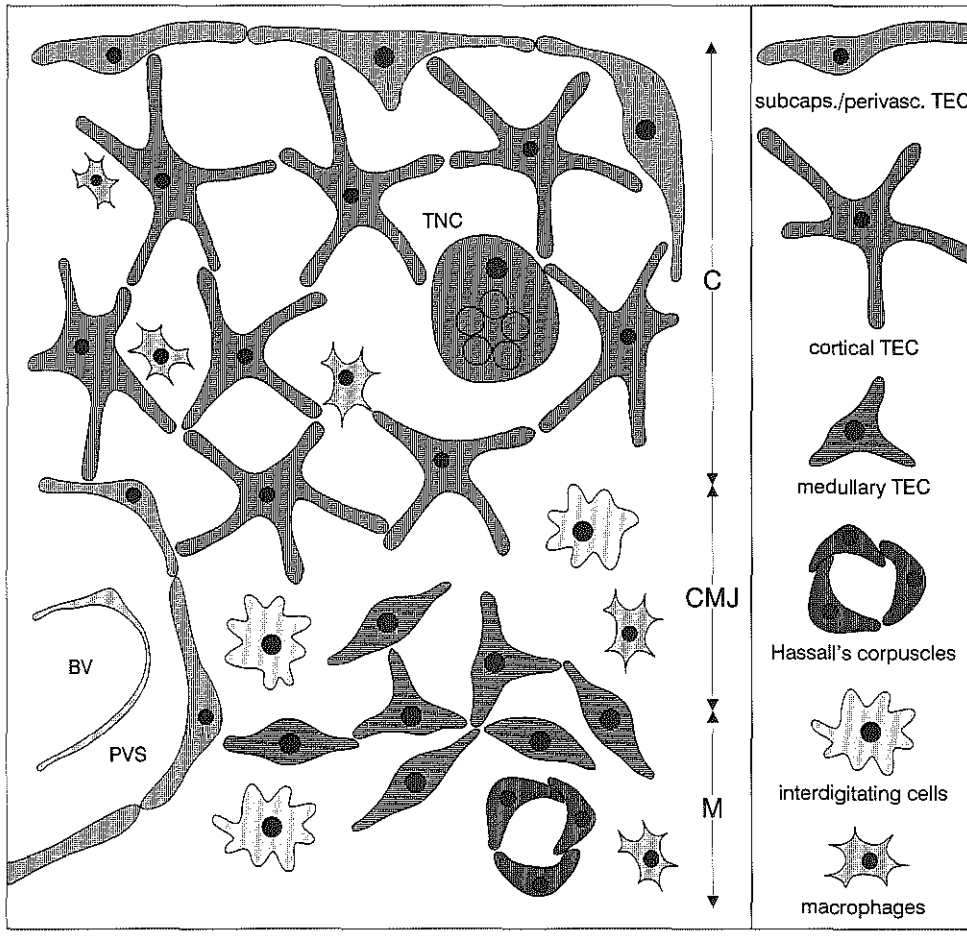

Figure 1. Schematic representation of the thymus architecture, based on the phenotypic characterization of 6 differerent thymic stromal cell types. Thymocytes have been omitted in this figure. This figure is a modified version of the figure in *Brekelmans and van Ewijk (1990) Semin Immunol 2:13-24* (14), printed as chapter 2 of this thesis.

network throughout the cortex. Both TEM and phenotypic analysis identified only one major TEC type in this compartment of the murine thymus (14,17). However, SEM clearly identified the heterogeneity of TEC in the cortex (19,20): (a) A specialized type of microenvironment was revealed in the subcapsular cortex, comprising "baskets" of epithelial cells filled with thymocytes; (b) the *in vivo* equivalents of "thymic nurse cells (TNC)" have been found in the outer cortex. TNC, comprising an epithelial cell that completely surrounds many thymocytes, were first described

in vitro after isolation from mouse thymus (22). SEM now shows that thymocytes can move freely in and out of TNC through small gaps in the surrounding TNC membranes; (c) In the deeper cortex, the epithelial cells are oriented perpendicular to the thymic capsule and seem to guide the migration from thymocytes from the outer cortex towards the medulla. In human thymus, three types of cortical TEC can be ultrastructurally defined, based on the electron lucency of their cytoplasm (23).

- *Medullary TEC* (mTEC) are mainly spindle-shaped cells with short cytoplasmic extensions, forming a network in the medulla. The medulla contains two additional epithelial cell types: globular cells, that contain, in the cytoplasm, either an extensive network of small vacuoles or a large vacuole lined with microvilli and/or cilia (17,18). Both cell types, detected with TEM, are only present in low numbers.
- *Hassall's corpuscles* (HC) are specific agglomerations of epithelial cells in the medulla, consisting of concentric arranged, highly keratinized epithelial cells. In the mouse these structures are very small and morphologically difficult to identify. However, they can be revealed after immunostaining with specific antikeratin antibodies (24).

So, heterogeneity typifies the major stromal cell type of the thymus. Such heterogeneity is not observed with bone marrow-derived stromal cells in the murine thymus. Macrophages, characterized by the presence of numerous lysosomes and phagolysosomes (that may contain apoptotic thymocytes), are present in both cortex and medulla of the thymus (25). Some morphological heterogeneity of Mø exists in the murine thymus, but this has neither been thoroughly studied nor identified with Mø-specific mAb (see chapter 2). In contrast, the thymus in rats contains cortical, cortico-medullary, and medullary Mø, that are distinguished by morphology, endogenous enzymes and antigenic phenotype, defined by mAb (26-29). IDC, identified by a very irregular surface with many digitations in close contact with adjacent cells, are only present in the medulla, predominantly near the cortico-medullary junction (19-21,25).

Maturing thymocytes migrate through this complex thymic architecture. Precursor T cells enter the thymus at the cortico-medullary junction, and migrate to the subcapsular cortex; after a period of intense proliferation immature thymocytes migrate from the outer cortex to the inner cortex and from there into the medulla. Here they leave the thymus as mature, selected T lymphocytes (30,31).

Considering this migration route of thymocytes through the stroma and the apparent heterogeneity of the stroma (as described above), it follows that differentiating thymocytes sequentially enter different microenvironments. Before determining the role of the various types of stromal cells in T cell development, the complicated steps in T cell differentiation need to be discussed.

Distinct stages of T cell differentiation are characterized by the expression of surface molecules (the 'phenotype' of the cell) and by their functional capacities. In the next

section these characteristics of thymocyte subpopulations will be described and ordered in a general scheme of T cell development. Subsequently, the role of different types of thymic stromal cells in the process of T cell differentiation will be discussed.

T cell differentiation

The thymus provides an environment, that is optimal for the differentiation of T lymphocytes (32,33). This process can be relatively easily monitored by analysis of the expression of the $\alpha\beta$TcR/CD3 complex and the expression of the coreceptor molecules CD4 and CD8 on the surface of the developing T cells. Based on the expression of various surface molecules, phenotypically distinct subpopulations have been identified and the lineage relationship between most of these thymocyte subpopulations has now been well established. A general pathway for the development of $\alpha\beta TcR^+$ thymocytes will be presented in the first part of this section.

Once the maturing cells in the thymus express an $\alpha\beta$TcR on their surface, they become subject to *selection*. The $\alpha\beta$TcR expressing thymocytes (of $CD4^+8^+$ phenotype) must pass through two selection filters: (1) *Positive selection* favors the development of thymocytes expressing TcRs that preferentially recognize foreign antigens in association with self-MHC molecules; (2) *Negative selection* eliminates or inactivates thymocytes bearing self-reactive TcRs. The impact of intrathymic selection on T cell development will be discussed in the second part of this section.

The thymus not only supports the development of $\alpha\beta$ T cells, but also of $\gamma\delta$ T cells as well as cells of a new lineage, the $CD4^-8^-$ $\alpha\beta$ T cells. The development and selection of these cells are the subject of the last two parts of this section.

T cell differentiation in the αβ T cell lineage

An outline of the main pathway of $\alpha\beta$ T cell development within the mouse thymus is presented in Fig. 2. It summarizes our current knowledge on the lineage relationship between phenotypically different thymocyte subpopulations that will be discussed below and has been extensively reviewed elsewhere (34-42). This scheme of T cell development is mainly based on the expression of the $\alpha\beta$TcR/CD3 complex and its coreceptors CD4 and CD8, essential molecules in the selection process of thymocytes. Other surface molecules, used to phenotype thymocyte subpopulations, include heat stable antigen (HSA), phagocytic glycoprotein-1 (Pgp-1 or CD44), the p55 chain of the interleukin-2 receptor (IL-2Rα or CD25), Thy-1, Lyt-1 (CD5) and the MHC class I antigens H-2K/D and Qa-2. Some of those molecules are included in the scheme of Fig. 2, because they allowed a refined analysis of the pathway of $\alpha\beta$ T cell development.

Immature CD4$^-$8$^-$3$^-$ thymocytes

The earliest precursor cells in the thymus have been considered to be within the CD4$^-$8$^-$3$^-$ or 'triple negative' (TN) population (37,42). However, a recently published study defined an even earlier population (0.05% of total thymocytes) of intrathymic precursors with a **CD4^{low}8$^-$3$^-$** phenotype (43). Upon intrathymic transfer these CD4^{low}8$^-$3$^-$ precursors reconstitute all thymocyte subpopulations, with an efficiency that is 50 to 80-fold higher than with CD4$^-$8$^-$3$^-$ precursors. The T cells produced include both the *αβ* and *γδ* T cell lineages (43,44). Based on phenotype and reconstitution ability, Wu et al. (43) conclude that cells in the CD4^{low}8$^-$3$^-$ precursor population are the direct progeny of the bone marrow hemopoietic stem cells. In contrast to multipotent bone marrow cells, such cells are already committed to the lymphoid lineage (43). However, it was reported that the CD4^{low}8$^-$3$^-$ precursor population also contained precursors of thymic dendritic cells (45).

The progeny of this CD4^{low}8$^-$3$^-$ precursor, the **CD4$^-$8$^-$3$^-$** thymocytes, can be divided into three subpopulations based on the expression of CD44 and CD25. The developmental order of these thymocytes, from **CD44$^+$25$^-$** through **CD44$^-$25$^+$** to **CD44$^-$25$^-$** TN cells, is based on the extent of rearrangement and expression of their TcR genes, their cell cycle status, and their thymus reconstitution capacity (46-48). In the fetal thymus, most CD25$^+$ cells also express CD44 and this **CD44$^+$25$^+$** TN population forms an intermediate stage, between CD44$^+$25$^-$ and CD44$^-$25$^+$ TN cells (49,50).

Two control points have been proposed within the TN stage of T cell development (35). The first one occurs around the CD4^{low}8$^-$3$^-$ stage and marks the first expansion phase. This phase starts with downregulation of CD4 and ends at the CD44$^-$25$^+$ TN stage. The receptor for stem cell factor (SCF), c-kit, is exclusively expressed by cells in this phase, indicating an important role for this factor in the early expansion phase (51).

The second control point occurs around the the CD44$^-$25$^+$ TN stage and considerable published evidence indicates it is a major control point in early T cell development. First, from this stage cells enter a major (second) phase of expansion that ends at the CD4$^+$8$^+$3$^-$ stage of T cell development (39). Second, a maturation arrest occurs around this stage of T cell development after IL-2 or anti-IL-2R treatment of thymus in both *in vitro* as well as *in vivo* modelsystems (52-56). Third, CD4$^-$8$^-$ *αβ*TcR$^+$ and CD4$^-$8$^-$ *γδ*TCR$^+$ cells directly differentiate from CD25$^+$ TN thymocytes in IL-7 treated cell cultures (57). Finally, the transition to CD44$^-$25$^-$ TN thymocytes is now also marked by phenotypic changes. It has been shown that downregulation of CD25 is parallelled by acquisition of CD4 and CD8 (58). CD44$^-$25$^-$ thymocytes are therefore not CD4$^-$8$^-$, but instead have a CD4^{low}8^{low} phenotype (not shown in Fig. 1; ref. 37,58). Moreover, even surface expression of CD3 and a TcR-*β* homodimer have be found on these cells (discussed in "Positive and negative selection"; ref. 37,59). Ligation of CD3

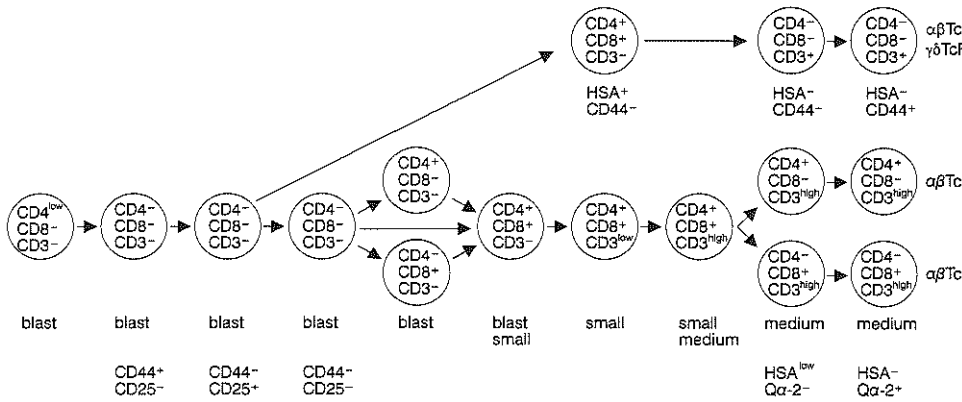

Figure 2. An outline of T cell development in the mouse thymus. The major lineage presented in this figure is the development of mature $CD4^+8^-$ and $CD4^-8^+$ $\alpha\beta$ T cells. Of this major lineage the cell size has been indicated in the figure. The surface molecules CD44, CD25, HSA and Qa-2 are indicated in this developmental scheme, when they identify distinct subpopulations. The distinct lineages of DN $\alpha\beta$ and $\gamma\delta$ T cells are not separately shown, as these lineages share an identical succession of subpopulations (based on expression of HSA and CD44). The DN $\alpha\beta$ and $\gamma\delta$ T cells branch of at the $CD44^-25^+$ TN stage of T cell development, as proposed by Suda et al. (57).

on these $CD4^-8^-$ cells accelerates the development of $CD44^-25^+$ into $CD4^+8^+$ cells, indicating a signal transduction function for CD3 at this stage of T cell development (59). Thus, when properly signalled, the $CD44^-25^-$ $CD4^{low}8^{low}$ population will progress to the $CD4^+8^+$ stage, by first upregulating either CD8 or CD4.

Immature $CD4^-8^+3^-$ and $CD4^+8^-3^-$ thymocytes

It is now well established that **$CD4^-8^+3^-$** and **$CD4^+8^-3^-$** thymocytes (0.7-2.4% and 0.5-1.0% of the total, respectively) are immature intermediates between $CD4^-8^-$ and $CD4^+8^+$ cells (60-67). They are clearly distinguishable from their mature ($CD3^+$) counterparts by their intrathymic location (outer cortex vs. medulla), their size (large vs small), their cell cycle status (rapidly cycling vs. non-cycling) and their phenotype (HSA^{high} vs. $HSA^{-/low}$). Both cell types have the precursor capacity to develop into $CD4^+8^+$ thymocytes, but there is no evidence that $CD4^+8^+$ cells derived from these two pathways show any preference for subsequent $CD4^+$ or $CD8^+$ lineages. Indeed, $CD4^-8^+3^-$ cells gave rise to both mature $CD4^+8^-$ and $CD4^-8^+$ cells (63). It has been observed that the balance between these two immature intermediates varies markedly between mouse strains, but a basis for the choice of one pathway over another is still not clear.

Similar to the $CD44^-25^-$ TN cells, it has been found that $CD4^+8^-3^-$ and $CD4^-8^+3^-$ cells express low levels of CD8, CD4, and CD3. After upregulation of CD8 or CD4, the

immature intermediate thymocytes develop into the typical (cortical) $CD4^+8^+$ stage of T cell development.

Nonmature $CD4^+8^+$ thymocytes

The **$CD4^+8^+$** or 'double positive' (DP) thymocytes constitute the major population within the thymus (80-85% of the total). For long, the most prominent characteristic of this cell population has been its massive intrathymic cell death, as kinetic studies indicated that only 3% of the daily produced DP cells developed into mature $CD4^+8^-$ or $CD4^-8^+$, 'single positive' (SP) thymocytes (39). Now we know this population is subject to strict selection mechanisms, occurring from the moment the $\alpha\beta$TcR-CD3 complex is expressed on the cell surface. Intrathymic cell death most probably results from a combination of non-productive TcR gene rearrangements and selection mechanisms operating on TcR expressing thymocytes (see "Positive and negative selection").

Two DP subpopulations are defined by expression of surface CD3, the **$CD4^+8^+3^-$** and the **$CD4^+8^+3^{low}$** thymocytes (68,69). All cycling DP blasts are present within the $CD4^+8^+3^-$ subpopulation. A phase of intense proliferation, that started at the $CD25^+$ TN stage, ends within this subset of large $CD4^+8^+3^-$ thymocytes. These results indicate that selection is a postmitotic event: cell division stops before the $\alpha\beta$TcR/CD3 complex is expressed (70,71). Of the DP thymocytes, only the large $CD4^+8^+3^-$ cells still have reconstituting ability (63). How these cells develop into mature SP cells is controversial. Shortman et al. (71,72) claim that small $CD4^+8^+3^-$ and small $CD4^+8^+3^{low}$ thymocytes are the apparent intermediates of this process. Guidos et al. (73), on the other hand, show that large $CD4^+8^+$ cells express low levels of CD3 and directly mature to SP cells, without small DP cells as intermediates. Both studies, however, confirm the presence of a **$CD4^+8^+3^{high}$** thymocyte population, characterized as an intermediate between $CD4^+8^+3^{low}$ and CD4 or CD8 SP thymocytes (48,74-77). The levels of CD4 and CD8 on these cells are distinct from those found on typical $CD3^{low}$ DP cells, in that most $CD3^{high}$ DP cells are downregulating one or the other marker (48,73,75). Upon *in vitro* culture these cells develop into mature cells by downregulating one or the other co-receptor and acquiring the potential to respond with proliferation to TcR-ligation (77).

Mature $CD4^+8^-3^{high}$ and $CD4^-8^+3^{high}$ thymocytes

The cells belonging to the medullary single positive (SP) thymocyte pool, the **$CD4^+8^-3^{high}$** and **$CD4^-8^+3^{high}$** cells, have been considered functionally equivalent to peripheral T cells. However, recent phenotypic characterization of these thymocytes has challenged this view (40,78-80). 30% of CD4 SP cells expressed a **$HSA^-Qa\text{-}2^+$** phenotype, resembling peripheral T cells, whereas 70% had a **$HSA^+Qa\text{-}2^-$** phenotype,

similar to the less mature $CD4^+8^+$ thymocytes (40,81). The latter population also expressed very low levels of CD8, indicating a close relation to its precursor, the $CD4^+8^+3^{high}$ thymocyte. The $HSA^+Qa\text{-}2^-$ thymocytes proved functionally immature: they did not respond (with proliferation and cytokine production) to TcR-crosslinking, like $HSA^-Qa\text{-}2^+$ CD4 SP thymocytes and peripheral CD4 SP T cells (81,82).

TcR mediated control of αß T cell development

The TcR repertoire, generated by random rearrangements of V(ariable), D(iversity), J(oining), and C(onstant) region genes, becomes subject to positive and negative selection as soon as the TcR is expressed at the cell surface of the thymocytes. These processes have been extensively reviewed (34,83-101) and so, only the essentials of these processes are presented below.

Recent studies on the expression of CD3 and the TcR-β chain in $CD4^-8^-$ thymocyte subpopulations indicate that these molecules probably control rearrangements of the TcR genes, guiding the development of "good" TcRs. These interesting findings will be discussed first.

Developmental control of αβTcR expression

Although the $\alpha\beta$TcR is not expressed before the $CD4^+8^+$ stage of T cell development, rearrangements of the TcR gene segments, coding for the V, D, J, and C regions of the chains, already start much earlier in T cell development (46). Rearrangements of the TcR-β chain genes occur at the $CD44^-25^+$ TN stage, whereas the first TcR-α chain rearrangements can be found in the $CD44^-25^-$ TN population (46). When proper rearrangements do not occur, for example in SCID mice (mice with a genetic recombinatorial defect; ref. 102), thymocytes do not develop beyond the DN stage (103-105). This suggests that the transition of $CD4^-8^-$ into $CD4^+8^+$ cells is controlled by TcR gene rearrangements. And indeed, introduction of a rearranged TcR-β transgene in SCID mice induced the development of $CD4^-8^-$ into $CD4^+8^+$ thymocytes (106-108). At the same time, it was discovered that $CD4^-8^-$ thymocytes expressed the TcR-β chain on their surface in the apparent absence of other TcR chains, either as a monomer (without CD3) or as a homodimer, atypically associated with CD3 (106-110). It has been suggested that the expression of this TcR-β/CD3 complex serves as control point, i.e. the second control point in early T cell development mentioned above (reviewed in ref. 83-85). Triggering would transduce intracellular signals which (1) stop further TcR-β gene rearrangements, (2) initiate TcR-α gene rearrangements, (3) control expansion, and (4) initiate CD4 and CD8 expression. The results, obtained with transgenic SCID mice, can be extrapolated to normal T cell development, as the TcR-β/CD3 complex was detected (although with much more

difficulty) on the surface of immature thymocytes in normal mice (83). Moreover, this view is supported by reports, demonstrating that anti-CD3 mAb, added to fetal thymus organ culture, induce the accelerated transition of $CD25^+CD4^-8^-$ cells to the $CD4^+8^+$ stage and block TcR-β chain production (59,111). Thus, it has been suggested (84), that thymocytes which express a 'good' TcR-β chain on their surface are then allowed to develop into $CD4^+8^+$ cells expressing the $\alpha\beta$TcR.

Cells that probably failed in the TcR-gene rearrangement process are represented by the small $CD4^+8^+3^-$ thymocytes. These failures may be due to nonfunctional rearrangements that produce no transcript, or to TcR-α or -β chain peptide products that failed to associate or migrate to the cell surface.

Positive selection

As soon as $CD4^+8^+$ thymocytes start expressing the $\alpha\beta$TcR at low levels, they enter a critical stage in T cell development at which the specificity of its TcR is screened. The development into mature SP thymocytes is absolutely dependent on the ability of the TcR to interact with MHC class I and class II molecules on stromal cells (92,93). In the absence of such an interaction, the cells remain $CD4^+8^+TcR^{low}$ and will die within 3-4 days. This process of selective survival is called *positive selection* or *MHC-restriction*, a third control point in T cell development. Positive selection also determines the direction of differentiation, the selected cells either enter the $CD4^+8^-$ T helper cell lineage or the $CD4^-8^+$ cytotoxic T cell lineage. For development of DP into CD4 SP, the interaction of both the TcR and CD4 with MHC class II molecules is obligatory, that is, CD4 SP cells are MHC class II restricted (112-119). In the same manner, CD8 SP cells are MHC class I restricted (119-126). It has been demonstrated that the restriction preference of the TcR, for either MHC class I or II, determines that a thymocyte will become a CD8 resp. a CD4 SP cell (92,93).

How the CD4 and CD8 SP lineages diverge, is still unclear. Different models of positive selection have been proposed, but recent studies (127,128) mainly seem to support an *instruction* model (as opposed to a *selective* model; for further reading on this subject, see ref. 94). In this instruction model, a DP thymocyte whose TcR recognizes MHC class I or class II receives a signal instructing it to become a CD8 resp. a CD4 SP cell. In this context, it was recently reported that the transmembrane region and/or the cytoplasmic tail of the CD4 molecule mediates the delivery of a specific signal that directs differentiation of immature thymocytes to the CD4 SP lineage (129).

As a result of positive selection, mature T lymphocytes develop whose TcRs will recognize foreign antigenic peptides presented by self-MHC molecules. Based on this reactivity of a TcR with a peptide/MHC complex, it was questioned whether positive selection in the thymus involved binding of the TcR to a self-peptide/MHC complex.

Sofar, there is not a satisfactory answer to this question. Experiments in $\alpha\beta$TcR transgenic mice have indicated that developing T cells are positively selected by thymic MHC molecules in the absence of the self-peptide recognized by the transgenic TcR (86,92,93). In contrast, positive selection was hampered in the thymus of mice carrying MHC molecules with mutations in the peptide-binding groove, that affected binding of self-peptides (130-134).

In determining the developmental stage of positive selection, it was found that the, newly defined, $CD4^+8^+3^{high}$ cells are post-selection intermediates (71,76,128). This was established by analyzing TcR-α gene rearrangements as well as expression of the recombination activating genes, RAG-1 and RAG-2, in thymocyte subpopulations (135,136). It was found that all cortical $CD4^+8^+3^{low}$ thymocytes were expressing the RAG genes and still continued to rearrange TcR-α genes, despite the presence of the $\alpha\beta$TcR on their cell surface. Mature SP thymocytes do not express these genes anymore. This finding suggests that it is not expression of the $\alpha\beta$TcR at the cell surface that will stop TcR-α gene rearrangements. Indeed, recently published studies demonstrate that the stop-signal is given by positive selection (84,136-138). It was shown that positive selection of thymocytes, occurring between the $CD4^+8^+3^{low}$ and $CD4^+8^+3^{high}$ stage of development in TcR transgenic mice, was accompanied by downregulation of RAG gene expression. Thus, it seems that thymocytes can try different α chains combined with its particular β chain until binding of TcR with MHC induces positive selection. Then, RAG gene expression is downregulated, preventing further TcR-α chain rearrangements, and cells become $CD4^+8^+3^{high}$.

Negative selection

A second process that shapes the T cell repertoire is termed *negative selection* or *self-tolerance*, a fourth control point in T cell development, and involves the removal of potentially autoreactive cells by *clonal deletion* (reviewed in ref. 34,83,86,88,89,93, 95,97-101). This process occurs when thymocytes express a TcR with high affinity for a deleting ligand, generally composed of a self-MHC molecule and a self-peptide. That clonal deletion is the mechanism for negative selection, was first demonstrated with the discovery of TcR-Vβ gene families that show specificity for endogenous superantigens, like the minor lymphocyte stimulating (Mls) antigens. In the presence of Mls antigen in the thymus, thymocytes expressing Mls-reactive TcR-Vβ were not allowed to mature (139-141). Comparable results were obtained with the various $\alpha\beta$TcR transgenic mice (142-144).

Clonal deletion affects MHC class I as well as MHC class II restricted autoreactive T cells. Similar to positive selection, negative selection also depends on the interaction of the TcR and its coreceptor with the same MHC molecule (145-149). This was demonstrated, for example, in MHC class I transgenic mice carrying a mutation in the

α3 domain of the MHC molecule, that prevents binding of CD8 (147-149). In the absence of the interaction of CD8 with MHC class I molecules, autoreactive cells were not subject to negative selection.

Clonal deletion of autoreactive thymocytes proceeds via apoptosis or programmed cell death (150-152). This type of cell death is characterized by distinct morphological changes, like rapid volume reduction and chromatin condensation. It was Wyllie (153) who showed that glucocorticoid-induced thymocyte apoptosis was associated with endogenous endonuclease activity and this event has served as the most characteristic biochemical feature of the process. Endonuclease activation during apoptosis results in the production of oligonucleosomal-length DNA, which can be resolved by agarose gel electrophorese, displaying a typical pattern of bands. Apoptosis could be induced *in vitro* and *in vivo* with anti-CD3/TcR mAb or staphylococcal enterotoxins, providing evidence that apoptosis is the major mechanism for the clonal deletion of potentially autoreactive cells (154-158).

Recently, *clonal anergy* was described as a second mechanism to induce negative selection within the thymus (97,98,159-162). Anergy is a non-deletional mechanism, that does not kill the cell subject to negative selection, but makes it unresponsive to activation (e.g. no response to TcR-crosslinking).

As for positive selection, the developmental stage where negative selection occurs was also determined. With cells expressing an Mls-reactive αβTcR, it was found that positive selection occurs in the transition of $CD4^+8^+3^{low}$ to $CD4^+8^+3^{int}$ cells, whereas negative selection occurs from the $CD4^+8^+3^{int}$ to the $CD4^+8^+3^{high}$ stage of T cell development (71,73,75,163). These observations indicate that positive and negative selection are successive processes. However, other studies show that clonal deletion could also be an early selection event, occurring before or even in the absence of positive selection (93,158,163-166). Early clonal deletion, observed in αβTcR transgenic mice and in mice treated with staphylococcal enterotoxins, resulted in the removal of at least half the $CD4^+8^+$ population. Thus, it was proposed that the developmental stage of negative selection is peptide dependent and probably ranges from the early $CD4^+8^+3^{low}$ to the late $CD4^+8^+3^{high}$ cells.

Comparing the characteristics of positive and negative selection, described above, it is evident that both these processes appear to depend on identical TcR-MHC interactions. To resolve this paradox, the *affinity* model has been proposed (167-170). This model suggests that the avidity of the interaction between thymocyte and stromal cell, based on TcR affinity (for MHC or MHC+peptide) and TcR/coreceptor/MHC densities, will determine the selection event. Low avidity will result in positive selection and high avidity will result in negative selection. One prediction of the affinity model is that increasing the avidity of the interaction between thymocyte and stromal cell should convert a positively selecting interaction into one that is negatively selecting. Indeed,

it was recently demonstrated that elevated levels of CD8 cause clonal deletion of TcR transgenic thymocytes, under conditions that otherwise would have supported positive selection of these cells (170,171). At the moment the affinity model is favored above other models (for further reading on these models, see ref. 167-170).

Thus, two rather complicated and yet not fully understood processes will shape the T cell repertoire, learning maturing thymocytes to distinguish self from non-self. The rules governing these processes have by and large been described for maturing cells in the $\alpha\beta$ T lineage, but it appears that, to some extent, they are also applicable to developing cells of the $\gamma\delta$ T cell lineage as well as the $CD4^-8^-$ $\alpha\beta$ T cell lineage. The development of these particular lineages will be discussed below.

T cell differentiation in the γδ T cell lineage

The thymus not only supports the development of $\alpha\beta$ T cells, but also of cells belonging to the $\gamma\delta$ T cell lineage (reviewed in ref. 172-179). Despite similarities in the overall structure of their TcR genes and polypeptide chains, $\gamma\delta$ T cells are quite differrent from $\alpha\beta$ T cells with respect to development, repertoire and extrathymic tissue distribution.

In the early fetal thymus $\gamma\delta$ T cells are the most prominent T cell lineage, since $\gamma\delta TcR^+$ cells are already present at gestational day 14, 2-3 days before $\alpha\beta TcR^+$ cells develop (180-182). It has been demonstrated that $\gamma\delta$ T cells develop in waves, that are identified by the expression of distinct TcR-Vγ gene segments: Vγ3 (nomenclature by Garman et al.; ref. 183) is expressed in the first wave, followed by overlapping waves using Vγ4 and Vγ2 (176,182,184). Cells from the first waves, expressing Vγ3 and Vγ4, are predominantly present in fetal thymus, whereas TcR-V$\gamma2^+$ cells constitute the major $\gamma\delta$ T cell population in adult thymus.

In fetal and adult thymus, $\gamma\delta$ T cells were mainly found within the $CD4^-8^-$ subpopulation, although a minor population of $\gamma\delta TcR^+$ $CD4^-8^+$ cells was also observed (185,186). It has been shown that the ability to generate fetal TcR-V$\gamma3^+$ (and probably also TcR-V$\gamma4^+$) T cells is a unique property of fetal thymic precursors developing in a fetal thymic microenvironment (187,188). Neither adult bone marrow precursors in fetal thymus nor fetal thymic precursors in adult thymus will produce TcR-V$\gamma3^+$ cells.

Within the thymus, the precursors for the $\gamma\delta$ T cells are present within the $CD4^{low}8^-3^-$ precursor pool (44). They will further develop until the point where the $\gamma\delta$ T cell lineage and the $\alpha\beta$ T cell lineage diverge. In adult thymus, this probably occurs at the $CD44^-CD25^+$ TN stage of T cell development (57,189).

In the periphery, $\gamma\delta$ T cells are not only present in blood and lymphoid tissues, but also within epithelial tissues. Moreover, the TcR-Vγ specificity seems to determine the destination of the $\gamma\delta$ T cell: V$\gamma3^+$ cells are targeted to the skin, as Thy-1^+ dendritic

epidermal cells; $V\gamma4^+$ cells localize to the female reproductive tract and the tongue; $V\gamma2^+$ cells are found in the lymphoid tissues; $V\gamma5^+$ cells preferentially home to the intraepithelial region of the gut (173,175,176,188). The development of $V\gamma3^+$, $V\gamma4^+$ and $V\gamma2^+$ cells is thymus dependent, whereas cells expressing $V\gamma5$ (190) and other $V\gamma$ gene segments can develop extrathymically.

The homing of the $\gamma\delta$ T cell subsets to the peripheral organs is not determined by the TcR itself, but probably by distinct homing receptors whose expression is coordinately regulated with TcR $V\gamma$ gene usage (176,191).

The nature of the antigens recognized by $\gamma\delta$ T cells, as well as the MHC(-like) molecules presenting these antigens have not been clearly established. Given the characteristics of the $\gamma\delta$ T cell, described above, it seems unlikely that $\gamma\delta$ T cells practise their immunological function like $\alpha\beta$ T cells do. Rather, it appears that these cell are able to recognize heat shock(-like) proteins, stress molecules induced as a response to a variety of cellular insults, including viral infection and transformation (179). In this context, Havran et al. (176,192) show that the $\gamma\delta$TcR of dendritic epidermal cells in the skin appears to recognize a keratinocyte-specific stress antigen. Therefore, they suggest that $\gamma\delta$ T cells function in "trauma signal surveillance", a primitive form of T cell immunity that provides a first line of defence, against local pathogens, in the epithelia. In particular, the autoreactive $\gamma\delta$ T cells within the epithelial layers may be capable of destroying altered epithelial cells and, by doing so, maintaining epithelial integrity (193).

The requirements for thymic selection in the determination of the $\gamma\delta$TcR repertoire have been studied in $\gamma\delta$TcR transgenic mice (reviewed in ref. 173-175,178). Using these particular mice, it was reported that $\gamma\delta$ T cells, like $CD8^+$ $\alpha\beta$ T cells, can be subject to both positive and negative selection, with MHC class I molecules as restricting element (194-198). However, it was doubted whether these selection mechanisms could be applied to all $\gamma\delta$ T cells, because normal development of $\gamma\delta$ T cells occurred in MHC class I deficient, β_2-microglobulin (β_2m) gene knockout mice (120,199). Therefore, it was suggested that only selection of cells belonging to the 'lymphoid' $V\gamma2^+$ subset (TcR-$V\gamma2$ was used by the transgenes) resembles the selection observed with $\alpha\beta$ T cells. The selection mechanism that, presumably, works on the other $\gamma\delta$ T cell subsets, esp. the $V\gamma3^+$ and $V\gamma4^+$ cells homing to the epithelial tissues, remains to be eludicated.

An important issue that concerns both the $\alpha\beta$ and $\gamma\delta$ T cells is their lineage relationship (reviewed in ref. 172,173,175). Since $\gamma\delta$ T cells appear prior to $\alpha\beta$ T cells in thymic ontogeny, it was initially proposed that γ and δ gene rearrangements play a regulatory role in the development of $\alpha\beta$ T cells (180). In this model, productive γ and δ rearrangements in a committed precursor T cell will result in further differentiation of these cells into mature $\gamma\delta$ T cells, whereas non-productive rearrangement

signals the cells to rearrange both the α and β genes. An alternative model proposes that $\alpha\beta$ and $\gamma\delta$ T cells are separate lineages, where the state of rearrangement of γ and δ TcR genes does not determine cell fate. The following evidence is in support of the second model. First, as the TcR-δ gene locus is embedded in the middle of the TcR-α gene segments, most of the excision products of TcR-α rearrangement will contain either rearranged (first model) or germline (second model) TcR-δ genes. Most of the excision products contained germline TcR-δ genes (172,200). Second, most transgenic mice with rearranged TcR-γ and TcR-δ genes exhibit continued development of $\alpha\beta$ T cells, although most or all $\gamma\delta$ T cells in the transgenics utilize the transgenic receptors (194,195,201,202). Finally, unique gene knockout mice have been generated that either lack the development of $\alpha\beta$ T cells (203,204) or the development of the $\gamma\delta$ T cells (205). In these mice, the development of $\gamma\delta$ T cells, resp. $\alpha\beta$ T cells proceeds undisturbed. These studies support a model of separate lineages, but still do not indicate how the $\alpha\beta$ and $\gamma\delta$ T cell lineages segregate. In this respect, interesting results were found in transgenic mice containing TcR-γ gene constructs of different lengths: long TcR-γ gene constructs were only expressed in $\gamma\delta$ T cells, whereas short TcR-γ gene constructs were expressed in both $\alpha\beta$ and $\gamma\delta$ T cells. Based on these observations, the presence of a putative TcR-γ gene silencer was proposed (202,206). Thus, precursor T cells will split into two cell lineages, independently of TcR gene rearrangements: in one lineage the γ silencer remains inactive while in the other it is activated. Further analysis of TcR-γ gene silencers, but also of other silencer and enhancer molecules (172,207), is necessary to understand the molecular events underlying this particular lineage segregation.

$CD4^-8^-\alpha\beta TcR^+$ thymocytes: a separate lineage

In addition to mature SP thymocytes, the thymus contains a third population of $\alpha\beta TcR^+$ cells, the **$CD4^-8^-\alpha\beta TcR^+$** thymocytes (208-210). These thymocytes (1) express intermediate levels of the $\alpha\beta$TcR, (2) appear after birth (10-30% of adult DN), and (3) phenotypically resemble $\alpha\beta TcR^+$ SP thymocytes ($HSA^-Qa\text{-}2^+$; ref. 210). They proliferate and produce cytokines in response to mitogens and TcR ligation, indicating the presence of a functional TcR (57,211). The $\alpha\beta$ DN cells express an unusual TcR repertoire, with an inexplicable overexpression of TcR-Vβ8.2 (210,212). The repertoire of the $\alpha\beta$ DN cells contains autoreactive TcR-Vβs, that, in the same animal, have been deleted from the repertoire of the mature $\alpha\beta$ SP T cells. The origin of this cell type is still controversial: are they mature T cells derived from a $CD4^+8^+$ precursor after downregulation of CD4 and CD8, a conclusion based on the demethylated state of their CD8 genes (210,212-214), or are they a second, distinct lineage of thymus-derived $\alpha\beta$ T cells (83,215)? This latter hypothesis is substantially supported by Suda

and Zlotnik (57), who show that *in vitro* $\alpha\beta TcR^+$ DN thymocytes develop from $CD25^+$ TN thymocytes. They demonstrate the presence of pre- and postselecting populations among $\alpha\beta TcR^+$ DN thymocytes, supporting the existence of positive and negative selection processes in the development of these cells. However, not all autoreactive TcR-Vβs in the TcR repertoire are subject to clonal deletion and this selection appears to be antigen dependent (57,210,212). The thymic $\alpha\beta$ DN cells phenotypically resemble the $\alpha\beta$ DN cells found in lymph node and a product-precursor relationship has been suggested (215-217). However, the completely unselected TcR repertoire of the lymph node $\alpha\beta$ DN cells seems to indicate otherwise. A function for these different $\alpha\beta$ DN cells has still to be determined.

Lymphostromal interactions

While migrating through the thymus, thymocytes constantly change from thymic mivroenvironment. As a consequence, thymocytes receive signals from a variety of thymic stromal cells. Cells are signalled through cell-cell interactions, e.g. necessary for selection, as well as growth factors, exerting their effect mainly on short range. The different types of interactions occurring between stromal cells and thymocytes will first be discussed. Subsequently, the available evidence on the specific role of the various stromal cells in promoting sequential stages of T cell development will be presented.

Molecules involved in lymphostromal interactions

During their development, thymocytes express a variety of surface molecules essential for interactions with stromal cells. The expression of these molecules changes with the developmental stage of the thymocyte, allowing it to have different interactions with different types of stromal cells. These molecules are roughly categorized in two groups, based on the type of lymphostromal interaction. The first group, involved with *cellular* interactions (Table 1; ref. 218,219), contains thymocyte surface molecules that have a receptor/ligand on the surface of the stromal cell. As has already been indicated, interactions of the TcR, CD4 and CD8 molecules on thymocytes with MHC molecules on stromal cells are essential for selection and maturation. Other thymocyte surface molecules involved with adhesion to thymic stromal cells include CD2, LFA-1 and Thy-1. CD2/LFA-3 and LFA-1/ICAM-1 interactions were first described for the interaction of human thymocytes with cultured human TEC (220,221). In mice, however, a role for the CD2/LFA-3 interaction in T cell development has not yet been established (222-225). On the other hand, the LFA-1/ICAM-1 interaction seems to be important for the development and selection

Table 1. Molecules involved in lymphostromal interactions

Type of interaction	Surface molecule[a]	Receptor/ligand[b]
cellular	TcR	MHC (TEC,IDC,Mø)
	CD4	MHC class II
	CD8	MHC class I
	CD2	LFA-3 (CD58; TEC)
	LFA-1 (CD11a/18)	ICAM-1 (CD54; TEC,IDC)
	Thy-1	? (TEC)
humoral	?	chemoattractants (sc/pvTEC): - thymotaxin (β2-microglobulin)
	Cytokine-R	IL-1, IL-2, IL-3, IL-4, IL-6 IL-7, IFN, TNF, TGF (thymo,TEC,Mø)
	OT-R	Neuropeptides (sc/pvTEC,TNC): - oxytocine (OT) - vasopressine (VP)
	?	thymic hormones (sc/pvTEC,mTEC,HC): - thymulin (FTS) - thymosin - thymopoietin
	?	hormones of extrathymic origin
matrix	VLA-4 (CD49d/CD29)	fibronectin (ECM, TEC line)
	VLA-5 (CD49e/CD29)	fibronectin (ECM)
	VLA-6 (CD49f/CD29)	laminin (ECM)

[a] ? indicates that a receptor for the respective growth factor has not yet been characterized, although an effect of the growth factor on the thymocytes, at least in some cases, was established.
[b] In brackets, the thymic stromal cells are indicated that carry the receptor or produce the ligand, summarized in this column.

of $CD4^+8^+$ cells (226,227). Thy-1 is also involved with mouse lympho-epithelial cell interactions, but its ligand on the surface of TEC is at present unknown (228). The adhesion molecules described here all display signal transducing capacities and activation through these molecules may result in cellular proliferation (219). Thus, adhesion receptors have various roles, contributing to differentiation, proliferation and, of course, adhesion.

The second group of thymocyte molecules is involved with *humoral* interactions and contains the receptors for a variety of soluble factors, like chemoattractants, cytokines,

neuropeptides, thymic hormones, and neuroendocrine hormones (Table 1; ref. 229-232). From these factors, only the role of cytokines in T-cell development is well established (229,233). Especially the development of DN thymocytes is under the control of various cytokines, such as IL-1 (234), IL-2 (52-56), IL-4 (235), and IL-7 (57,236-238). Of these cytokines, IL-1 and IL-7 are produced by TEC, whereas IL-2 and IL-4 are produced by thymocytes themselves, indicating that thymocytes can also influence their own development (229). The role of the other soluble factors is not that well established. Chemoattractants, produced by TEC, are involved with the immigration of T cell precursors into the thymus, but receptors for these molecules are still not known (239-241). Neuroendocrine control of T cell development is substantiated by reports showing that neuropeptides, thymic hormones, and extrathymic hormones can have effects on T cell development (230-232; see also chapter 2). These factors influence T cell development, either by direct binding to thymocytes or indirectly by controlling TEC function.

It should be noted that both types of lymphostromal interactions probably not only mediate signals from stromal cells to thymocytes but also vice versa. There is recent evidence that supports this notion. For example, it has been shown that binding of thymocytes to a cultured thymic stromal cell line induces phosphorylation of epithelial adhesion molecules (242). More importantly, the thymic medulla is absent when TcR-expressing thymocytes do not develop, as was found in SCID mice, that lack mature $\alpha\beta$ T cells due to a genetic defect (243). However, normal development of the medulla occurs as soon as non-SCID bone marrow cells, injected into SCID mice, repopulate the thymus and develop into mature $\alpha\beta$ T cells (243; see also the discussion on the role of the medulla in "Different levels of lymphostromal interactions"). These data indicate that a symbiotic relationship exists between maturing epithelial cells and developing thymocytes.

A third group of thymocyte molecules is involved with binding to the extracellular matrix (ECM; the *matrix* group in table 1). They belong to the integrin family of adhesion molecules and most of them are β_1 integrins also referred to as VLA antigens (244,245). The first integrin identified on thymocytes was VLA-4, a fibronectin (Fn) receptor (246-248). It was shown that VLA-4 is mainly expressed by immature, cycling $CD4^-8^-$ and $CD4^-8^+3^-$ thymocytes, indicating that adhesion to ECM is important for early T cell development. Moreover, these immature thymocytes used the VLA-4 integrin to bind to a Fn-expressing thymic stromal cell line (248,249). Thus, cell-cell interactions can also be mediated by ECM molecules. At the same time, VLA-5, a fibronectin receptor, and VLA-6, a laminin receptor, were also identified as thymocyte surface molecules (250). It has been suggested that adhesion of thymocytes to the ECM or to thymic stromal cells through VLA integrins is important for migration and differentiation of the thymocyte (244,245).

Different levels of lymphostromal interactions

In this section, the role of the different thymic stromal cells in supporting T cell development will be discussed, while following the T cells on their route through the adult thymus (30,31). The distinct stages of this pathway are depicted in figure 3.

Entering the thymus: a role for chemoattractants (stage 1)

T cell precursors enter the adult thymus at the corticomedullary junction (30). These precursors are recruited from the bloodstream by means of chemoattractants produced by thymic epithelial cells (239-241). In this context, thymotaxin, an 11 kDa polypeptide produced by a rat thymic epithelial cell line, has been characterized as a thymic chemoattractant for a bone marrow subpopulation that contains hemopoietic stem cells already committed to the lymphoid lineage (241,251,252). Thymotaxin was later identified as β2-microglobulin, the light chain of MHC class I molecules (253). An antibody against thymotaxin indeed labels the subcapsular and perivascular TEC, indicating the exclusive role of this TEC subpopulation in attracting T cell precursors.

To enter the thymus, precursor T cells have to be able to attach to the endothelium of thymic blood vessels and to migrate into the surrounding tissue, similar to what has been described for the interaction of mature lymphocytes and endothelial cells in peripheral lymph nodes (254,255). During the contact between lymphocytes and endothelial cells, a variety of adhesion molecules, including integrins, selectins, and CD44, is used. Interestingly, it has been shown that entry of precursor T cells into the thymus can be inhibited by CD44 mAb (256,257). Moreover, a novel adhesion molecule on vascular endothelium has been described, involved in the homing of precursor T lymphocytes to the thymus (258).

The precursor T cells, attracted from the bloodstream, have a $CD4^{low}8^{-}3^{-}$ phenotype. After their immigration into the inner cortex, they downregulate CD4 and start dividing while migrating outwards on their way to the subcapsular cortex (30).

Cortical macrophages: a first contact (stage 2)

The seeding of distinct thymic microenvironments has been studied by analyzing distinct complexes of stromal cells with thymocytes (31,259). These complexes can be isolated from the thymus after enzymatic digestion and reflect an association between thymocytes and stromal cells that preexists within the thymus. They include rosettes of Mø and IDC with thymocytes (designated Mø-ROS resp. DC-ROS; ref. 260-262) and thymic nurse cells, a lympho-epithelial structure (see below). It has been found that the Mø-ROS are the earliest discernible lymphostromal complexes in T cell development, present before $CD4^{+}8^{+}3^{low}$ thymocytes develop in the thymus (31,259,263,264). This indicates that a macrophage is one of the first stromal cells that by cellular interaction

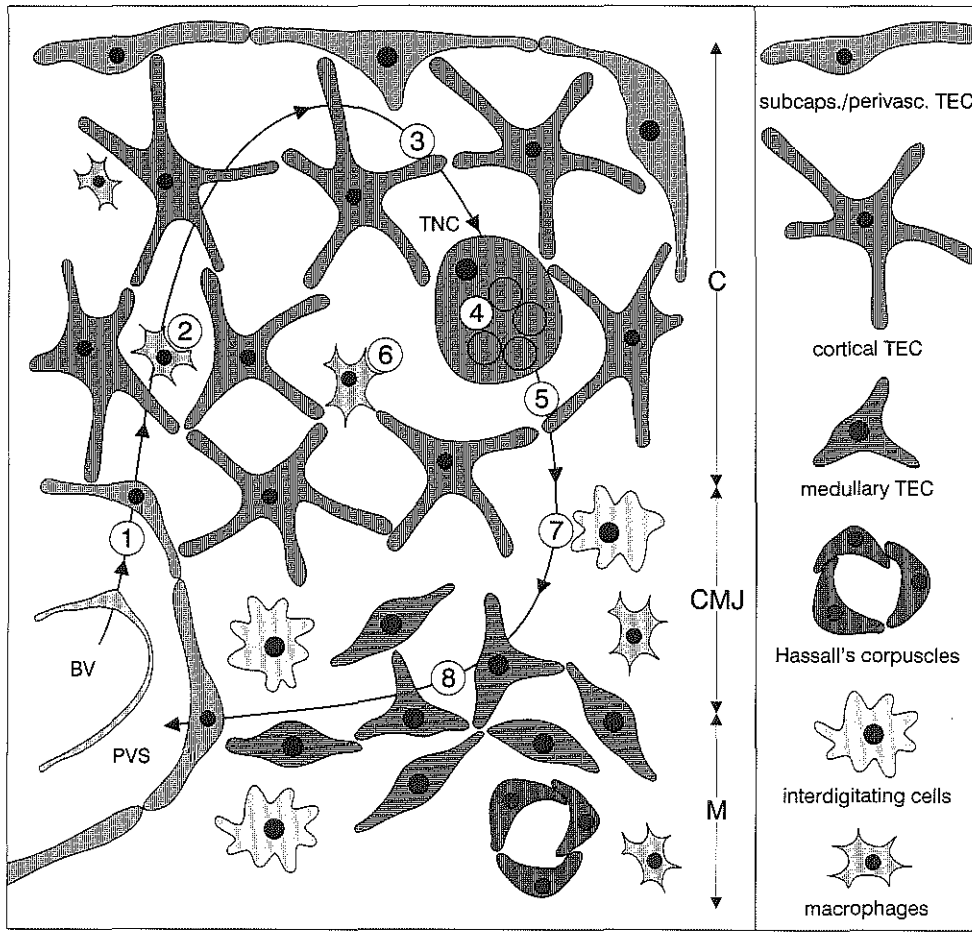

Figure 3. Different thymic microenvironments, passed by maturing thymocytes while migrating through the thymus. Distinct stages of lymphostromal interactions are numbered and discussed in the text.

supports T cell development. Its target cell is the immature $CD4^-8^-3^-$ thymocyte. As $CD4^-8^-3^-$ thymocytes are actively cycling, it seems likely that the cortical macrophage supports the expansion of these cells. In this respect it has been shown that phagocytic cells of the thymic reticulum (P-TR), cells with macrophage characteristics, support the survival and proliferation of $CD4^-8^-$ thymocytes *in vitro* (265-267). The interaction of cycling cells with a cortical macrophage can already occur at the inner cortex, just after precursor T cells entered the thymus, since these cells proliferate while migrating to the outer cortex.

The cortical macrophage could therefore be the proliferation inducing stromal cell

of the first control point. Nevertheless, they can also support proliferation of $CD4^-8^-3^-$ thymocytes in the subcapsular region.

The subcapsular cortex: diverging T cell lineages (stage 3)

The presence of large TN, $CD4^-8^+3^-$ and $CD4^+8^+3^-$ thymocytes in the subcapsular cortex indicates that stromal cells in this location have an important regulating function in the early phase of T cell development. Here, cells diverge into the different lineages of $\alpha\beta$ and $\gamma\delta$ T cells. Development into the main $\alpha\beta$ T cell lineage is accompanied by proliferation and, indeed, most cycling thymocytes are found in the subcapsular cortex (30,61,268-270). A close interaction with TEC is suggested by the presence of TEC with basket-like morphology and the presence of thymic nurse cells. This latter cell population has been subject of particular interest, because its closed structure hints to a specialized microenvironment for T cell development. However, the physiological relevance of these structures *in vivo* has still not been eludicated (discussed in "Thymic nurse cells: a suggestive microenvironment (stage 4)"). Cell cultures of lymphostromal complexes have been carried out as a first step to understand the role of stromal cells in supporting T cell development.

It is now clear that proper thymic stromal cell cultures can only be obtained by using serum-free culture media, containing factors generally beneficial for epithelial cell growth, and ECM-coated culture flasks (271-275). These primary cultures of thymic stromal cells have the ability to promote the development of both αβ and γδ T cells (272,276). However, as these cultures are heterogeneous in stromal cell composition, the specific role of a particular thymic stromal cell type at distinct stages of T cell development can not be determined. Therefore, murine TEC lines capable of inducing T cell differentiation have been developed over the last years.

Some of the new TEC lines are involved with differentiation inductive signals that occur around the second control point at the $CD44^-25^+$ stage of T cell development. It was found that two TEC lines, which express markers of cortical TEC, produce IL-7 and after being injected into spleens of young athymic nude mice support the in vivo generation of $CD4^+8^-$ αβ T cells (ET line) or both $CD4^+8^-$ and $CD4^-8^+$ αβ T cells (EA2 line; ref. 272,277). At the same time, both cell lines also supported the generation of γδ T cells, indicating that cortical TEC are involved with the divergence of the αβ and γδ T cell lineages. IL-7 plays an important role at this point as it has been shown to induce the development of γδ T cells (57,238).

The cell line MRL104.8a (278), of unestablished phenotype, supports the differentiation of TN thymocytes, through $CD4^-8^+3^-$ and DP intermediates, to mature $CD4^+$ SP thymocytes (279,280). It was also established that different TN thymocyte subpopulations could be induced to develop to mature αβ T cells (280). The study revealed that the cell line failed to induce the differentiation of $CD44^+25^-$ TN cells, whereas $CD44^-25^+$ TN cells developed into $CD4^-8^+3^-$ cells. In contrast, only the $CD44^-25^-$ TN cells were supported by this cell line to differentiate to mature SP thymocytes. These results indicate that various TN subpopulations have differential requirements for thymic stromal cells.

The studies with these cell lines suggest that the complete development of late $CD44^-25^-$ TN to SP thymocytes can be supported by the same cortical epithelial cell. Earlier stages of T cell development require additional thymic stromal cells.

The results found with these cell lines were comparable to observations done in

reaggregate fetal thymus organ cultures, a new *in vitro* model system to study the role of individual thymic stromal cell components in T cell development (281,282). Using this modelsystem, it was found that the development of TN T cell precursors into mature $CD4^+8^-$ and $CD4^-8^+$ $\alpha\beta$ T cells requires the presence of both MHC class II expressing cortical TEC and fetal mesenchymal cells (282). The mesenchymal cells are only required at the early stages of T cell development, because the maturation of DP to SP cells can be supported by epithelial cells alone. Thus, at least in the fetal thymus both epithelial cells and mesenchymal cells are required to support the development of TN thymocytes in the subcapsular cortex, whereas subsequent development to mature SP cells only requires a cortical epithelial cell. These data suggest that mesenchymal cells of the thymic capsule provide the additional differentiation inducing signals.

Thymic nurse cells: a suggestive microenvironment (stage 4)

Thymic nurse cells (TNC) are lympho-epithelial complexes that were first identified *in vitro* after enzymatic digestion of the thymus (22,283,284). They appear to be closed structures, with thymocytes completely enclosed within the epithelial cells. However, kinetic studies on TNC development *in vivo* and *in vitro* have indicated that complete envelopment of the thymocytes is an artefact caused by enzymatic digestion (283-285). Nevertheless, TNC represent the *in vitro* correlate of a pre-existing *in vivo* interaction between thymocyte and epithelial cells. Moreover, *in vivo* equivalents of TNC have been identified using SEM, revealing an open structure that allowed thymocytes to move freely in and out (19).

TNC are mainly found in the outer cortical region (19,283), which is reflected in the cortical phenotype of TEC (286) and the cortical $CD4^+8^+$ phenotype of the enclosed thymocytes. The adhesion in these lymphoepithelial complexes was found to be mediated by interaction of the TcR and its coreceptor CD4 or CD8 with MHC class II resp. class I molecules (263,264,284,287,288). This observation, in combination with TNC morphology, suggests that TNC can provide a special microenvironment for positive selection of thymocytes. Indeed, chicken TNC seemed to provide an optimal microenvironment for positive selection: here, TNC lymphocytes had been subject to positive but not yet to negative selection (289). However, these results are "chicken specific" and comparable preferential development of intra-TNC thymocytes (as compared to extra-TNC thymocytes) has not been demonstrated in other species (284).

Together, these observations indicate that the physiological relevance of thymic nurse cells *in vivo* still remains to be resolved. However, the interaction of $CD4^+8^+$ thymocytes with MHC expressing cortical epithelial cells, including TNC, is essential for the maturation of the thymocyte. This interaction occurs while the DP thymocytes

migrate from the outer to the inner cortex.

Cortical epithelial cells: a selective microenvironment (stage 5)

It is now well established that positive selection of thymocytes is a major function of cortical TEC. Initially, this was established in MHC chimeric mice, that expressed different MHC molecules on the different types of thymic stromal cells (for further reading, see ref. 98,290-294). Now, elegant studies, using transgenic mice that expressed MHC class II molecules either on cortical TEC or on medullary TEC (295), confirm that positive selection only occurs when MHC molecules are expressed on cortical TEC (113,296-299). Whether all cortical TEC or just a subpopulation of TEC induce positive selection has not been identified. As stated above, cortical epithelial cells already in the outer cortex (TNC) are capable of inducing positive selection. However, the divergence into $CD4^+8^-$ and $CD4^-8^+$ thymocytes, a result of positive selection, occurs shortly before or after thymocytes enter the medulla. Therefore, it has been proposed that the inductive signal for positive selection can be given in the outer cortex, whereas positive selection may become manifest at a later differentiation stage (16,99).

A role for cortical TEC in negative selection is less well defined. Different studies show that TEC can induce clonal deletion and clonal anergy of self-reactive thymocytes, but these studies do not distinguish between cortical and medullary TEC (160,300-303). However, using an *in vitro* clonal deletion system (304), it was reported that cortical TEC lines as well as freshly isolated cortical TEC (i.e. TNC) were able to induce antigen-specific deletion of $CD4^+8^+$ thymocytes (305,306). Whether this reflects the *in vivo* situation remains to be established.

$CD4^+8^+$ thymocytes, that are positively selected, will migrate to the cortico-medullary region where IDC are waiting to screen maturing thymocytes for auto-reactivity. Unselected thymocytes are removed by cortical macrophages.

Cortical macrophages: removing unselected cells (stage 6)

Most $CD4^+8^+$ thymocytes are not selected for development into mature cells and it has been proposed that these cells die within the thymus. However, this proposition seems highly controversial, as massive cell death is not visible in thymus sections. Their absence is probably best explained if one assumes that cells destined to die are rapidly cleared from the thymic microenvironment by macrophages. Indeed, *in vitro* it was observed that immature $CD4^+8^+$ thymocytes are phagocytosed by thymic macrophages (307-309). Moreover, only $CD4^+8^+3^{low}$ thymocytes are selectively cleared by macrophages *in vitro*, indicating that maturing thymocytes that are not positively selected will be removed before they eventually die (307). These macrophages have the morphology of "tingible body macrophages", present in germinal

centers and involved with removing B cells. It should be noted that this macrophage type is only present in low numbers in thymus sections, suggesting that other clearance mechanisms might be active.

Negatively selected thymocytes will undergo apoptosis or programmed cell death. In general, apoptotic cells are recognized by phagocytes and ingested while still intact, thus protecting tissue from the potentially harmful contents of dying cells (310). Different mechanisms for recognition of apoptotic cells have been identified and one of these mechanisms, found with apoptotic thymocytes, involves the surface expression of phosphatidylserine as "eat me" marker for phagocytes (311).

Interdigitating cells: rejecting the autoreactive cells (stage 7)

Interdigitating cells (IDC) are mainly involved with negative selection of autoreactive thymocytes: thymocytes are removed by clonal deletion (97-99,312-314). These stromal cells are located in the medulla close to the cortico-medullary junction. As indicated before (see "Negative selection"), early $CD4^+8^+3^{low}$ to late $CD4^+8^+3^{high}$ thymocytes can be subject to negative selection. Indeed, DP thymocytes proved to be the major thymocyte subpopulation found adhering to IDC (261,262). Mature $CD4^+8^-3^{high}$ and $CD4^-8^+3^{high}$ also bound to the surface of the IDC. They probably represent cells that passed selection and just downregulated either CD4 or CD8.

Phenotypic characterization of IDC indicated that this thymic stromal cell population is more heterogeneous than expected (315,316). Surprisingly, an IDC subpopulation expressing CD8 on the cell surface has recently been reported. This appeared to be authentic CD8, and was produced by the IDC themselves, since they contained CD8 mRNA (315). The high expression of CD8 on IDC suggests a functional role, which remains to be established. It has been suggested that CD8 is used by a cell to exhibit a veto function, that is, binding of CD8 to MHC class I molecules on T cells can result in deletion (317).

Clonal deletion of autoreactive cells results in apoptosis or programmed cell death. Macrophages present at the cortico-medullary junction are probably involved with removing these dying cells. Cells that were not deleted will now enter the medulla for their final stage of T cell development.

The medulla: a true thymic microenvironment? (stage 8)

Different studies, using cell cultures of cortical TEC, have shown that the development of DP into SP thymocytes requires only cortical TEC (280,282), suggesting that medullary TEC are not essential for T cell development. Rather, it appears that maturing thymocytes are essential for the development of medullary TEC. Studies with SCID mice and Cyclosporin A-treated mice have shown that the medulla is absent when mature TcR^+ thymocytes do not develop (243,318-320). In CsA treated mice it

was observed that both medullary TEC and IDC were almost completely absent (319,320). However, the architecture of the medullary microenvironment is restored as soon as TcR^+ cells are introduced. In CsA treated mice, this occurs after cessation of the treatment (319,320). In SCID mice, the medulla is restored after the mice were reconstituted with allogeneic bone marrow cells resulting in repopulation of the thymus (243). The induction of a thymic medulla by maturing thymocytes suggests that maturing thymocytes need a thymic medulla. A function of the medulla might be as simple as providing a microenvironment for T cells to stay in good condition while waiting to leave the thymus.

One function for medullary TEC has been reported. Like IDC, medullary TEC are also involved with negative selection of autoreactive cells. For example, negative selection by clonal anergy was observed when a self-molecule was solely expressed by a small subpopulation of medullary TEC (162). This finding suggests that that the function of TEC in negative selection, reported in various studies, might be attributed to medullary TEC.

Recently, a novel MHC class II molecule, H-2O, was identified and it was reported that in thymus only medullary TEC expressed the new MHC molecules (321,322). Refined phenotypic characterization of medullary TEC indicated that two distinct subpopulations exist, one expressing the new H-2O molecules, the other the conventional H-2I-A/E molecules (323). The conventional MHC class II expressing medullary TEC are involved with negative selection as indicated above. The function of the H-2O molecules, and consequently the function of the $H\text{-}2O^+$ medullary TEC, is presently unknown.

Thus the medulla is a heterogeneous compartment whose functions are not yet fully understood. However, the relation between the medulla and the maturing thymocytes is symbiotic, with still unidentified signals. It is from this compartment, that the mature SP thymocytes enter the bloodstream in order to exert their function in the peripheral immune system.

Introduction to the experimental work

As outlined in this chapter, T cell differentiation in the thymus is a complex multi-step process. By phenotypic and functional characterization, distinct thymocyte subpopulations have been identified and a lineage relationship between these thymocyte subpopulations is now well established. Sequential stages of T cell development are promoted by different thymic stromal cells, since maturing thymocytes follow an elaborate migration pattern through the thymus. This thesis focuses on two facets of this complex differentiation process: (1) *phenotypic*

characterization of thymic stromal cells, and (2) *lymphostromal interactions*.

Thymic epithelial cells can now be characterized by various TEC-specific monoclonal antibodies. By comparative immunohistological analysis of these mAb, five main clusters of thymic epithelial staining (CTES) were established. In **chapter 2**, the CTES mAb as well as other mAb, available for the characterization of thymic stromal cells are reviewed. The applications of these mAb, e.g. in the characterization of thymic microenvironments under various experimental conditions and the phenotyping of thymic epithelial cell lines are also reviewed in this chapter.

Directly related to chapter 2, the development and staining patterns of new anti-stromal antibodies are described in **chapter 7**. These mAb were raised against an anti-Pgp-1 mAb, with the intention to identify the putative ligand for Pgp-1 on the surface of thymic epithelial cells with anti-idiotypic mAb. Although the true anti-idiotypic nature of the new mAb could not be established, the antibodies identified new subpopulations of thymic epithelial cells.

During their development, thymocytes express a variety of surface molecules essential for interaction with thymic stromal cells. These molecules either bind ligands on thymic stromal cells (cellular interaction) or cytokines (humoral interaction). Since expression of surface molecules changes with their developmental stage, thymocytes will automatically interact with different types of stromal cells. The role of such lymphostromal interactions at distinct stages of T cell development can be studied in fetal thymus organ cultures (FTOC). This *in vitro* model system was employed in chapters 4, 5 and 6. FTOC supports the development of immature $CD4^-8^-$ cells into mature $CD4^+8^-$ and $CD4^-8^+$ $\alpha\beta$ T cells and also the development of $\gamma\delta$ T cells (324). Incubation of the FTOC with either mAb, directed against cellular adhesion molecules or cytokine receptors, or cytokines, may result in a blockade of T cell development. Analysis of the phenotype of the thymocytes in such cultures will indicate the stage of T cell development controlled by specific cellular interactions or cytokines.

The transferrin receptor (CD71) is mainly expressed by cycling cells. In **Chapter 3** the expression of CD71 by murine thymocytes is described. Phenotypic analysis revealed that CD71 is expressed on immature cycling cells in early T cell differentiation. **Chapter 4** describes the results of FTOC treated with a CD71 mAb. As a result of CD71 treatment, both proliferation and differentiation of immature thymocytes were inhibited.

Interaction of both the $\alpha\beta$TcR and the coreceptor CD4 with MHC class II molecules are important for positive selection of $CD4^+8^-$ cells. These lymphostromal interactions were analyzed by treating FTOC with a mAb against MHC class II molecules, as described in **chapter 5**. It was found that, under these conditions, (1) mature $CD4^+8^-$ thymocytes did not develop, and (2) CD4 was upregulated on the surface of $CD4^+8^+$ thymocytes.

It was recently shown that the thymic medulla is absent when mature T cells can not develop. This finding indicates that developing T cells influence the integrity of this thymic compartment. Since cytokine treatment of thymus, esp. IL-2 and IL-4, inhibits T cell development, a profound effect of these cytokines on thymic stromal cells was expected. **Chapter 6** describes the effects of IL-2 and IL-4 on the development of thymic epithelial cells in FTOC. Profound effects were only observed in IL-2 treated FTOC, where cortical epithelial cells were almost completely absent, but medullary epithelial cells expanded.

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CHAPTER 2

Phenotypic characterization of murine thymic microenvironments*

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Contents

Summary

The thymus provides the necessary microenvironments for the differentiation of T lymphocytes. Thymic non-lymphoid cells, such as epithelial cells, macrophages and interdigitating cells are thought to promote sequential stages in T cell differentiation. However, their specific role in each step of T cell differentiation remains to be established. With the development of new monoclonal antibodies it has now become possible to characterize the different thymic stromal cell types. In this review, various aspects of thymic stromal cells and their functions in T cell differentiation are discussed, such as (1) phenotypic analysis of stromal cells in situ; (2) the application of new 'chimeric' monoclonal antibodies which 'link' developing thymocytes and stromal cells; (3) perturbation of thymic microenvironments after cyclosporin A treatment; (4) perturbation of thymic microenvironments in new transgenic mouse lines; (5) phenotypic analysis of in vitro growing stromal cell lines.

Introduction

Thymic stromal cells play a crucial role in the differentiation of immunocompetent T lymphocytes. It is generally assumed that education signals are provided by non-lymphoid (stromal) cells that compose the thymic microarchitecture (1-3). There are three major non-lymphoid cells in the thymus: thymic epithelial cells (TEC), macrophages (Mø) and interdigitating cells (IDC).

Within the thymic microarchitecture committed T cell progenitors differentiate towards mature T cells, which express a functionally rearranged T cell receptor (TcR) associated with the CD3 complex. In addition, these cells are characterized by the presence of co-receptors, either CD4 or CD8. Somewhere along the differentiation pathway T cells 'learn' to recognize antigen in the context of self major histocompatibility complex (MHC) molecules (*MHC restriction* or *positive selection*; ref. 2,4). Furthermore, T cells 'learn' to be tolerant to self-peptides (*tolerance induction* or *negative selection*; ref. 5-7). These peptides are most probably presented by self-MHC molecules. As a result, potentially autoreactive cells are deleted.

It is well known that different stages of T cell development are found in three different thymic compartments (8,9): (1) the subcapsular region, a compartment characterized by baskets of epithelial reticular cells, contains most of the $CD4^-8^-$ immature lymphoblasts; (2) the cortex, composed of spider-shaped and sheetlike epithelial cells, is densely packed with small $CD4^+8^+$, $TcR^{-/low}$ immature thymocytes; (3) the medulla, consisting of a network of short stubbed epithelial cells, is loosely filled with $CD4^+8^-$ and $CD4^-8^+$, TcR^{high} mature thymocytes. Other stromal cell types, such

Table 1. Clusters of thymic epithelial staining patterns (CTES)

Cluster	Main staining pattern				Variations
	sc/pv[a]	cort	med	HC	
I	+	+	+	+	
II	+	s	+	+	Only in man to be divided in: (a) more than one layer of subcapsular TEC (b) only one layer of subcapsular TEC
III	–	+	–	–	(a) cortical TEC only (b) + subset medullary TEC (c1) + macrophages or IDC (c2) + thymocytes
IV	–	–	+	+	
V	–	–	–	+	(a) HC only (b) + thymic myeloid cells (c) + associated medullary TEC (d) as c, + small number of thymic leukocytes (e) as c, + subcapsular TEC
XX	Miscellaneous, e.g. majority of subcapsular/cortical TEC and minority medullary TEC				

[a] sc: subcapsular; pv: perivasculair; cort: cortex; med: medulla; HC: Hassall's corpuscles. –: all TEC are negative; s: a subpopulation of TEC is positive; +: majority (≥ 75%) of TEC is positive. This table is a modification of the table in the paper by Kampinga et al. (13).

as interdigitating reticular cells (IDC) are also located in this latter compartment. IDC, however, are most conspicuous at the cortico-medullary junction (CMJ). Macrophages are present in all three compartments.

The anatomical distribution of lymphoid and non-lymphoid cells indicates that different thymic stromal cells promote different stages of T cell differentiation. However, the specific role of each stromal cell type in the promotion of sequential stages of T cell development is poorly understood. The main obstacle here is the difficulty to isolate *in vitro* growing stromal cell lines at a clonal level. Furthermore, with the exception of MHC proteins, the cell surface molecules of thymic stromal cells involved in interactions with T cells are not well defined. With the development of monoclonal antibodies (mAb) against thymic stromal cells, the heterogeneity of the non-lymphoid cell populations and the complexity of thymic microenvironments became well accepted.

This review will in the first part focus on the identification of thymic microenvironments, characterized by new monoclonal antibodies. Next, we will report on perturbed thymic microenvironments as they occur in cyclosporin-A-treated mice, SCID mice, and most particularly, in various types of transgenic mice. Finally, we will discuss some functional data concerning the role of cell surface molecules on stromal cells in

T cell differentiation.

Phenotypic characterization of thymic microenvironments

Antibodies against MHC antigens

Monoclonal antibodies (mAb) which were first noticed to label non-lymphoid cells of the thymus were mAb raised against Major Histocompatibility Complex (MHC) antigens. On frozen sections these mAb reveal a characteristic staining pattern: a reticular meshwork in the cortex and a more confluent staining pattern in the medulla (10,11). This staining pattern is observed with mAb detecting I-A, I-E and H-2D determinants. Anti-H-2K mAb reveal a confluent staining pattern in the medulla, but only a very weak reticular staining pattern in the cortex (8). Using immuno-electron microscopy it has been shown that epithelial reticular cells were the major MHC positive stromal elements in the thymic cortex (11). Also, IDC in the medulla and a proportion of macrophages in both cortex and medulla are found to express MHC determinants (11,12).

Antibodies against thymic epithelial cells

Over the years, a wide variety of mAb against TEC has been produced. Some of these antibodies have been compared in a recent immunohistological study in order to characterize subpopulations of TEC (13). The results of this particular study are briefly summarized below. Furthermore, the presence of specific keratins in the cytoplasm of TEC will be discussed in this section as well as the reactivity of mAb to hormones and neuropeptides.

Clusters of thymic epithelial staining (CTES)

At a recent meeting "The Thymus: Histophysiology and Dynamics in the Immune System" (organized in Rolduc, the Netherlands, March 1989) a first part of a study was completed, aiming at the characterization of the specific determinants on TEC (13). The reactivity of a panel of 25 mAb raised against TEC of man (2 mAb), mouse (13 mAb) and rat (10 mAb) in immunohistology was assessed. The aim of this immunological study was to group mAb according to their distinctive staining patterns on frozen section of the young adult thymus of the species against which these mAb were raised ("reference thymus"). In addition, mAb were also comparatively tested on young adult thymuses of other species (man, mouse, rat, chicken, goat, guinea-pig, calf and axolotl).

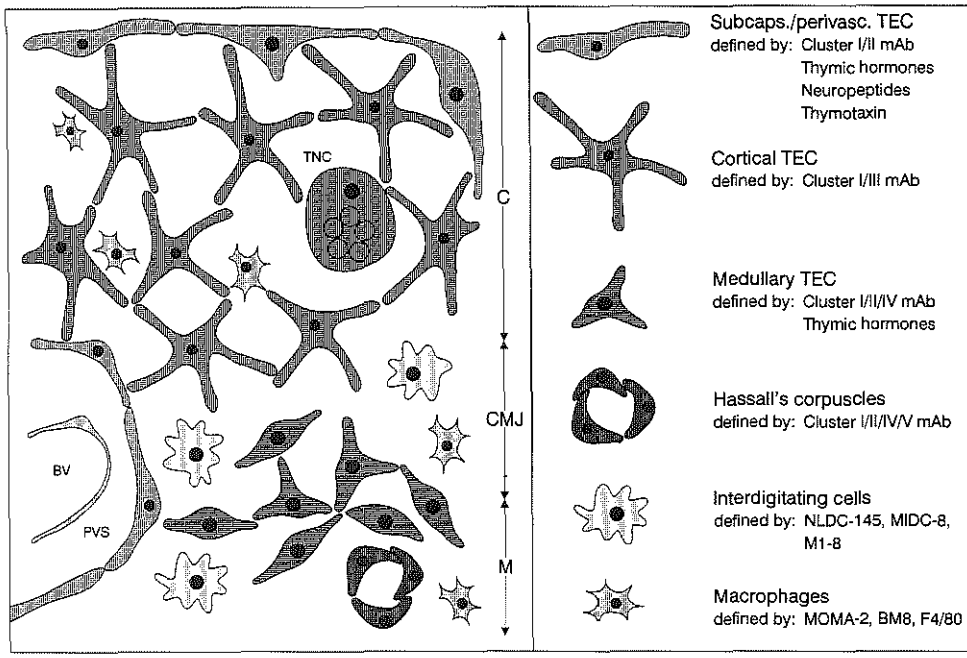

Figure 1. Schematical representation of the thymic stroma, showing the localization of the different types of thymic stromal cells, identified by mAb. TNC: thymic nurse cell; PVS: perivasculair spaces; BV: blood vessel; C: cortex; M: medulla; CMJ: cortico-medullary junction.

Based on the thymic staining patterns of mAb against human TEC (which were classified at the "Third International Workshop and Conference on Human Leucocyte Differentiation Antigens" in Oxford, 1986; ref. 14), clusters of mAb were established, defining subpopulations of TEC (13). At least five staining patterns were discerned (Table 1):

- cluster I mAb stain all TEC;
- cluster II mAb stain subcapsular and perivascular TEC (only one cell layer), in addition medullary TEC and Hassall's Corpuscles (HC) are stained;
- cluster III mAb stain virtually all cortical TEC;
- cluster IV mAb stain medullary TEC and HC, exclusively;
- cluster V mAb stain HC.

Antibodies that do not fit into the main five staining patterns were temporarily grouped in cluster XX, of which one pattern is shown in Table 1.

Based on the thymic staining patterns of mAb against TEC, at least four different subpopulations of TEC can be distinguished (Figure 1):

- subcapsular and perivascular TEC, characterized by CTES cluster II mAb;

Table 2. Monoclonal antibodies against murine thymic epithelial cells[a]

Antibody	MW[b]	Staining pattern					CTES[c]	Reference
	(kDa)	sc	cort	med	HC	TNC		
MTS-1		+	+	+	*		I	23
MTS-5		+	+	+	*		I	23
MTS-10		+	–	+	*		II	23
IVC4		+	–	+	*		II	22
ER-TR5		+	–	+	*	–	II	21
MD1		s	–	+	*	–	II	19
B6TS-1		+	–	+	*	+	II	17
Th-3		?	+	–	–	+	III.A?	15
6C3	160	–	+	–	–	+	III.A	18
CDR1	160	–	+	–	–	+	III.A	19
ER-TR4		–	+	s	*	+	III.B	21
4F-1E4		–	+	s	*		III.B	22
MTS-9		–	+	s	*		III.B	23
NLDC-145	145	?	+	IDC	*		III.C.1?	20
Th-4		–	–	+	*	–	IV	15
TMF	200	–	–	+	*	–	IV	16
MD2		–	–	+	*	–	IV	19
AKTS-1		+	+	s	–	–	XX	17
MTS-7		–	s	s	*		XX	23
MTS-33		–	–	s	*		XX	23

[a] Staining patterns are the result of immunohistological studies on murine thymus sections.
[b] MW: molecular weight; sc: subcapsular; cort: cortex; med: medulla; HC: Hassall's corpuscles; TNC: thymic nurse cell; CTES: clusters of thymic epithelial staining patterns. –: all TEC are negative; s: a subpopulation of TEC is positive; +: majority (≥ 75%) of TEC is positive; ?: difficult to deduce reactivity from the description in the original manuscript; *: since Hassall's corpuscles are small in mice, it is difficult to distinguish HC from other medullary TEC by staining with a mAb that recognizes most medullary TEC.
[c] The antibodies ER-TR4, ER-TR5, IVC4, 4F-1E4 and the antibodies of the MTS series were included in the panel to establish CTES patterns; the staining patterns of the antibodies shown here, are the patterns that were established at the recent thymus meeting in Rolduc (13) and may slightly differ from the pattern presented in the original papers (21-23).

- cortical TEC, characterized by CTES cluster III mAb;
- medullary TEC, characterized by CTES cluster IV mAb;
- Hassall's Corpuscles, characterized by CTES cluster V mAb.

All presently available mAb against murine TEC are listed in Table 2 (15-23). Most mAb received a CTES-number, based on the staining patterns presented in the original papers. Because molecular analysis of the determinants detected by the mAb has not yet been performed, and because these determinants are only clustered based on immunohistological data, it is presently not yet possible to establish a C(luster) of D(ifferentiation) nomenclature. Therefore the established CTES patterns can only form a temporary nomenclature.

Antibodies to cytokeratins

Cytokeratins (CK) are characteristic for cells of epithelial origin. In TEC, they form the major components of the cytoskeleton. In the human species, at least 19 cytokeratin polypeptides have been defined on the basis of molecular weight (ranging between 40-68 kDa) and isoelectric point (range 5-8). CK are numbered according to the catalogue of human cytokeratins by Moll et al. (reviewed in ref. 24). The "basic" subfamily ranges from CK1 to CK8 and the "acidic" subfamily ranges from CK9 to CK18. In each subfamily the molecular weight decreases from low to high CK number. Antibodies against CK are routinely used to characterize epithelial cells under normal as well as pathological conditions (reviewed in ref. 24 and 25). A number of rules govern the expression of CK. Firstly, CK are expressed in specific pairs, consisting of an acidic and a basic unit (25). Secondly, within each pair, the two keratins have identical "size ranks" in their respective subfamilies and appear to follow similar rules for expression. Thus, high molecular weight CK pairs, e.g. CK 3 and 10, are typically expressed in (keratinized) stratified epithelia, while low molecular weight CK pairs, e.g. CK8 and CK18, are solely expressed by simple epithelia (25).

MAb against cytokeratins clearly show that CK expression in the thymus is heterogeneous. Subpopulations of TEC can be defined accordingly (Table 3; ref. 26-32). Overall, the data in Table 3 indicate that cortical TEC resemble "simple" epithelia, while part of the medullary TEC correlate to more "complex" epithelia, as far as CK expression is concerned.

Antibodies to neuroendocrine factors

Over the years several hormone-like polypeptides have been extracted from the thymus. Three of these peptides, thymosin $\alpha 1$, thymopoietin and thymulin, have now been isolated, fully characterized and chemically synthesized (reviewed in ref. 33). Only the last two fulfil the physiological and endocrinological criteria of true thymic hormones: thymopoietin and thymulin are "exclusively" produced in the thymus, they induce T cell markers on T cell precursors (Thy-1 and Lyt-1), and they stimulate various T cell functions. Using polyclonal and monoclonal antibodies, it has been shown that these hormones are produced by TEC in the medulla and in the subcapsular region (Table 4; ref. 34-41).

The antibody A2B5, which recognizes peptide secreting endocrine cells, and also neurons and neural crest-derived cells, reacts both with TEC in the medulla and the subcapsular region (Table 4; ref. 37-39). TEC in these thymic compartments also synthesize oxytocin and arginine-vasopressin (39). During ontogeny the thymus recruits T cell precursors the bloodstream. In this context, thymotaxin, an 11 kDa polypeptide produced by a rat thymic epithelial cell line, has been characterized as a thymic chemo-attractant for a subpopulation of bone marrow-derived hemopoietic

Table 3. Antibodies against cytokeratin polypeptides recognize subpopulations of thymic epithelial cells[a]

Monoclonal antibody[b]		Staining pattern		References[c]
Code	Specificity	Cortex	Medulla	
35βH11	CK8	+	+	26, 30
RPN 1166	CK8	+	–	27, 31
RPN 1160	CK18	+	–	27, 31
KL1	CK3/10	–	s	28, 32
RPN 1165	CK19	–	s	29, 31
34βE12	CK5,6	–	+	26, 30

[a] Staining patterns are the result of immunohistological studies on murine thymus sections. –: all TEC are negative; s: a subpopulation of TEC is positive; +: majority (≥ 75%) of TEC is positive.
[b] These mAb were originally prepared and screened using human cytokeratins. Cytokeratins were numbered according to the catalogue of human cytokeratins (Moll et al.; ref. 24). The RPN mAb were obtained from Radiochemical Centre, Amersham, UK.
[c] The first reference describes the preparation of the antibody and the second describes the staining pattern of the antibody on murine adult thymus.

Table 4. Localization of thymic hormones and neuropeptides in thymic epithelial cells[a]

Peptide	Staining pattern				References
	sc	cort	med	TNC	
Thymosin α1	+	s	+		34
Thymulin	+	s	+		35
Thymopoietin	+	–	+		36
GQ gangliosides	+	–	+	+	37-39
Oxytocin	+	–	+	+	39
Vasopressin	+	–	+	+	39
Thymotaxin	+	–	–		40, 41

[a] Staining patterns are the result of immunohistological studies on murine thymus sections. sc: subcapsular; cort: cortex; med: medulla; TNC: thymic nurse cell; –: all TEC are negative; s: a subpopulation of TEC is positive; +: majority (≥ 75%) of TEC is positive.

precursors in rats, mice and humans (40). An antibody against thymotaxin labels the subcapsular and perivascular TEC (reported in ref. 41).

Together, these data indicate that TEC both in subcapsular and medullary compartments synthesize hormones, neuropeptides and chemo-attractants. Both compartments (in particular the subcapsular thymic compartment) also harbour immature thymocytes. It is therefore tempting to speculate that such factors play a role early in T cell differentiation. Immature $CD4^-8^-$ lymphoblasts are probably the main target cells for the neuro-endocrine factors, since immature $CD4^+8^+$ thymocytes do not respond to these factors (42).

Table 5. Monoclonal antibodies recognizing thymic bone marrow derived cells[a]

Antibody	MW[b] (kDa)	Macrophages		IDC	References
		cort	CMJ/med		
Mac-1	95/170	+	+	+	12, 43
Mac-2	32	+	+	+	12, 44
ER-BMDM1	160	s	+	+	45
F4/80	160	+	+/+	-	46
BM8	125	+	+/s	-	47
MOMA-2	-	+	+	-	48
NLDC-145	145	-	-	+	20
MIDC-8	-	-	-	+	49
M1-8	15	-	-	+	50
ER-TR6	-	-	+	+	21

[a] The staining patterns were obtained by immunohistology or immuno-electronmicroscopy on thymus sections. -: all TEC are negative; s: a subpopulation of TEC is positive; +: majority (≥ 75%) of TEC is positive; in some cases the authors specified staining in the CMJ and the medulla separately.
[b] MW: molecular weight; cort: cortex; CMJ: cortico-medullary junction; med: medulla.

Antibodies against thymic bone marrow derived stromal cells

Thymic macrophages and interdigitating cells can be easily identified at the light and electron microscopical level. As yet no specific mAb against thymic bone marrow derived stromal cells have been raised; mAb which react with thymic Mø and IDC were developed against these particular cell types isolated from peripheral lymphoid organs. Incubation of thymus sections with these mAb shows three major staining patterns (Table 5; ref. 43-50). MAb either stain Mø alone or IDC alone, or both Mø and IDC (Figure 1, Table 5). Although mAb against Mø probably react with all Mø in the thymus, Mø in the cortex, in the medulla and at the CMJ are morphologically distinct from each other (45,46).

Antibodies against thymic reticular fibroblasts

Reticular fibroblasts in the thymus are recognized by the antibody ER-TR7 (21,51). This antibody recognizes fibroblasts and extracellular matrix components in the connective tissue of the capsule and septa as well as in the connetive tissue that surrounds bloodvessels. This staining pattern allows recognition of perivascular spaces in the medulla and at the CMJ.

Antibodies against components of the thymic extracellular matrix

In the majority of organs studied, the extracellular matrix (ECM) is a well organized complex of collagens, glycosaminoglycans and glycoproteins such as fibronectin and laminin. In order to exert their function, most epithelial cells and endothelial cells depend on the interaction with the ECM of basement membranes. In the human thymus, collagens, laminin and fibronectin are found in the connective tissue of the capsule, septa and bloodvessels (52). Collagens and fibronectin are present in connective tissue at the CMJ, whereas Fn is also present in fibers adjacent to medullary TEC. Laminin is mainly expressed in the medulla, inside TEC as well as in adjacent fibers. Thymic epithelial cell cultures produce collagen type IV, laminin and fibronectin (52). A perturbed ECM was detected in pathological thymuses (52). In the mouse, no studies on the thymic ECM are available as yet. However, recently it was shown that a subpopulation of thymocytes carries a fibronectin receptor (FnR; ref. 53). The FnR^+ thymocyte fraction was enriched for $CD4^-8^-$ thymocytes and dividing cells. This suggests that fibronectin is essential for thymocyte proliferation at an immature stage of development.

Hybrid antibodies

The production of hybrid antibodies (HAb) provides a new impulse for functional studies *in vivo* and *in vitro*, where antibodies are used to interfere with T cell differentiation. Zepp and Staerz (54) recently probed T cell differentiation, by culturing murine fetal thymuses with HAb of which one Fab-site recognized $V\beta8^+$ TcR and the other either MHC class I or class II antigens. Thymocytes exposed to either HAb in an early stage of maturation responded with a significant increase in the frequency of $V\beta8$ carrying cells. In contrast, addition of the HAb at a later stage in the culture, when $CD4^+8^+$ cells had developed, resulted in the deletion of $V\beta8^+$ thymocytes. Major conclusions from this study are that (1) the hybrid antibodies can link developing thymocytes to the stroma, and (2) positive selection starts before negative selection. A HAb, with one Fab site recognizing $V\beta8^+$ TcR and the other the cortical TEC antigen CDR1, was prepared and used for *in vivo* treatment of newborn mice (B. Kyewski, unpublished observations). This experiment resulted in an increased appearance of $V\beta8^+$, preferentially $CD4^+8^-$ thymocytes. This observation indicates that $V\beta8$ positive thymocytes are positively selected by cortical epithelial cells.

Application of monoclonal antibodies against thymic stromal cells

An important notion in T cell differentiation is the fact that at different stages of

maturation, thymocytes are located within different thymic compartments (8). This observation suggests that each thymic compartment may play a role in the promotion of various sequential stages in T cell development. An abnormal or blocked T cell differentiation might therefore be correlated with changes in or loss of thymic compartments. In the next part of our paper we will discuss some immunohistological and functional data on the thymus of mice with an abnormal or a blocked T cell differentiation.

Perturbed thymic microenvironment: effect of Cyclosporin A

Cyclosporin A (CsA) acts as a powerful immunosuppressive agent, but it can also, when given in repeated doses, cause T cell-dependent graft-versus-host disease and organ-specific autoimmune disease (55). Two studies by Gao et al. (56) and by Jenkins et al. (57) reveal that CsA affects T cell differentiation and deletion of autoreactive cells in the thymus. CsA inhibits the development of mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes that express the T cell receptor and CD3 at high levels. Secondly, it causes an incomplete deletion of cells bearing self-reactive TcR's in the population of single positive thymocytes that do develop. Therefore, CsA seems to interfere with the processes of negative and positive selection.

Not only thymocytes, but also thymic stromal cells are targets for CsA. In CsA treated mice, the thymic medulla is almost completely absent and, concomitantly, the number of cells expressing MHC class II antigens in this compartment is greatly reduced (55). These data were recently confirmed by Kanariou et al. (22). In addition, these authors showed that medullary TEC (ER-$TR5^+$, $IVC4^+$), IDC (MHC class II^+, CK^-) and macrophages were equally diminished by CsA. However, such cells could still be found after the treatment. By contrast, the cortical TEC (ER-$TR4^+$, 4F-$1E4^+$) were normally present. MHC class II expression remained high during the treatment, showing a reticular staining pattern in the cortex and a confluent staining pattern in the medullary remnant. No thymocytes were found in the medulla, which caused the medullary blood vessels, visible by staining with ER-TR7, to appear more densely packed (22). The observed medullary atrophy, although dramatic, is not permanent. After cessation of CsA treatment, the various cellular components of the medulla reappear and the thymic architecture returns to normal.

The fact that all medullary cells, stromal cells as well as thymocytes, are affected raises the question which cell type is the primary target for CsA. Colombani et al. (58) have shown that CsA binds to calmodulin and inhibits the calcium-dependent activation of T lymphocytes. Therefore, binding of CsA to thymocytes might interfere with the Ca-dependent signal transduction after MHC-TcR interactions, which in turn might abrogate positive and negative selection. However, a direct effect on thymic

stromal cells cannot be excluded. When 2 weeks after CsA treatment mice were irradiated and reconstituted with bone marrow cells, mature $CD4^+8^-$ thymocytes developed. These cells were, however, functionally inactive (Fukuzawa, et al.; ref. 59).

Perturbed thymic microenvironment: SCID mice

Mice homozygous for the severe combined immune deficiency (SCID) mutation (SCID mice) lack functional B and T cells (60). These cells do not develop because of a defect in the enzyme DNA-recombinase (61). Therefore, mature thymocytes are not present. In the SCID mouse, the medulla (ER-TR5) is virtually absent (62). However, reconstitution of SCID mice with normal bone marrow cells restores T cell differentiation to a certain extent (Shores et al.; ref. 63). In such bone marrow reconstituted mice, ER-$TR5^+$ medullary TEC were found to develop (62). Noteworthy, this observation indicates that the medullary microenvironment itself is influenced by the presence of developing mature thymocytes.

Perturbed thymic miocroenvironments: transgenic mice

Besides CsA treated mice and manipulated SCID mice, transgenic mice are important new tools for the study of T cell differentiation. Rüther et al. (64) studied the function of the *c-fos* proto-oncogene in transgenic mice. Mice expressing the *c-fos* transgene develop an abnormal composition of lymphoid populations and thymic microenvironments. Thus compared to non-transgenic littermates, *c-fos* transgenic mice contain 10-50% thymocytes, relatively more mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes and less $CD4^+8^+$ immature thymocytes. The cause of this abnormal T cell distribution most likely lays within the stromal microenvironment, since transfer of hemopoietic cells from *c-fos* transgenic mice into normal syngeneic irradiated mice does not cause any defects in T cell development in the normal recipient thymus.

In a recent immunohistological study, Kyewski et al. (65) showed that the total number of keratin positive cells in the thymus of *c-fos* trangenic mice was increased by at least 2- to 4-fold. This increase was caused by an expansion of the medullary TEC ($MD1^+$, $MD2^+$, ER-$TR5^+$). Medullary IDC (MHC class II^+ and ER-$BMDM1^+$) were progressively lost. The cortex had decreased in size, but TEC could still be identified with antibodies specific for cortical TEC. The peripheral immune function of these trangenic mice was impaired at both the T and the B cell level. Together, the date indicate a lack of T helper cells. This lack of T helper cells might be caused by inappropriate selection mechanisms, but a causal link between the altered stromal cell composition and the aberrant thymocyte differentiation remains to be established.

Mice transgenic for the activated *Ha-Ras* oncogene linked to the human β-interferon

promoter develop an extreme thymic hyperplasia in the early postnatal peria (65). The size of the thymus, and concomitantly the number of thymocytes increases 10-fold compared to control littermates. The numerical increase in thymocytes proportionally affects all four thymocyte subsets. Interestingly, in *Ha-Ras* transgenic mice the cortical TEC marker CDR-1 is lost, while the NLDC-145 and the MHC class II antigens are normally expressed. Cortical TEC also loose their typical reticular staining pattern. Similarly, the medullary TEC marker MD1 is progressively lost, while the expression of the ER-TR5, MD2 and MHC class II antigens is retained. Medullary IDC (MIDC-8^+) are reduced in number. The authors conclude that, although TEC antigens are lost both in the thymic compartments of cortex and medulla, T cell differentiation remains normal.

Ferrick et al. (66) recently characterized transgenic mice carrying a functional TcR Cγ4 gene. The active transcription of the Cγ4 transgene can influence expression of endogenous Cγ4, Cγ1 and Cγ2 genes, whereas the ultimate expression of other TcR δ, α and β chain genes, as well as the adult T cell response, are relatively unaltered. These transgenic mice show an accelerated developing immune system compared to control littermates. At two weeks of age the peripheral lymphoid tissues were as much as 100-fold more immunoreactive, as measured by Con A response and alloreactivity. These striking differences became less obvious in adult mice. Slight but consistent increases in the percentage of $CD4^-8^+$ and $CD4^+8^-$ thymocytes were found at the expense of the $CD4^+8^+$ thymocytes. Immunohistological analysis showed that the thymic medulla of the Cγ4 transgenic mice was greatly expanded. This expansion was probably not caused by an increase in the number of ER-$TR5^+$ medullary TEC, but by an increase in size of the TEC themselves, since medullary TEC in these transgenic mice showed long and branched cytoplasmic extensions (Ferrick et al.; ref. 67). In addition, Hassall's Corpuscles, recognized by an antibody against CK 10 (RKSE 60; ref. 68), were enlarged and more numerous. Within the expanded medulla, the perivascular spaces were also enlarged, as shown by staining with ER-TR7, indicating increased cell traffic from the thymus. No apparent changes in cortical TEC (ER-$TR4^+$), Mø (ER-$BMDM1^+$) or IDC (ER-$BMDM1^+$) were observed. Thus, the observed expansion of the medulla seems to be mainly caused by a raise in the number of medullary thymocytes and not by an increase in the number of medullary TEC. The reason for this phenomenon remains unclear at present. However, an extended network of epithelial cells in the medulla might affect T cell maturation in a positive way.

The role of MHC class II expression in different thymic microenvironments was studied in I-E_α transgenic mice. Introduction of the E_α^k transgene in C57Bl mice (these mice contain a silent E_α gene, caused by a deletion in the promoter region of E_α) results in complete expression of I-E molecules on B cells, antigen-presenting cells and thymic stromal cells (69). It appears that mutagenesis of short conserved motifs

(X and Y box) in the promoter of the E_α gene influences E_α transcription in a cell type-specific fashion (70). Van Ewijk et al. (71) showed that at the immunohistological level, deletion of the X box (ΔX mice) results in I-E expression in the medulla, but not in the cortex. By contrast, ΔY mice showed the reverse pattern: I-E was, by and large, expressed only on cortical TEC. A major conclusion arising from this work was that different promoter elements regulate MHC class II expression in different cell types. Given this apparent differential I-E expression in the thymus of ΔX and ΔY mice, it was obviously interesting to know in which thymic microenvironment positive and negative selection occurred. Recently, two very elegant studies by Benoist and Mathis (72) and by Berg et al. (73), using the ΔX and ΔY mutant transgenics, showed that expression of I-E molecules on cortical TEC is essential for positive selection in T cell differentiation (reviewed in ref. 74).

Negative selection occurred both in ΔX and ΔY mice (74). Hence a precise localization of this step in T cell differentiation remains unclear. In this context, experiments from the group of Von Boehmer (Basel) have shown that negative selection in HY specific $\alpha\beta$ TcR transgenic mice may occur already at a very early stage of T cell differentiation (7). Recently performed immunohistological analysis of these transgenic mice revealed down regulation of CD4 and CD8 already in subcapsular lymphoblasts. Interestingly, the cortical compartment in the thymus of male (HY^+) but not of female (HY^-) mice was affected. In particular, the deep cortex showed a closely packed meshwork of ER-$TR4^+$ TEC, instead of a fine reticular pattern. The expression of $H\text{-}2D^b$ (the restriction element for T cells which react with HY) was also grossly aberrant in male but not in female mice (van Ewijk et al.; ref. 75).

Thymic stromal cell lines

Primary cultures of thymic stromal cells have demonstrated their ability to promote the proliferation and differentiation of thymocytes (76). However, since thymic stromal cells have demonstrated their ability to promote the proliferation and differentiation of thymocytes. However, since thymic stromal cell cultures are heterogeneous in composition the development of continuously growing stromal cell lines is essential for a study of the role of one specific stromal cell type in T cell differentiation. In this context, murine TEC lines capable of inducing thymocyte proliferation, have been developed over the last few years (reviewed in ref. 41). However, although most of these TEC lines were typed as epithelial in origin, a systematic study using markers which define the epithelial, phagocytic or fibroblastic origin of the cells has not been carried out. Sofar, Savino et al. (77) reported the characterization of four different putative TEC lines. The authors demonstrated that, based on CK expression, thymulin

production and the production of the extracellular matrix proteins collagen type IV and fibronectin, only one of them was indeed epithelial. This indicates that, although thymic tissue was used to prepare TEC lines, an extensive phenotypic characterization is required to conclude a real epithelial origin. Since at least four subpopulation of TEC can be defined with mAb, a particular TEC line should reflect its origin based on the phenotypic characterization. In this respect the following criteria are essential for ultimate typing of TEC lines: (1) presence or absence of desmosomes and tonofilaments, studied by EM; (2) detection of CK with mAb; (3) detection of TEC-specific antigens with mAb; and (4) absence of antigens specific for Mø, IDC and fibroblasts, identified with mAb.

In the mouse three TEC lines have been described that, more or less, meet the proposed criteria. Farr et al. (78) reported the generation of two cloned TEC lines, TE-71 and TE-75, which resemble medullary TEC by a number of criteria ($A2B5^+$, $ER\text{-}TR5^+$, CK^+, but $ER\text{-}TR4^-$). Medium conditioned by TE-71 and TE-75 exhibited colony-stimulating activity for bone marrow cells and IL-1-like activity which could be neutralized with anti-IL-1 antibodies. However, no induction of T cell differentiation by these cell lines has been reported as yet. The TEC line E5, described by Potworowski et al. (79) also resembles medullary TEC. This TEC line reacts with mAb against cytokeratins and medullary TEC (TMF, ER-TR5), but not with a mAb against cortical TEC (ER-TR4). Thymocytes and (day 16) fetal thymocytes adhere to this cell line. These interactions can be blocked by an antibody against TMF or by TMF antigen (80,81). Furthermore, the adherence of thymocytes to E5 was not MHC-restricted (82). Predominantly, $CD4^+8^+$ thymocytes bound to E5, whereas no adherence of $CD4^-8^-$ thymocytes could be detected. Upon coculture with E5, a proportion of the adherent thymocytes underwent a phenotypic switch towards a more mature, $CD4^+8^-$, stage (P.Hugo, unpublished observations).

One paper on the functional aspects of thymic stromal cells merits attention. In this context, Kosaka et al. (83) showed that a thymic stromal cell clone (of which the epithelial nature could not be established) could be used as a model for clonal deletion. This line, MRL104.8a, expresses MHC class I and II antigens after exposure to γ-interferon and produces a thymic stroma derived T cell growth factor (TSTGF). TSTGF is distinct from other known interleukins and is capable of promoting the growth of Ag-specific T helper clones (Th) without requiring specific antigen or IL-2. When a Keyhole Limpet hemocyanin (KLH)-specific, $I\text{-}E^k$ restricted Th clone was cultured on the ($I\text{-}A^k$ and $I\text{-}E^k$ expressing) stromal cell line, massive proliferation of the Th clone was induced. In contrast, the addition of KLH resulted in total growth inhibition of the Th clone. The death of the Th clone could be prevented by the addition of anti-$I\text{-}E^k$ or anti-CD3 mAb. When the same Th clone was cultured in the presence of KLH on another thymic stromal cell clone (MRL28.8a, also $I\text{-}E^{k+}$, but

producing a marginal amount of TSTGF), Th cells did not die. However, a lethal effect could be induced by adding TSTGF to the culture. In a control experiment, a KLH-specific, I-A^b restricted Th clone, cocultured with the MRL104.8a clone was induced to proliferate, whether or not the KLH was present. These results indicate that TSTGF-producing and Ia-expressing thymic stromal cells induce either continuous proliferation or selective elimination of each T cell clone, depending on whether the T cell receptor recognizes the relevant antigen associated with Ia molecules expressed on the stromal cell surface. Unfortunately, the exact phenotype of this stromal clone has not yet been established.

Concluding remarks

An important conclusion from this review is the notion that, at this moment, we stand only at the beginning of unraveling the role of thymic microenvironments in T cell differentiation. Although we recognize thymic microenvironments more precisely by the newly developed anti-stromal antibodies, biochemical analysis of the recognized antigens and, more importantly, functional studies of the stromal cells recognized by these antibodies is a major future challenge. An important obstacle in assaying the role of thymic microenvironments *in vitro* is the poor growth capacity of thymic stromal cells. Improved culture and immortalization techniques for stromal cells are clearly needed. It may therefore well be that in the near future progress in research on the immunological contribution of thymic microenvironments will come from the development of new transgenic mouse models. Thus, although the progress is made, the thymus and its various thymic microenvironments remain a "black box" for a while.

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CHAPTER 3

Transferrin receptor expression in murine T cell differentiation I. Characterization of transferrin receptor expressing thymocytes*

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Summary

Dividing cells require iron and, therefore, most cells express the transferrin receptor (CD71) on the cell surface during cell cycle. In this paper, we analyzed the expression of CD71 on fetal, neonatal and adult thymocytes in correlation to cell size, cell cyclus, and expression of CD3, CD4, CD8, $\alpha\beta$TcR, and $\gamma\delta$TcR.
Phenotypic analysis revealed that only the large, immature $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ in fetal, neonatal and adult thymus expressed CD71. In addition, $CD71^+$ large adult thymocytes were all cycling, as determined by DNA-analysis. Together, these data indicate that CD71 is expressed on immature cycling cells in early T cell development. Immunohistology showed that $CD71^+$ cells were found in the subcapsular region and scattered throughout the cortex of the adult thymus.

Downregulation of CD71 occurs when proliferation ceases, i.e. within the $CD4^+8^+3^-$ thymocyte subpopulation. The gradual changes in size and CD71 expression suggest a developmental lineage from large $CD71^+$ via small $CD71^\pm$ to small $CD71^-$ cells. As a consequence, CD71 was not expressed on mature TcR^+ thymocytes. However, cycling cells can be found within specific TcR^+ subpopulations in thymus, like the fetal $\gamma\delta TcR^+$ cells and the neonatal $\alpha\beta TcR^+$ $CD4^+8^-$ cells. CD71 was also expressed by these subpopulations.

Introduction

The transferrin receptor (TfR) is expressed on many cells that require iron for their development (1,2). Cellular iron uptake starts with the binding of transferrin-bound iron to the receptor. Via clathrin-coated pits and vesicles, internalization occurs as a receptor-ligand complex. Once inside, iron molecules are exchanged to intracellular ferritin and the transferrin and its receptor are recycled to the cell surface. The human transferrin receptor molecule is a 190 kDa glycoprotein composed of two disulfide linked chains of 95 kDa, each capable of binding a transferrin molecule (3,4). In the mouse, this homodimer has a 200 kDa molecular weight with two chains of 100 kDa (5).

Proliferation of many cell types requires an increased iron uptake by the cell. The cells respond to this need by increasing the surface expression of the transferrin receptor. Therefore, TfR expression is related to cell proliferation. It is not only evident for probably every cell line in culture, but also for many neoplastic tissues (2,6). However, TfR expression is also associated with cell function, e.g. tissue macrophages use iron-dependent enzymes, like peroxydases (1,6,7).

With the assignmemt of a cluster designation, i.e. CD71, the transferrin receptor was

recognized as a human leucocyte differentiation antigen (2). CD71 mAb did not react with resting peripheral blood lymphocytes and monocytes, but when activated, both cell types showed significant CD71 expression. For this reason CD71, like e.g. CD25, was characterized as an activation antigen (8). Virtually all permanent hemopoietic cell lines, regardless of their cell lineage, as well as many lymphomas and leukemias, expressed a TfR (2). This indicates that, also in the hemopoietic system, transferrin receptor expression correlates to cell proliferation.

An intensive proliferation is part of the T cell differentiation process in the thymus, both in the fetus (9) and in the adult (10,11). In the fetal thymus proliferation occurs between day 13 and day 18 of gestation, when cells expand at least a thousand times in cell number (9). In the adult thymus, even an overall expansion of around 10^5-fold was calculated when a daily entry of 100 prothymocytes was assumed (10). Distinct thymocyte subpopulations, with an immature $CD4^-8^-$, $CD4^-8^+$ and $CD4^+8^+$ phenotype, have been defined as the major cycling cell populations (10,12-15). Do cycling thymocytes express the transferrin receptor? Flow cytometric analysis of adult human thymocytes showed a 14% mean reactivity with CD71 mAb (2). In addition, staining of thymus sections with CD71 mAb gave a strong labeling of cells in the subcapsular area, where immature thymocytes are located (16). This staining was especially prominent in thymus specimens from newborns and early childhood (3,16). In contrast, flow cytometric analyses of *murine* thymocytes with CD71 mAb showed ample or no reactivity with the TfR (17,18). This does not seem to be in agreement with the estimation that $\approx$ 20% of all thymocytes are actually dividing cells (10,12). A new CD71 mAb, i.e. ER-MP21 (19), developed in our laboratory, showed a 20-30% reactivity with adult murine thymocytes (this manuscript). But so far, no data are available that indicate the cell cycle status as well as the developmental stage of CD71-expressing thymocytes.

In the present study, we examined whether CD71 expression could be used as a new differentiation marker, identifying immature cycling cells in the early stages of T cell development. To that purpose, we analyzed the expression of CD71 on fetal, neonatal and adult thymocytes, using three-color flow cytometric analysis. Our data indicate that CD71 is expressed on large immature thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes. DNA-analysis of adult thymocytes revealed that large $CD71^+$ thymocytes were all cycling cells. CD71 is downregulated before the CD3/$\alpha\beta$TcR complex is expressed on the cell surface. However, CD71 could be expressed on TCR^+ cells, **only** when they were cycling, as observed for fetal $\gamma\delta TcR^+$ and neonatal $\alpha\beta$TcR $CD4^+8^-$ thymocytes.

In an accompanying study (20), we show that proliferation and differentiation of $\alpha\beta$TcR-positive, but not $\gamma\delta$TcR-positive thymocytes are inhibited in fetal thymus organ cultures treated with a CD71 mAb.

Materials and Methods

Mice

Male and female BALB/c (H-2^d) mice were bred and maintained in the animal facilities of our department. For timed pregnancies, the appearance of a vaginal plug was considered to be day 0 of gestation. Pregnant mice were killed by cervical dislocation and fetuses were dissected from the uterus on different days of gestation. Fetal thymuses were isolated from embryos of day 14-19 of gestation. In our animal facilities pregnant mice litter at the end of day 19, beginning of day 20. Neonatal mice were used within the first 24 hours after birth. Adult mice were 6-10 weeks old.

Antibodies

The antibodies used in this study are listed in Table 1. They were purified from hybridoma culture supernatant by affinity chromatography, which yielded always pure antibodies, i.e. only heavy and light chains were detected by PAGE. For flow cytometric analyses of the thymocytes mAb conjugated with FITC, biotin or R-Phycoerythrin were used. FITC and biotin conjugates were prepared in our laboratory, whereas the R-Phycoerythrin conjugate was commercially obtained as indicated in the legend of Table 1. The properties of the monoclonal antibodies used, were described in detail in the indicated references (19,21-28). To avoid aspecific staining, all conjugates were carefully titrated.

The mAb ER-MP20 detects a differentiation antigen on macrophage precursors (28) and identifies only a small number of thymic cells (unpublished observations; this manuscript). Here, we used this antibody as an isotype control mAb for ER-MP21; both are of the IgG2a isotype.

In this study the mouse transferrin receptor was identified using the rat mAb ER-MP21 (19). This mAb precipitates an antigen of the same apparent molecular mass as the murine TfR, precipitated by mAb H129.121 (5,19). Both mAb recognize a complex of 200 kDa, consisting of two identical chains of 100 kDa. Moreover, ER-MP21 inhibits the cellular uptake of the Fe-transferrin complex (19).

The mAb ER-MP20 and ER-MP21 have been developed in our department by Pieter Leenen (19,28) and are now commercially available from BMA, Augst, Switzerland.

Immunofluorescence and flow cytometric analysis

For flow cytometric analysis, thymocyte suspensions were prepared in PBS containing 0.5% BSA and 2 mM sodium azide. 10^6 cells (in a volume of 10-20 μl) were incubated on ice for 30 min with 25 μl of

Table 1. Characteristics of the monoclonal antibodies

CD[a]	Antigen	Clone	Conjugation[b]	Reference
CD3	CD3	KT3	B	21
CD4	L3T4	GK1.5	PE	22
CD8	Lyt-2	53-6.7	F	23
CD25	IL2R	PC61	F	24
CD44	Pgp-1	IM7.8.1	B	25
CD71	TfR	ER-MP21	F	19
TcR1	$\gamma\delta$TcR	GL3	B	26
TcR2	$\alpha\beta$TcR	H57-597	B	27
-	-	ER-MP20	F	28

[a] Monoclonal antibodies are ordered by the Cluster of Differentiation (CD) nomenclature, when possible.
[b] MAb were used unconjugated or conjugated with FITC (F), biotin (B), or R-Phycoerythrin (PE). R-Phycoerythrin-conjugated anti-L3T4 was obtained from Becton Dickinson, Mountain View, CA, USA.

the appropriate mAb or mixture of mAb, FITC-, biotin-, or R-Phycoerythrin conjugated. After three washes with the PBS-BSA-NaN3 buffer, cells were further incubated for 30 min on ice, with appropriate dilutions of R-Phycoerythrin or Tricolor conjugated to Streptavidin (Caltag, San Francisco, CA, USA). Finally, cells were again washed three times and collected in a small volume for flow cytometric analysis. For two-color staining FITC (green) and R-Phycoeryhtrin (orange) were used as fluorochromes, while for three-color staining Tricolor (red) was used as third color. To identify dead cells in samples with two-color staining, Propidiumiodide (PI) was added just before acquisition at a final concentration of 5 μg/ml. Background fluorescence was determined by staining cells with second step antibodies only or with the FITC-conjugated mAb ER-MP20.

Samples were analyzed for light scatter and fluorescence on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with a 488 nm argon laser and interfaced to a Hewlett-Packard computer running the FACScan software. Calibration of the cytometer was performed by eye, using thymocytes that were unlabeled, labeled with CD8-FITC, labeled with CD4-R-Phycoerythrin, or labeled with CD4-biotin followed by Streptavidin-Tricolor. Forward light scatter and perpendicular light scatter were analyzed with linear amplification, while all three fluorescence channels were subject to logarithmic amplification. In most cases 10,000 cells were analyzed. Dead cells were excluded during data analysis on the basis of forward and perpendicular light scatter (for three-color staining) or a combination of forward light scatter and PI-staining (for two-color staining). All data were analyzed using the FACScan, Paint-a-Gate or LYSYS software (Becton Dickinson, Mountain View, CA, USA). Data are presented in this paper as one-parameter histograms or as two-parameter dotplots, with a four log-decade fluorescence scale when appropriate. Specific subpopulations of cells in dotplots were quantified by quadrant or window analysis as indicated in the figure legends.

Cell cycle analysis of sorted thymocytes

Cell cycle analysis was performed on total adult thymocytes and sorted large, CD71$^+$ thymocytes. The latter population was obtained by staining thymocytes with 10 μg/ml purified ER-MP21, followed by FITC-conjugated anti-rat IgG (Cappel/Organon Technika, Oss, the Netherlands) and subsequent sorting with a FACS Vantage (Becton Dickinson) interfaced to a Hewlett-Packard computer running the LYSYS II software. For DNA analysis, cells were fixed with 70% ethanol and stained with 40 μg/ml Propidium-iodide, in the presence of 100 μg/ml RNase A. Cells were analyzed on a FACScan with linear amplification in the fluorescence channel. The proportion of cells in G_0/G_1, S and G_2/M was calculated using the DNA Cell-Cycle Analysis Software (Becton Dickinson).

Immunohistology

Immunohistology was performed on cryostat tissue sections of adult thymus, essentially as described before (29). In the present study, frozen sections of the adult thymus were incubated with supernatant of the rat hybridoma ER-MP21 (CD71) or with PBS and developed with a Peroxydase conjugate of rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark). For better localization of CD71 expression in the cortex of the thymus, sections were stained with a mixture of purified mAb from the hybridomas ER-MP21 (5 μg/ml) and ER-TR5 (40 μg/ml). ER-TR5 specifically recognizes thymic medullary epithelial cells (30).

Results

The aim of our study was to characterize the CD71 expressing thymocytes. The presence of CD71 on immature and cycling thymocytes has been indicated, but the correlation with cell cycle status and developmental stage of thymocytes (based on CD4, CD8 and TcR expression) has not been accurately established by flow cytometric

analysis. Here, we present these data for adult murine thymus. We also analyzed CD71 expression on developing fetal thymocytes, because their gradual development in ontogeny makes it easier to identify changes in CD71 expression on thymocyte subpopulations.

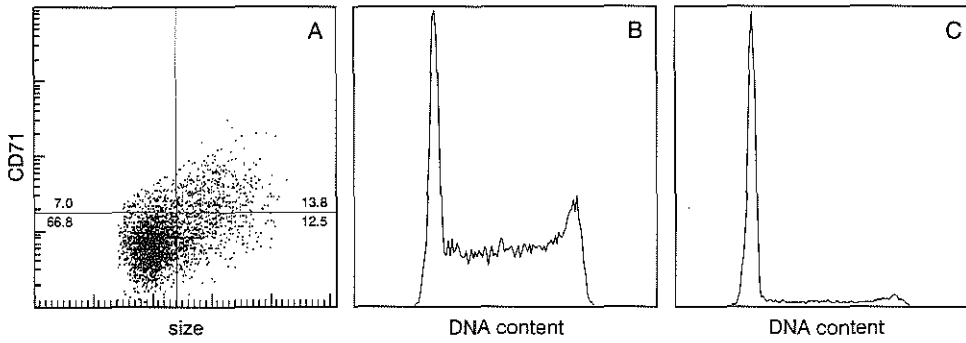


Figure 1. Cell cycle analysis of large CD71$^+$ adult thymocytes. Adult thymocytes were stained with CD71-FITC and CD71 expression is correlated to cell size (A). Sorted large CD71$^+$ cells were compared with unfractionated cells for their cell cycle status. The DNA content, measured as PI-fluorescence, of large, CD71$^+$ (B) and unfractionated thymocytes (C) is shown in histograms with a linear scale. In DNA histograms, the left peak, the lower mid-section, resp. the right peak represent the G_0/G_1, S and G_2/M phases of the cell cycle (in B: 25/64/11% of the cells; in C: 81/14/5% of the cells).

The transferrin receptor is expressed on large-sized dividing thymocytes

The expression of the transferrin receptor was analyzed on thymocytes by use of the CD71 mAb ER-MP21 (19). 20-25% of adult thymocytes expressed CD71 with a weak to moderate intensity and most of these cells had a large cell size, as determined by forward light scatter (Fig. 1A). We performed cell cycle analysis on sorted large CD71$^+$ thymocytes, because large thymocytes are mainly cycling cells (31) and CD71 expression on most cell types strongly correlates to proliferation of the cell. About 64 and 11% of the large CD71$^+$ cells were in the S and G_2/M phases, respectively (Fig. 1B). Since the S-phase occupies 50-70% of the thymocyte cell cycle (13,32), this indicates that all large CD71$^+$ cells are cycling. In contrast, unfractionated thymocytes contained only 14 resp. 5% cells in the S and G_2/M phases (Fig. 1C).

The transferrin receptor is expressed on immature adult thymocytes

The adult thymus contains four major thymocyte subpopulations, defined by CD4 and CD8 expression (Fig. 2A). These subpopulations differ from each other in cell size, i.e. the immature CD4$^-$8$^-$ cells are large, the nonmature CD4$^+$8$^+$ cells are mostly small,

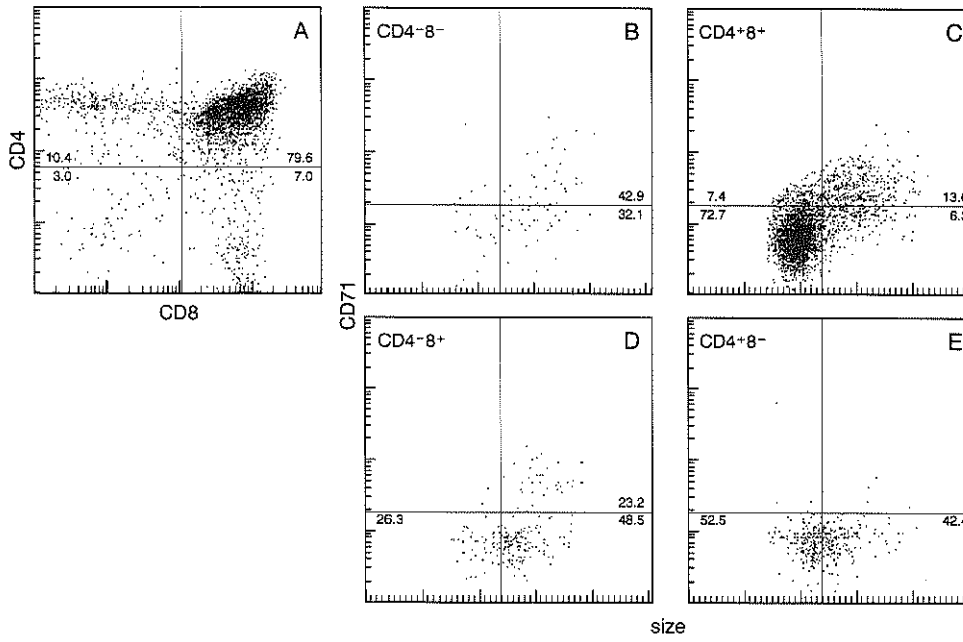


Figure 2. CD71 expression on adult thymocytes. Adult thymocytes were phenotyped with CD71-FITC, CD4-R-Phycoerythrin, and CD8-biotin, followed by streptavidin-Tricolor. A CD4/CD8 dotplot is shown for total thymocytes (A) and CD71/size dotplots for thymocyte subpopulations: $CD4^-8^-$ (B), $CD4^+8^+$ (C), $CD4^-8^+$ (D), and $CD4^+8^-$ (E) cells. CD4/CD8 defined subpopulations were quantified by quadrant analysis (A) and size of the thymocytes was measured as forward light scatter. In CD71/size dotplots, marker lines separate small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within the quadrants.

and the mature $CD4^+8^-/CD4^-8^+$ cells have an intermediate cell size (31). Both, the number of large cells and the number of $CD4^-8^-$ cells suggest that CD71 is also expressed on other thymocyte subpopulations. To that purpose, we analyzed CD71 expression and cell size of CD4 and CD8 defined thymocytes, using three-color flow-cytometry. We observed, that large $CD4^-8^-$ thymocytes expressed CD71 with variable intensity, ranging from negative to high surface expression (Fig. 2B). The $CD4^+8^+$ thymocytes contained a major population of small $CD71^-$ cells and a minor population of large $CD71^+$ cells (Fig. 2C). The $CD4^-8^+$ thymocytes also consisted of two populations, $CD71^-$ thymocytes of intermediate cell size and $CD71^+$ thymocytes with a large cell size (Fig. 2D). $CD4^+8^-$ thymocytes were CD71 negative (Fig. 2E).

The $CD4^+8^+$ and $CD4^-8^+$ thymocytes can not only be subdivided by size and CD71 expression, but also by expression of the CD3/$\alpha\beta$TcR complex (Fig. 3A). $CD4^+8^+$ thymocytes are either negative or express this complex with low intensity (33,34),

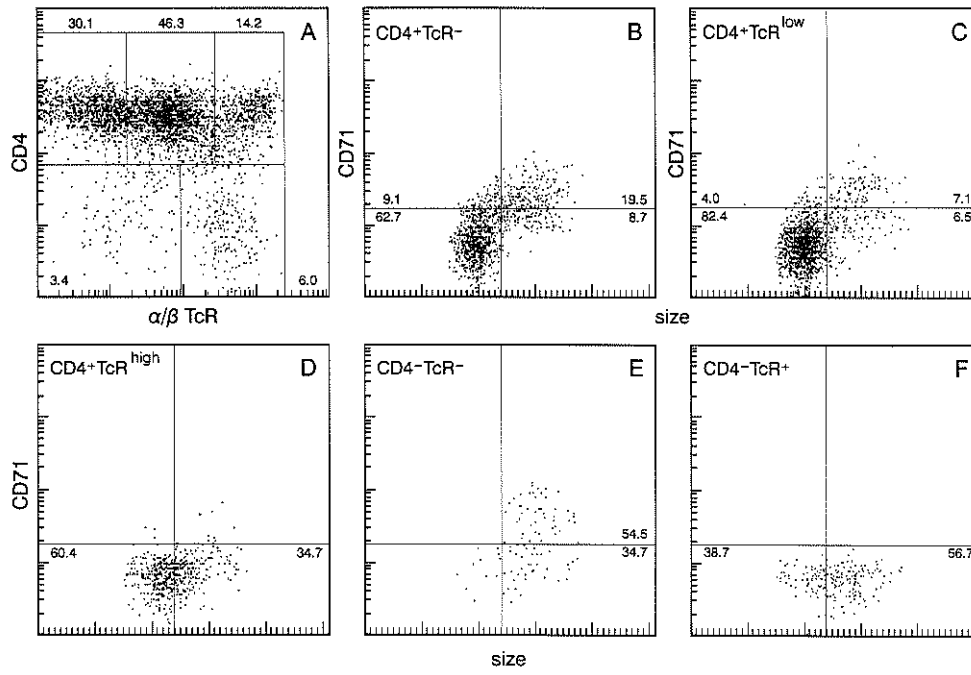


Figure 3. CD71 expression on CD4/$\alpha\beta$TcR defined adult thymocyte subpopulations. Cells were stained with CD71-FITC, CD4-R-Phycoerythrin, and anti-$\alpha\beta$TcR-biotin, followed by streptavidin-Tricolor. $\alpha\beta$TcR expression of adult thymocytes is shown in combination with CD4 (A) and 5 thymocyte subpopulations were defined by window analysis: $CD4^+\alpha\beta TcR^-$ (B), $CD4^+\alpha\beta TcR^{low}$ (C), $CD4^+\alpha\beta TcR^{high}$ (D), $CD4^-\alpha\beta TcR^-$ (E), and $CD4^-\alpha\beta TcR^+$ (F) cells. CD71/size dotplots are shown, with marker lines separating small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within these quadrants.

whereas $CD4^-8^+$ thymocytes are either mature $\alpha\beta TcR^+$ or immature $\alpha\beta TcR^-$ cells (15,35). We analyzed cell size and CD71 expression of these subpopulations. $CD4^+TcR^-$ cells, representing the $CD4^+8^+TcR^-$ thymocytes, contained small $CD71^-$ cells as well as large $CD71^+$ cells (Fig. 3B). In addition, small cells with an intermediate CD71 expression could be identified. The $CD4^+TcR^{low}$ thymocytes, representing the $CD4^+8^+TcR^{low}$ thymocytes, were mainly small and $CD71^-$, however, a small but distinct population of large $CD71^+$ cells was also present (Fig. 3C). The mature TcR expressing $CD4^+8^-$ thymocytes, identified as $CD4^+TcR^{high}$ cells, were $CD71^-$ as was also observed in figure 2E (Fig. 3D). $CD4^-TcR^-$ cells, mainly representing $CD4^-8^-$ cells, are large cells with variable CD71 expression (Fig. 3E). The $CD4^-TcR^+$ cells, representing the $CD4^-8^+TcR^{high}$ as well as the $CD4^-8^-TcR^+$ ($\alpha\beta$ and $\gamma\delta$) thymocytes, also did not express CD71 (Fig. 3F). This indicates that the $CD4^-8^+$ $CD71^+$ thymocytes, as identified in figure 2D, are immature TcR-negative thymocytes.

Together, our results indicate that (1) in adult thymus CD71 is expressed on large immature $CD4^-8^-$, $CD4^-8^+$ and $CD4^+8^+$ thymocytes and (2) downregulation of CD71 on $CD4^+8^+$ thymocytes correlates to increased expression of the CD3/$\alpha\beta$TcR complex on the cell surface.

All immature thymocytes in ontogeny express a transferrin receptor

Because the gradual development of T cell subpopulations in the ontogeny allows easier identification of $CD71^+$ thymocytes, we analyzed CD71 expression in fetal and neonatal thymus. CD71 was expressed on all fetal thymocytes of day 14, 16 and 17 of gestation, with the same intensity (Fig. 4A,C,E). From day 14 to day 17 of gestation, $CD4^-8^-$ thymocytes (Fig. 4B,D) developed through $CD4^-8^+$ intermediate into $CD4^+8^+$ thymocytes (Fig. 4F). Also, $CD4^{low}8^-$ and $CD4^-8^{low}$ cells could be seen (especially in Fig. 4B and 4D). In addition, $CD71^+$ cells were all large in size and did not express the $\alpha\beta$TcR (data not shown). Thus, immature CD4/CD8 defined subpopulations in fetal thymus of day 14 to 17 of gestation expressed the TfR.

Around day 18 of gestation, $CD4^+$ thymocytes developed with lower expression of CD71, indicated by a reduction in the relative number of positive cells and a reduction of the staining intensity (Fig. 4G). These thymocytes had a $CD4^+8^+$ phenotype (Fig. 4H), and only a small part of this population expressed the $\alpha\beta$TcR at a low level (Fig. 5A). At this stage, mature $CD4^-8^+$ had not yet developed, whereas $CD4^+8^-$ thymocytes with high $\alpha\beta$TcR expression started to appear (Fig. 4H and 5A). Analysis of CD71 expression and size in the $CD4^+8^+$ thymocyte population revealed that the observed reduction in the staining intensity of CD71 occurred on the surface of small $CD4^+8^+$ thymocytes (Fig. 5B). Within this population of small $CD4^+8^+$ cells, $\alpha\beta TcR^-$ cells showed a higher surface expression of CD71 compared to $\alpha\beta TcR^{low}$ cells (Fig. 5C,D). All other CD4/CD8 defined subpopulations were large in cell size and CD71-positive (data not shown).

Thus, during fetal T cell development CD71 expression gradually diminishes as the $\alpha\beta$TcR appears on the cell surface, confirming our observations in the adult thymus. Does this also apply to cells of the $\gamma\delta$ T cell lineage present in fetal thymus? We detected $\gamma\delta$TcR expressing thymocytes at gestational days 16, 17, and 18 (Fig. 6A-C). As figure 6 shows, all fetal $\gamma\delta$TcR positive thymocytes expressed the transferrin receptor.

Together, these ontogenetic results show that immature thymocytes express a transferrin receptor until the onset of development of small $CD4^+8^+$ thymocytes with low $\alpha\beta$TcR expression. By contrast, all cells of the $\gamma\delta$ T cell lineage, in the fetus, show surface expression of the transferrin receptor.

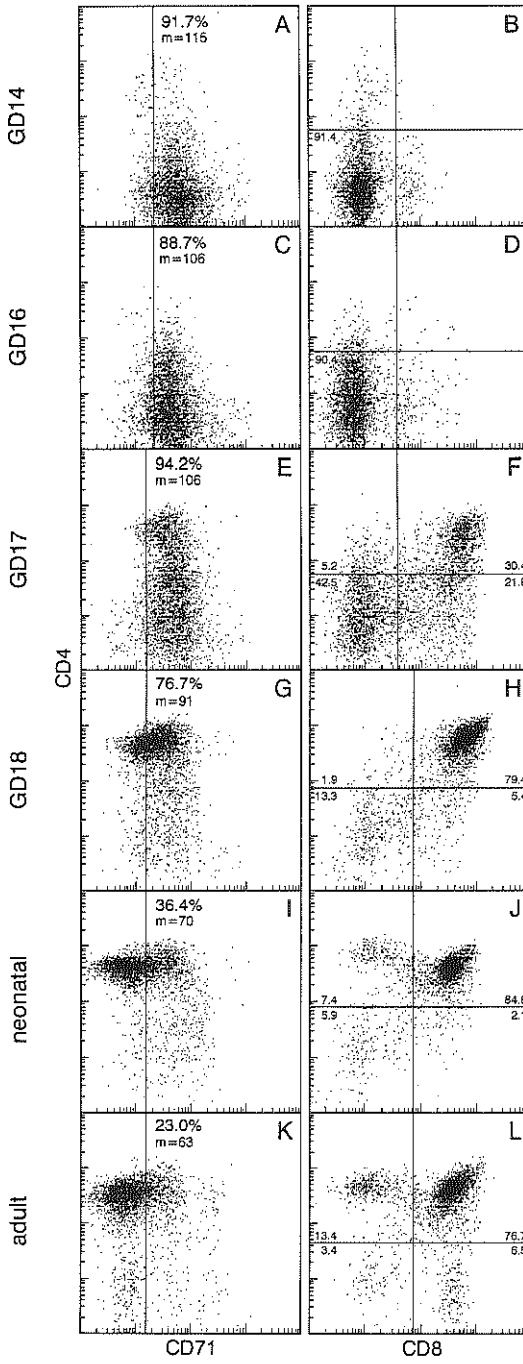


Figure 4. CD71 expression and developmental status of fetal thymocytes in ontogeny. Cells from the indicated stages of fetal, neonatal, and adult thymus were stained with CD71-FITC and CD4-R-Phycoerythrin (A,C,E,G,I,K), or with CD8-FITC and CD4-R-Phycoerythrin (B,D,F,H,J,L). The marker line in the CD4/CD71 dotplots separates CD71$^+$ from CD71$^-$ cells; figures indicate the relative number and mean fluorescence intensity (by channel number on a full scale of 256 channels). CD4/CD8 defined thymocyte subpopulations were quantified by quadrant analysis and relative numbers of cells are indicated within the quadrants.

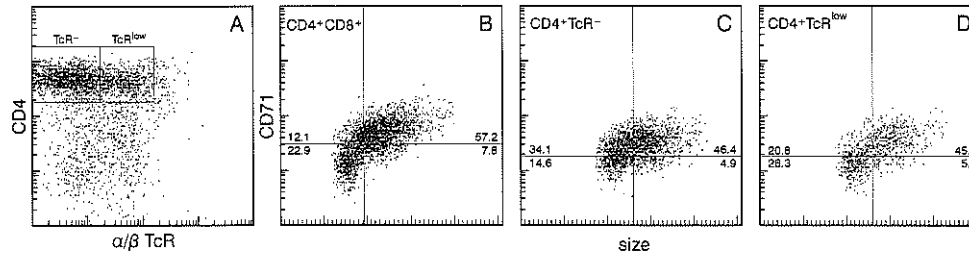


Figure 5. CD71 expression on day 18 fetal thymocytes. Cells were stained with anti-$\alpha\beta$TcR-biotin and CD4-R-Phycoerythrin, followed by streptavidin-Tricolor (A). Thymocyte subpopulations were defined by window analysis for CD4/TcR$\alpha\beta$ dotplots. CD71 expression on thymocyte subpopulations was performed as indicated in the *legends of figures 2 and 3* for resp. fig. 5B and 5C,D. CD71/size dotplots are shown for $CD4^+8^+$ (B), $CD4^+\alpha\beta TcR^-$ (C), and $CD4^+\alpha\beta TcR^{low}$ (D) thymocytes, with marker lines separating small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within these quadrants.

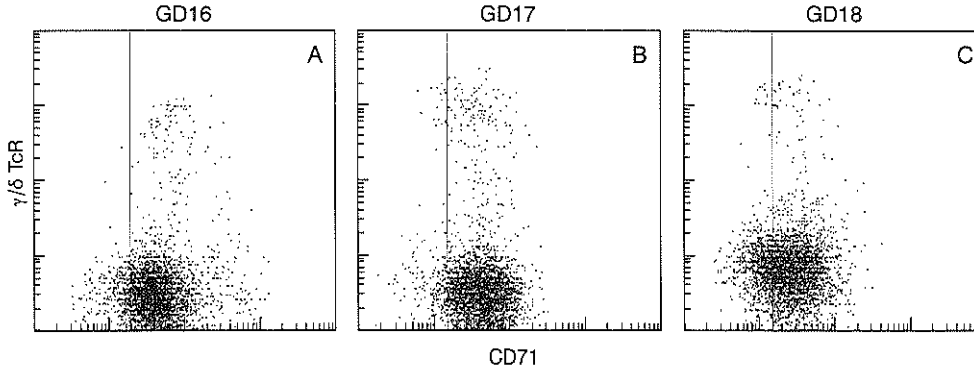


Figure 6. CD71 expression on $\gamma\delta$TcR positive fetal thymocytes. Fetal thymocytes of gestational days 16 (A), 17 (B), and 18 (C) were stained with CD71-FITC and anti-$\gamma\delta$TcR-biotin, followed by streptavidin-R-Phycoerythrin.

The neonatal thymus contains large TfR^+, $\alpha\beta TcR^+$ $CD4^+8^-$ thymocytes

Around birth, CD71 expression could only be observed on a minority of the cells, similar to the CD71 expression on adult thymocytes (compare fig 4I and 4K). At this stage in ontogeny, $CD4^+8^-$ cells with high $\alpha\beta$TcR expression had developed (compare Fig. 4J and 7A), whereas mature $CD4^-8^+$ cells, also identified as $CD4^-\alpha\beta TcR^+$ cells, were only detected in the adult thymus (compare Fig. 4L and 3A).

It has been shown that, in contrast to adult $CD4^+8^-$ cells, $CD4^+8^-$ thymocytes in late fetal and early postnatal thymus contain significant numbers of cells in cell cycle (36). Therefore, we analyzed CD71 expression on neonatal $CD4^+$ thymocytes. CD71

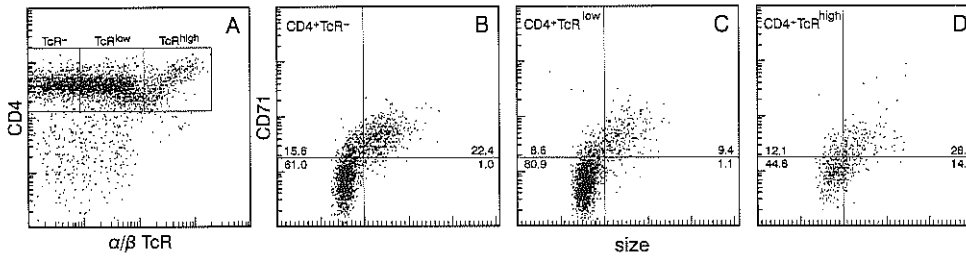


Figure 7. CD71 expression on neonatal thymocytes. Neonatal thymocytes were stained and analyzed as indicated in the *legend of figure 3.* $\alpha\beta$TcR expression is shown in combination with CD4 (A) and 3 thymocyte subpopulations were defined by window analysis: $CD4^+\alpha\beta TcR^-$ (B), $CD4^+\alpha\beta TcR^{low}$ (C), and $CD4^+\alpha\beta TcR^{high}$ (D) cells. CD71/size dotplots are shown, with marker lines separating small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within these quadrants.

expression and cell size of neonatal $CD4^+TcR^-$ ($\simeq CD4^+8^+$ $\alpha\beta TcR^-$) and $CD4^+TcR^{low}$ ($\simeq CD4^+8^+$ $\alpha\beta TcR^{low}$) thymocytes was comparable with the results for the adult populations (compare figures 7B,C with 3B,C). However, the $CD4^+TcR^{high}$ cells, representing the mature $CD4^+8^-$ thymocytes, were not completely negative, as observed in the adult population, but clearly contained a subset of large $CD71^+$ cells (compare fig. 7D and 3D). Thus, CD71 identifies the cycling cells within the neonatal $\alpha\beta TcR^+$ $CD4^+8^-$ thymocyte subpopulation.

TfR^+ thymocytes are predominantly localized in the subcapsular region

Immature, cycling thymocytes in adult thymus are predominantly localized in the subcapsular area of the cortex, but also scattered throughout the cortex (11,31,35,37,38). Our CD71 mAb specifically recognized these immature cycling thymocytes, and is therefore a suitable marker to study the localization of cycling cells in the various microenvironments of the thymus. To that purpose we performed immunohistology on adult thymus. For accurate localization of CD71 expressing cells in the cortex, sections were at the same time stained with ER-TR5 (30), recognizing the medullary epithelial cells (Fig. 8). Staining with the CD71 mAb was observed in the subcapsular region, where the outermost thymocytes in the range of 4 to 15 cell layers were stained. In the remainder of the cortex, $CD71^+$ cells were observed as scattered single cells or small clusters. In the medulla, CD71 expression on thymocytes was not observed (data not shown). Within the stromal cell population of the thymus, only cortical macrophages showed CD71 expression (Fig. 8). Our results indicate that a CD71 mAb is useful marker to study the presence of proliferating cells in thymus, using immunohistology.

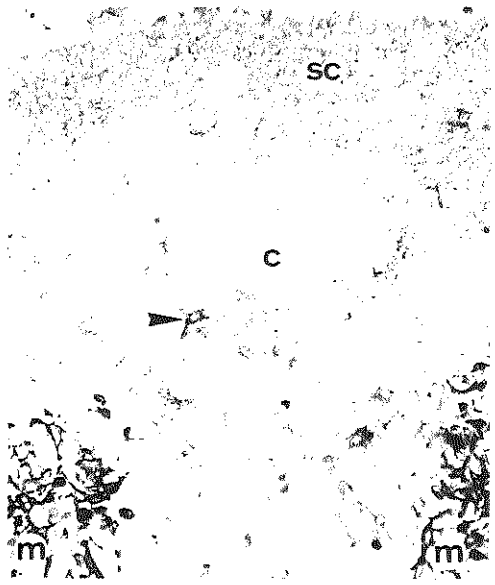


Figure 8. Localization of CD71 expressing cells in the cortex of adult thymus. The section was stained with a mixture of the CD71 mAb ER-MP21 and ER-TR5. ER-TR5 identifies the medullary area (M) by strongly labeling epithelial cells (*lower left and right corners*). The less intense staining of CD71 can be seen on thymocytes, in the subcapsular region (SC) and scattered in the cortex (C), and on cortical macrophages (arrowhead). Magnification: x 180.

Discussion

Dividing cells require iron which is taken up by the cell via internalization of transferrin-bound iron by the transferrin receptor (CD71). Therefore, CD71 expression is associated with cell proliferation. However, the inverse reasoning that CD71 expressing cells are necessarily in a state of proliferation does not hold: the most simple example against this argument are the non-dividing, CD71 expressing tissue macrophages (1,6,7). Hence, it has been concluded that CD71 expression is probably more closely regulated by intracellular iron levels than by proliferation *per se* (2,39). From this perspective we analyzed CD71 expression on murine thymocytes, as they show strong proliferation during their development in fetal and adult thymus.

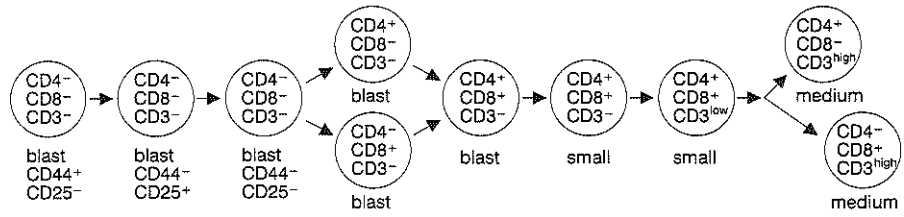
Indeed, our results illustrate that CD71 expression on thymocytes is closely related to proliferation of the cells. First of all, we observed that around 20-25% of adult thymocytes expressed CD71, which corresponds to the estimated number of cycling cells reported by others (10,12,13). The lower number of $CD71^+$ thymocytes observed with other CD71 mAb (17,18) is most likely caused by low antibody titers of the mAb preparations used. Second, the $CD71^+$ cells were large in cell size and we showed that large $CD71^+$ cells were all cycling. Our data agree with reports showing that the population of large thymocytes contains practically all cycling cells in the thymus (31,40). Third, our more refined analysis of thymocyte subpopulations showed that CD71 is present on $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ immature blasts. Cell cycle

analysis studies have shown that cycling cells in adult thymus are confined to these subpopulations (10,12-15). Around 60% of $CD4^-8^-$ cells, most $CD4^-8^+3^-$ cells and around 80% of large $CD4^+8^+3^-$ cells, in adult thymus, have been estimated to be in cell cycle (10,12,13,15,35). Our present study shows comparable frequencies of CD71 expressing cells within these three subpopulations. In fetal thymuses of GD14-17, *all* large, immature $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+$ thymocytes expressed CD71 at the cell surface. At these timepoints of fetal development, more than 80% of the cells were actively cycling (9). Fourth, we observed CD71 expression by fetal $\gamma\delta$ T cells and neonatal $CD4^+8^-$ $\alpha\beta$ T cells. Both populations have been reported to contain cycling cells (36,41). Finally, in an accompanying manuscript (20) we show that proliferation of fetal thymocytes in organ culture can be inhibited by CD71 treatment.

Together these data indicate that (1) cycling thymocytes express CD71 and (2) CD71 can be used as a differentiation marker for immature cycling cells. Especially in adult thymus, CD71 is only expressed on large cycling immature thymocytes with $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes (Fig. 9). Thymocytes with low and high CD3/TcR expression do not express CD71: CD71 is downregulated before this complex appears at the cell surface. The adult $CD4^-8^-3^+$ population, containing both $\alpha\beta$ and $\gamma\delta$ T cells (44,45), is probably also CD71-negative, as cycling cells were not identified within this subpopulation (10,13,46).

The preferential staining of immature thymocytes is reflected in the staining pattern observed after staining thymus sections with our CD71 mAb. We observed that $CD71^+$ cells were mainly, but not exclusively, located within the subcapsular region of the thymus. This confirms the finding that $CD4^-8^-$, $CD4^-8^+$ and $CD4^+8^+$ blast cells can be found in this region (11,31,35,37,38). $CD71^+$ thymocytes could, however, also be seen scattered throughout the cortex, either as single cells or as small clusters of cells. A similar staining pattern in thymus was observed when cycling thymocytes were labeled with bromodeoxyuridine *in vivo* (38). Cycling cells in the regenerating thymus were first located in the inner cortex, indicating that immature cells migrating to the outer cortex are cycling cells (11). CD71 is expressed on these migrating, cycling cells.

Expression of CD71 marks a major expansion phase in early T cell development that starts within the $CD4^-8^-$ cell population and ends at the level of the $CD4^+8^+3^-$ cells (10). The start of this expansion phase is initiated within $CD44^-25^+$ $CD4^-8^-3^-$ cell population, one of three subpopulations identified by expression of CD44 and/or CD25 and sequentially ordered by their maturation stage (see Fig. 9; ref. 12,13). Both the $CD44^-25^+$ and, its direct progeny, the $CD44^-25^-$ subpopulations contain cycling cells (40% resp. 100%), suggesting that CD71 is accordingly expressed on these two adult $CD4^-8^-3^-$ populations. The end of this expansion phase is marked by downregulation of CD71 and occurs within the $CD4^+8^+3^-$ subpopulation. Within this subpopulation we identified three subsets based on CD71 expression and cell size: large $CD71^+$, small



Transferrin receptor expression

adult	⊢——variable——⊣			+	+	–	–	– –
neonatal	⊢——variable——⊣			+	+	–	–	–/+ np
fetal	+	+	+	+	+	±	–	np np

Figure 9. Transferrin receptor expression in murine T cell differentiation. The development of thymocytes in the $\alpha\beta$ T cell lineage is shown, with emphasis on the early T cell development, based on our results and results reported by others (10,12,13,42,43). CD71 expression of fetal, neonatal and adult thymocyte subpopulations is presented below the differentiation scheme. The indicated subpopulations contain cells, positive (+) or weakly positive (±) for CD71, or no (–) CD71 expressing cells at all. For fetal thymus the development of CD71 expression on cells from day 14-18 fetal thymus is given. Some of the mature thymocytes were not present (n.p.) in neonatal or fetal thymus.

CD71$^{\pm}$, and small CD71^{-} cells. The gradual change in CD71 expression and cell size of the three subsets (see figures 3B, 5C and 7B) suggests a lineage, with large, CD71^{+} cells developing through small, CD71$^{\pm}$ cells into small CD71^{-} cells (Fig. 9). This finding indicates that CD71 is downregulated as cell division ceases, before the CD3/$\alpha\beta$TcR complex is expressed (13,42,47).

In this study we show that CD71 expression by thymocytes strongly correlates with cell division of the cells. CD71 is present on the surface of cycling CD4^{-}8^{-}, CD4^{-}8^{+}3^{-}, and CD4^{+}8^{+}3^{-} immature thymocytes. Sofar, we cannot conclude from this study whether thymocytes use iron exclusively for cell division or also for cell functions not linked to proliferation. To study the role of iron in proliferation and maturation of thymocytes, we treated fetal thymus organ cultures with a CD71 mAb. The results, presented in the accompanying manuscript (20), indicate that iron is taken up by the thymocyte not only for proliferation, but also for other, differentiation-dependent, cell functions.

Acknowledgements

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CHAPTER 4

Transferrin receptor expression in murine T cell differentiation II. Inhibition of proliferation and differentiation during early T cell differentiation by anti-transferrin receptor antibody treatment*

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Summary

In the accompanying paper, we showed that cycling thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes could be characterized by expression of the transferrin receptor (CD71). This receptor mediates the cellular uptake of iron, an essential nutrient for proliferating cells. In this study, we analyzed whether iron is essential for proliferation and maturation of thymocytes. Fetal thymi of gestational days 14 and 16 were cultured in the presence of a CD71 mAb.

Both proliferation and differentiation of thymocytes were inhibited, when day 14 fetal thymus was treated with CD71 for seven days. Cell recovery was reduced by 50%, but cells still expanded 10-fold during the culture. Thymocytes with low or high CD3/$\alpha\beta$TcR expression did not develop and only few cells reached the $CD4^+8^+3^-$ stage of T cell development. However, only $CD44^-25^+$ $CD4^-8^-3^-$ thymocytes and its precursors developed in normal numbers, indicating that the $CD44^-25^+$ cells or its direct progeny were affected by CD71 treatment. Differentiation and proliferation were not inhibited when day 16 organ cultures were treated with CD71. Because day 16 fetal thymus contains mainly $CD44^-25^+$ and $CD44^-25^-$ $CD4^-8^-3^-$ thymocytes, we conclude that the transition between these cell types is probably most sensitive for CD71 treatment. $\gamma\delta$TcR cells develop before this transition, because they are unaffected by CD71 treatment.

We found evidence that iron, taken up by the thymocyte, is not only used for cell proliferation, but also for differentiation-dependent cell functions.

Introduction

The thymus provides a unique environment for the development of T lymphocytes, supporting both precursor cell proliferation and maturation (1). These precursor cells, with a $CD4^-8^-3^-$ phenotype, differentiate in the thymus into mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes, expressing the CD3/$\alpha\beta$TcR complex at the cell surface (2,3). Cells with a $CD4^-8^+3^-$, $CD4^+8^+3^-$, and $CD4^+8^+3^{low}$ phenotype are the sequential intermediate populations in this $\alpha\beta$ T cell lineage (2,4-6). In addition, the $CD4^-8^-3^-$ cells are heterogenous and can be subdivided into three subpopulations by the expression of phagocyte glycoprotein 1 (Pgp-1 or CD44) and the p55-chain of the interleukin-2 receptor (IL-2Rα or CD25): $CD44^+CD25^-$ cells develop via $CD44^-CD25^+$ into $CD44^-CD25^-$ cells (7,8). Within this scheme of T cell development, proliferation occurs before the CD3/$\alpha\beta$TcR complex becomes expressed on the surface of the $CD4^+8^+$ thymocyte (3,9,10).

In this early phase of T cell differentiation and proliferation cytokines play an

essential role (11). Adult and fetal $CD4^-8^-$ thymocytes respond with proliferation when stimulated by IL-1, IL-2, IL-3, IL-4, IL-6, IL-7 or TNF-α, mainly in the presence of mitogens, as lectins and phorbol esters. The lack of T-cell differentiation during *in vitro* culture reflects the requirement for additional signals of an intact microenvironment. The fetal thymus organ culture (FTOC) provides an accessible *in vitro* modelsystem to study the requirement of cytokines in T cell proliferation and differentiation (11). The intact microenvironment of this modelsystem supports the differentiation of cells of the $\alpha\beta$ and $\gamma\delta$ T cell lineages (12). Using this modelsystem, the cytokines IL-2, IL-4 and IL-7 were identified as growth factors, important for early T cell development (11).

A growth factor that also might play a role in the early T cell development is transferrin, an iron-transporting molecule that delivers iron to the cell via the transferrin receptor (CD71). In the accompanying paper (13), we have shown that the transferrin receptor is expressed on $CD4^-8^-3^-$, $CD4^-8^+3^-$ and $CD4^+8^+3^-$ cells, both in fetal and adult T cell differentiation. These subpopulations also contain almost all cycling cells present within the thymus (3,5,8,9,14) and we have found evidence that CD71 expression on thymocytes is closely correlated to proliferation. However, CD71 is not only linked to cell proliferation, but can also be expressed by cells that require iron for other cell functions, like the iron-dependent enzym peroxydase in tissue macrophages. In a similar way, the expression of CD71 on thymocyte subpopulations, that are not only actively cycling, but also differentiating, suggests that iron might also be necessary for the differentiation of thymocytes. We used the FTOC to analyse the role of the transferrin receptor and the uptake of transferrin-bound iron in T cell development.

In this study, we cultured fetal thymuses of day 14 of gestation for seven days (FTOC day 14+7) in the presence of the CD71 mAb ER-MP21 (15). We observed that both T cell proliferation and differentiation were affected by the anti-CD71 treatment. Differentiation in the $\alpha\beta$ T cell lineage was completely abrogated, because thymocytes did not develop beyond the $CD4^+8^+3^-$ stage. Cells of the $\gamma\delta$ T cell lineage, on the other hand, were not affected by the treatment. Surprisingly, no effect could be observed when FTOC day 16+5 was treated with the CD71 mAb. Since the fetal thymus of gestational day 16 mainly contains $CD4^-8^-$ cells, our data suggest that CD71 treatment has a major effect on the $CD4^-8^-3^-$ cells.

Materials and Methods

Mice

Male and female BALB/c ($H\text{-}2^d$) mice were bred and maintained in the animal facilities of our department. For timed pregnancies, the appearance of a vaginal plug was considered to be day 0 of gestation. Pregnant mice were killed by cervical dislocation and fetuses were dissected from the uterus at different days of gestation. Fetuses of day 14 and day 16 of gestation were used for fetal thymus organ culture.

Antibodies

The antibodies used in this study are listed in Table 1. They were purified from hybridoma culture supernatant by affinity chromatography, which yielded always pure antibodies, i.e. only heavy and light chains were detectable by SDS-PAGE. For flowcytometric analyses of organ-cultured thymocytes mAb conjugated with FITC, biotin or R-Phycoeryhtrin were used. FITC and biotin conjugates were prepared in our laboratory, whereas the R-Phycoerythrin conjugate was commercially obtained as indicated in the legend of Table 1. The properties of the monoclonal antibodies used, were described in detail in the indicated references (15-22). To avoid aspecific staining, all conjugates were optimally titrated.

The mAb ER-MP20 and ER-MP21 have been developed in our department by Pieter Leenen (15,22) and are now commercially available from BMA, Augst, Switzerland.

Table 1. Characteristics of the monoclonal antibodies

CD[a]	Antigen	Clone	Conjugation[b]	Reference
CD3	CD3	KT3	B	16
CD4	L3T4	GK1.5	PE	17
	MT4	H129.19	B	18
CD8	Lyt-2	53-6.7	F	19
CD71	TfR	ER-MP21	F	15
TcR1	$\gamma\delta$TcR	GL3	B	20
TcR2	$\alpha\beta$TcR	H57-597	B	21
-	-	ER-MP20	F	22

[a] Monoclonal antibodies are ordered by the Cluster of Differentiation (CD) nomenclature, when possible.
[b] MAb were used unconjugated or conjugated with FITC (F), biotin (B), or R-Phycoerythrin (PE). R-Phycoerythrin-conjugated anti-L3T4 was obtained from Becton Dickinson, Mountain View, CA, USA.

Fetal thymus organ culture

Fetal thymus lobes were removed from fetuses of day 14 or day 16 of gestation and placed in organ culture, according to the methods of Mandel (23) and Jenkinson (24). Briefly, four to five single thymus lobes were placed on the surface of polycarbonate filters (0.8 μm pore size; Poretics, Livermore, CA, USA) supported, either on 1 mm thick squares of gelatin foam sponge (Sterispon no.2; Allen and Hanbury, London, UK) in 2 ml of medium in 60 mm plastic petri dishes (Falcon; Becton Dickinson, Plymouth, UK), or on 7 mm thick gelatin foam blocks (Upjohn Co., Kalamazoo, MI, USA) in 2 ml of medium in 35 mm plastic petri dishes (Falcon). The culture medium consisted of Iscove's modified Dulbecco's medium containing 25 mM HEPES (GIBCO Life Technologies Ltd, Paisley, UK) and supplemented with penicillin, streptomycin, L-Glutamin and 10% FCS (Hyclone, Logan, Utah, USA), heat inactivated (30 min, 56°C). The cultures were grown in a humidified incubator with 8.5% CO_2 in air at 37°C. Day 14 and day 16 fetal thymuses were cultured for seven (FTOC day 14+7), respectively five days (FTOC day 16+5).

At the end of the culture period the lobes were harvested and viable thymocyte suspensions were prepared, by putting four to five lobes in a small volume of PBS containing 0.5% BSA and 2mM sodium azide and disrupting the tissue using a small potter homogenizer. Cells were washed, and subsequently counted using a Coulter counter (model ZB1, Coulter Electronics Ltd, Luton, England).

Antibody blocking

Fetal thymuses of day 14 of gestation were cultured for seven days in the presence of purified ER-

MP21, a CD71 mAb (15), in concentrations of 25, 50 and 100 μg/ml. Control cultures received PBS only, or the IgG2a isotype control antibody ER-MP20 (22) at a dose of 100 μg/ml. The mAb ER-MP20 detects a differentiation antigen on macrophage precursors (22) and identifies only a small number of thymic cells (unpublished observations). All cultures received the same amount of PBS and the contribution of PBS to the cultures never exceeded 10%.

In some experiments we analyzed the thymocytes at day 3, 4, 5, 6, 7, and 12 of the day 14 FTOC. In these experiments we used only ER-MP21 and ER-MP20 at a concentration of 100 μg/ml. In FTOC day 14+12, mAb containing culture medium was replenished at day 6. FTOC day 16+5 was, also, only treated with ER-MP21 and ER-MP20 at a concentration of 100 μg/ml.

Immunofluorescence and flow cytometric analysis

For flow cytometric analysis, thymocyte suspensions were prepared in PBS containing 0.5% BSA and 2 mM sodium azide. 10^6 cells (in a volume of 10-20 μl) were incubated on ice for 30 min with 25 μl of the appropriate mAb or mixture of mAb, FITC-, biotin-, or R-Phycoerythrin conjugated. After three washes with the PBS-BSA-NaN3 buffer, cells were further incubated for 30 min on ice, with appropriate dilutions of R-Phycoerythrin or Tricolor conjugated to Streptavidin (Caltag, San Francisco, CA, USA). Finally, cells were again washed three times and collected in a small volume for flowcytometric analysis. A different staining strategy was followed when for two-color staining a mAb supernatant had to be combined with CD4-R-phycoerythrin. In this procedure, cells were successively incubated with mAb supernatant, a FITC-conjugated second step antiserum, normal rat serum, and CD4-R-phycoerythrin. For two-color staining FITC (green) and R-Phycoeryhtrin (orange) were used as fluorochromes, while for three-color staining Tricolor (red) was used as third color. To identify dead cells in samples with two-color staining, Propidiumiodide was added just before acquisition at a final concentration of 5 μg/ml. Background fluorescence was determined by staining cells with second step streptavidin-conjugated antibodies only or with the FITC-conjugated mAb ER-MP20.

Most likely, the transferrin receptors on fetal thymocytes in organculture are all labeled with ER-MP21, present in the medium. This could be detected by staining thymocytes with FITC-conjugated Rabbit anti-rat IgG (Cappel/Organon Technika, Oss, the Netherlands) only. In some experiments, this staining was combined with a R-Phycoerythrin-conjugated CD4 mAb. In the two-color staining, cells were successively incubated with anti-rat-FITC, normal rat serum, and CD4-R-Phycoerythrin.

Samples were analyzed for light scatter and fluorescence on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with a 488 nm argon laser and interfaced to a Hewlett-Packard computer running the FACScan software. Calibration of the cytometer was performed by eye, using thymocytes that were unlabeled, labeled with CD8-FITC or labeled with CD4-R-Phycoerythrin. Forward light scatter and perpendicular light scatter were analyzed with linear amplification, while all three fluorescence channels were subject to logarithmic amplification. In most cases 10,000 cells were analyzed. Dead cells were excluded during data analysis on the basis of forward light scatter and PI-staining. All data were analyzed using the FACSCAN or Paint-a-Gate software (Becton Dickinson, Mountain View, CA, USA). Data are presented in this paper as one-parameter histograms or as two-paramater dotplots, with a four log-decade fluorescence scale when appropriate.

Results

Development of CD71 expression in fetal thymus organ culture

The aim of this study was to determine whether proliferation and/or differentiation of thymocytes depended on transferrin receptor mediated iron uptake. To that purpose we cultured fetal thymuses of gestational day 14 and 16 in the presence of the CD71

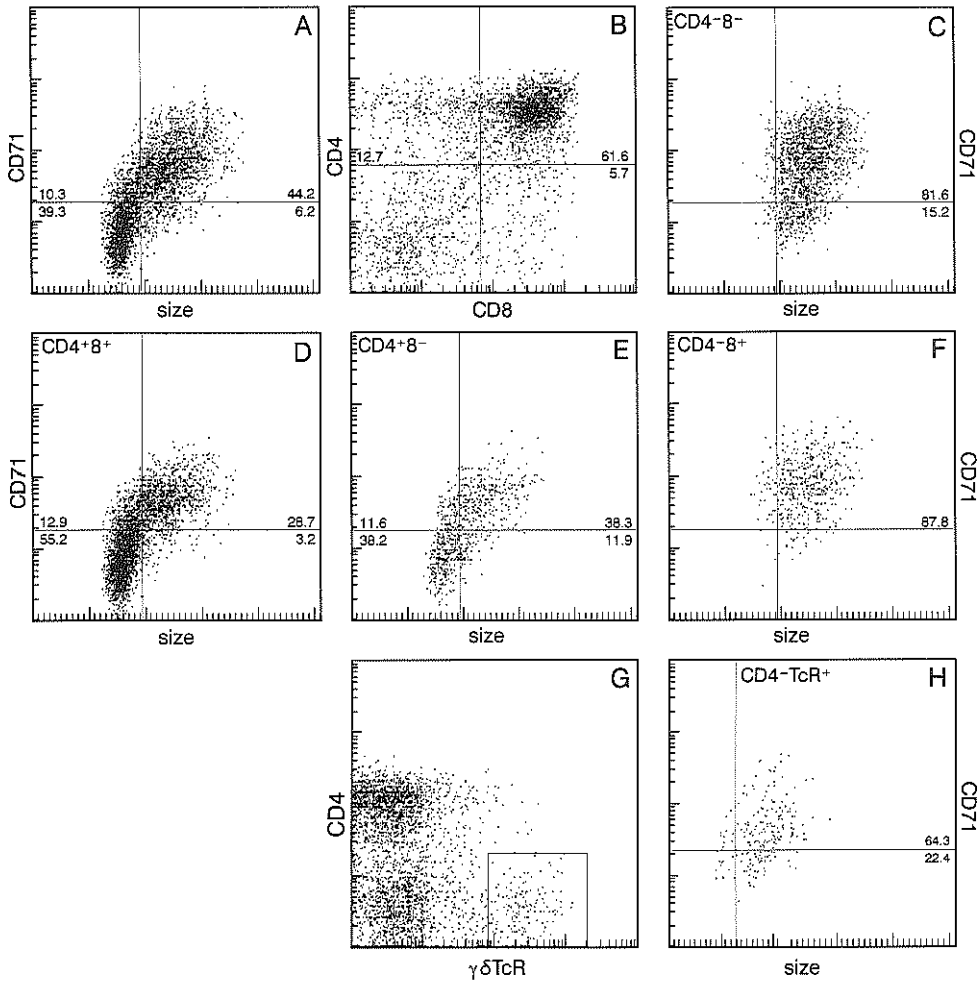

Figure 1. CD71 expression and size of thymocyte subpopulations in FTOC day 14+7. Organcultured thymocytes were stained with CD71-FITC, CD4-R-Phycoerythrin, and either CD8-biotin (A-F) or anti-γδTcR-biotin (G,H), followed by streptavidin-Tricolor. CD71 expression and size are shown for total (A), $CD4^-8^-$ (C), $CD4^+8^+$ (D), $CD4^+8^-$ (E), and $CD4^-8^+$ (F) thymocytes, with marker lines separating small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within the quadrants. The CD4/CD8 subpopulations were defined by window analysis as indicated in figure B. $\gamma\delta TcR^+$ thymocytes were identified as $CD4^-\gamma\delta TcR^+$ cells (G) and characterized by cell size and CD71 expression (H).

mAb ER-MP21. However, we first identified the phenotype of CD71 expressing thymocytes *in vitro*, in normal fetal thymus organ culture. In the accompanying paper (13), we showed that, *in vivo*, immature thymocytes of large cell size and with a

$CD4^-8^-$, $CD4^-8^+$, and $CD4^+8^+$ phenotype expressed CD71, during both fetal and adult T cell development. Size analysis of CD71-expressing thymocytes showed that also in FTOC day 14+7 large cells (but not small cells) expressed CD71 (Fig. 1A). After seven days of culture, all CD4/CD8 defined subpopulations had developed (Fig. 1B). From our staining results with mAb against $\alpha\beta$TcR, CD3, CD4, and CD8, we found that $\alpha\beta TcR^+$ thymocytes in FTOC day 14+7 developed only in very low numbers (25). Under these experimental conditions, the $CD4^+8^-$ cells contained a subpopulation (15-25%) that expressed the $\alpha\beta$TcR, whereas mature $\alpha\beta$TcR expressing $CD4^-8^+$ cells had not yet developed (data not shown).

After triple staining thymocytes with mAb against CD4, CD8, and CD71, we analyzed CD71 expression and cell size of each CD4/CD8 defined subpopulation. Most $CD4^-8^-$ cells appeared large and CD71-positive, but a minor population of large cells with negative to low surface expression of CD71 was also present (Fig. 1C). $CD4^+8^+$ thymocytes contained a major population of small $CD71^-$ cells and a minor population of large $CD71^+$ cells (Fig. 1D). Similarly, the $CD4^+8^-$ thymocytes also contained two populations, one was $CD71^-$ with small to intermediate cell size and the other was $CD71^+$ and large (Fig. 1E). The $CD4^-8^+$ thymocytes were all large $CD71^+$ cells (Fig. 1F).

Our data indicate that in FTOC CD71 was expressed on immature $CD4^-8^-$, $CD4^-8^+$, and $CD4^+8^+$ blast cells, as we observed *in vivo* in fetal and adult T cell development (13). The presence of $CD71^+$ $CD4^+8^-$ cells, also present in neonatal thymus *in vivo*, suggests that T cells developed in FTOC to the developmental stage observed in neonatal thymus. Also, all $\gamma\delta$TcR thymocytes were large $CD71^+$ cells (Fig. 1G,H), similar to the $\gamma\delta$TcR thymocytes observed in fetal thymi of days 16-18 of gestation (13).

Proliferation of thymocytes in FTOC day 14+7 is inhibited by CD71 mAb treatment

Since the transferrin receptor is mainly expressed on proliferating cells, we analyzed whether our CD71 mAb could inhibit proliferation in FTOC day 14+7. Day 14 fetal thymuses were cultured in the presence of purified ER-MP21 in doses of 25, 50 and 100 μg/ml, whereas control cultures received either an isotype control mAb ER-MP20 in a dosis of 100 μg/ml or PBS only. In both control cultures around 400,000 cells per thymus lobe were recovered (Fig. 2). Cell recovery in the CD71 treated cultures was severely reduced, but this reduction of 50% seemed to be independent of the concentration of mAb used. However, cells still proliferated and expanded at least tenfold since the start of the organ culture, when the thymus contained not more than 20,000 cells. Thus, CD71 treatment causes an inhibition but not a complete abrogation

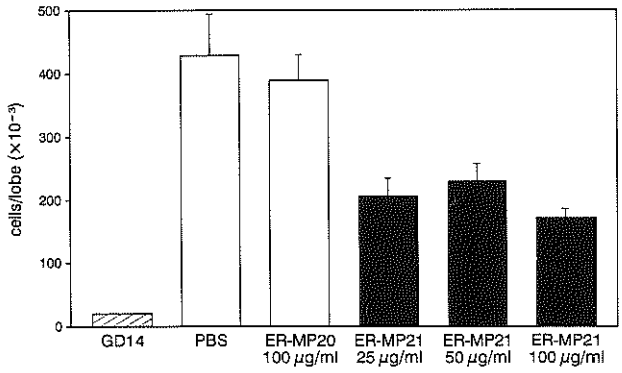

Figure 2. Effect of CD71 treatment on cell recovery in FTOC day 14+7. Organ cultures were treated with saline (PBS), the control antibody ER-MP20 (100 µg/ml) or different doses of the CD71 mAb ER-MP21 (25, 50, and 100 µg/ml). Bars represent the mean ± SEM of 4-5 experiments. For comparison, the number of cells at the start of the culture (≤20,000 cells/lobe), i.e. gestational day 14 (GD14), is shown.

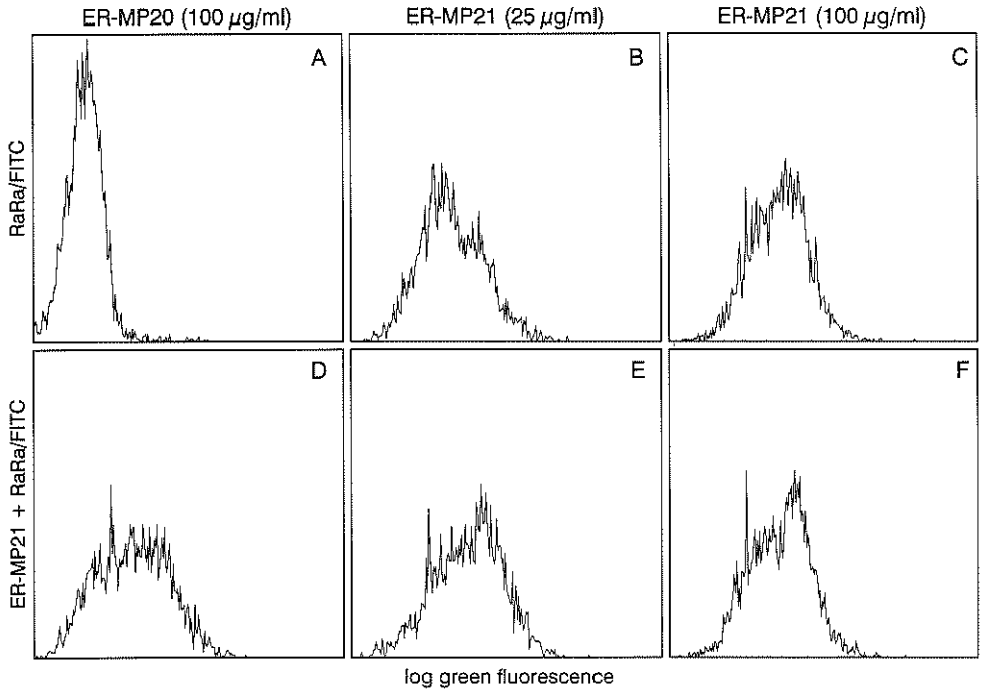

Figure 3. Detection of CD71 mAb on the surface of thymocytes in FTOC day 14+7. Cells were stained with either anti-rat-FITC alone (A-C), or with supernatant of the CD71 mAb, followed by anti-rat-FITC (D-F). Cells from the culture, treated with the control mAb ER-MP20 (A,D), were compared with cells from the CD71 treated cultures, in the presence of either 25 (B,E) or 100 (C,F) µg/ml of ER-MP21.

of cell proliferation in the organ cultures.

The cell recovery data do not indicate the optimal dose of the ER-MP21 mAb. However, the optimal dose can also be determined by establishing the dose of ER-MP21 mAb that results in complete saturation of the CD71 molecule. Therefore, we analyzed the effect of the mAb on the surface expression of CD71 on thymocytes in antibody treated FTOC. In the culture, the mAb binds to its antigen and may cause complete saturation of the CD71 molecules on the cells surface. However, modulation of CD71, leading to reduced surface expression is also conceivable (26). In order to detect whether the mAb ER-MP21 was present on the cell surface or not, thymocytes from CD71 treated cultures were stained with a FITC-conjugated anti-rat antiserum. Thymocytes from a control culture did not stain with the FITC-conjugated antiserum (Fig. 3A). In the ER-MP21 treated cultures, however, the FITC-conjugated antiserum clearly stained the thymocytes, indicating that ER-MP21 in the culture is bound to CD71 at the surface of the thymocytes (Fig. 3B,C). The strongest staining was observed in the culture treated with the highest dose of mAb (Fig. 3C). Figures 3E and 3F show that, when the same cell samples were additionally stained with ER-MP21 followed by the FITC-conjugated antiserum, complete saturation was only found in the cultures incubated with a high dose of 100 μg/ml ER-MP21 (Fig. 3F), and not at lower doses (Fig. 3E and data not shown). The surface CD71 expression was comparable to that seen in the control culture (compare fig. 3D and 3F). These results indicate that (1) CD71 was not modulated from the cell surface and (2) complete saturation by CD71 mAb was only observed at the highest dose. In the remainder of this study we concentrated on cultures treated with ER-MP21 and ER-MP20, in a dose of 100 μg/ml.

Differentiation of αβTcR$^+$, but not γδTcR$^+$ thymocytes in FTOC day 14+7 is inhibited by CD71 mAb treatment

We also expected an effect on T cell differentiation, because CD71 is expressed on three immature thymocyte subpopulations, linked to each other by a proliferation driven differentiation process. The size of the cells gave a first indication of an effect of the mAb treatment. In the CD71 treated cultures all cells were of large size, whereas in the control culture both small and large cells were present (Fig. 4A, *upper panels*). When thymocytes were simultaneously stained with CD4 and CD8 mAb, a dramatic inhibition of T cell differentiation was observed (Fig. 4A, *lower panels*). CD4$^+$8$^+$ and CD4$^+$8$^-$ thymocytes developed in relatively very low numbers, causing a substantial (relative) increase in the number of CD4$^-$8$^-$ thymocytes as compared to the control culture. In absolute numbers, CD4$^+$8$^+$ and CD4$^+$8$^-$ cells were drastically reduced (Fig. 4B). The reduction of the CD4$^+$8$^+$ thymocytes was not only caused by the almost complete absence of small CD4$^+$8$^+$ cells, but also by a reduction in the number of

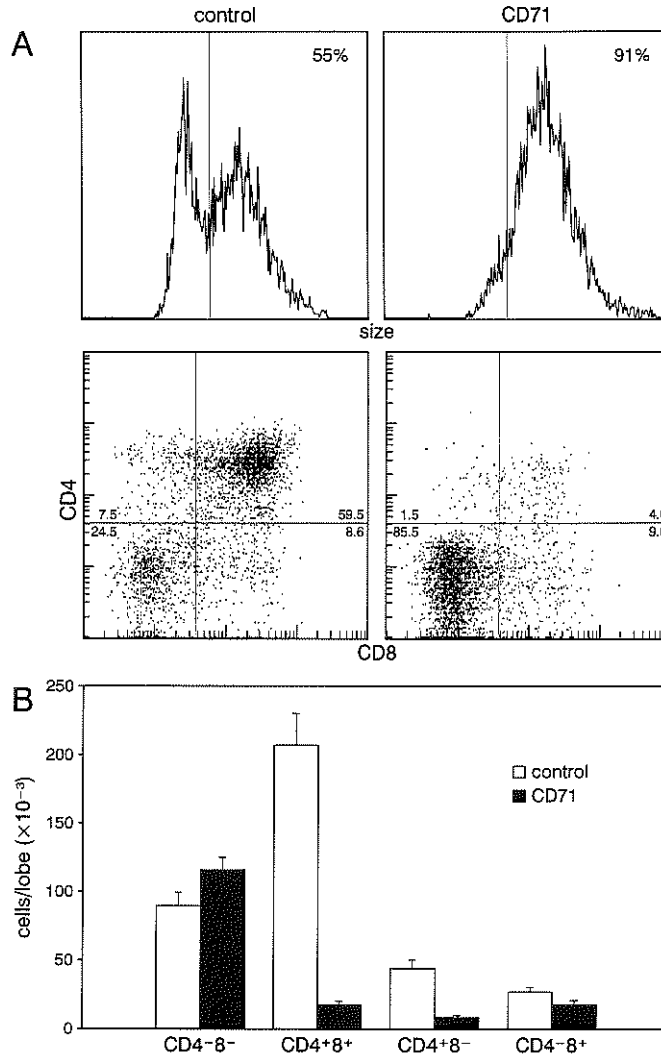

Figure 4. Effect of CD71 treatment on the development of CD4/CD8 defined thymocyte subpopulations. Cells from control and CD71 treated cultures were analyzed for cell size (A) and for CD4/CD8 expression (A,B). Cell size, as measured by forward light scatter, is depicted in histograms, showing the relative number of large cells (A, the *upper panels*). For CD4/CD8 phenotyping, cells were stained with CD4-biotin and CD8-FITC, followed by Streptavidin-R-Phycoerythrin. Subpopulations were quantified by quadrant analysis and the relative number indicated in the two-color dotplots (A, *lower panels*). In figure B the absolute numbers have been depicted in a bar graph, with different bar patterns for the control and CD71 treated cultures. Bars represent the mean ± SEM for 8 (control) or 9 experiments.

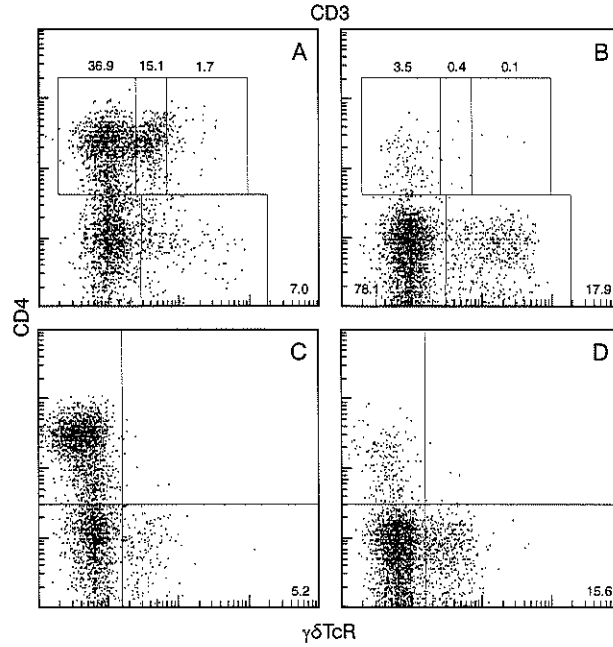

Figure 5. Effect of CD71 treatment on the development of $\alpha\beta$TcR and $\gamma\delta$TcR expressing thymocytes in FTOC day 14+7. Thymocytes from the control culture (A,C) or the CD71 treated culture (B,D) were used for two-color analysis. Cells were stained by successive incubations with, either CD3 (A,B) or anti-$\gamma\delta$TcR (C,D) supernatant, anti-hamster-FITC (Caltag, San Francisco, USA), normal rat serum, and CD4-R-Phycoerythrin. Subpopulations were quantified by window analysis for CD4/CD3 dotplots (A,B) and by quadrant analysis for CD4/$\gamma\delta$TcR dotplots (C,D).

large $CD4^+8^+$ cells (data not shown). The number of $CD4^-8^+$ cells, however, was only mildly reduced, whereas more $CD4^-8^-$ cells were recovered from CD71 treated cultures compared to control cultures. IL-$2R^+$ cells, representing mainly the $CD44^-25^+$ $CD4^-8^-3^-$ thymocytes, were also recovered in slightly increased numbers (data not shown).

Sofar, these data indicate that, under the influence of CD71 treatment, T cell differentiation in the $\alpha\beta$ T cell lineage is severely impaired. Indeed, $CD4^+8^-$ $\alpha\beta TcR^{high}$ and $CD4^+8^+$ $\alpha\beta TcR^{low}$ thymocytes, identified as $CD4^+3^{high}$ resp. $CD4^+3^{low}$ cells, were completely absent in the mAb treated cultures (Fig. 5A,B). Remaining $CD4^+8^+$ thymocytes were TcR^-. In contrast, there was a relative increase in the number of $CD4^-3^+$ thymocytes. As figures 5C and 5D indicate, these cells all expressed a $CD4^-$ $\gamma\delta TcR^+$ phenotype. Such a relative increase in the number of $\gamma\delta TcR^+$ thymocytes was observed in four different experiments (Table 2). However, in absolute numbers, the $\gamma\delta TcR^+$ population was similar in size in both CD71 treated and control cultures

Table 2. Recovery of γδTcR-positive thymocytes in CD71 treated FTOC day 14+7.

	Relative (%)		Absolute (x 10^{-3}/lobe)	
	ER-MP20	ER-MP21	ER-MP20	ER-MP21
Exp. 1[a]	5.2	14.7	13.0	16.2
Exp. 2	5.4	12.8	26.1	24.6
Exp. 3	12.7	36.3	42.2	45.0
Exp. 4	5.7	13.6	26.8	20.6

[a] The relative number of γδTcR$^+$ thymocytes was determined from histograms; the absolute recovery was calculated using the relative number and total cell recovery of the organcultures.

(Table 2).

Together our results indicate that (1) T cell differentiation in the αβ T cell lineage is completely abrogated by CD71 mAb treatment and (2) thymocytes of the γδ T cell lineage are not affected. Within the CD4$^-$8$^-$ compartment, cells seem to develop normally, at least up to the IL-2R$^+$ stage.

Inhibition of differentiation in FTOC day 14+7 occurs independent of the inhibition of proliferation

The question arises whether the inhibition of differentiation is a mere consequence of the inhibition of proliferation, or whether differentiation requires iron-uptake by the cells. When inhibition of differentiation is a mere consequence of the inhibition of proliferation, one can expect that a CD71 treated culture and a control culture with identical cell number are both of the same developmental stage. Such an identical cell recovery is found by comparing the organ culture, treated with CD71 for seven days, with a control FTOC cultured for a relative shorter period. As figure 6A shows, both cultures increased in cell number with time, but the CD71 treated culture increased more slowly in cell number. The control culture at day 4 and the CD71 treated culture at day 7 contained comparable cell numbers, 164,000 resp. 178,000 cells per lobe (see asterisks in fig. 6A). We analyzed the developmental status of the thymocytes in these particular samples by staining the cells with CD4 and CD8 mAb. Equal numbers of CD4/CD8 defined thymocyte subpopulations would indicate that inhibition of differentiation was caused by the inhibition of proliferation. As figure 6B shows this was not the case. The CD71 treated culture contained more CD4$^-$8$^-$ and CD4$^-$8$^+$ cells, but less CD4$^+$8$^+$ cells. In addition, cells of the CD71 treated culture were all large in size, whereas the control culture contained both small and large cells (data not shown).

In a second experiment, under identical experimental conditions, we cultured fetal thymi up to 12 days. Again, we compared cultures with similar cell yields. In this

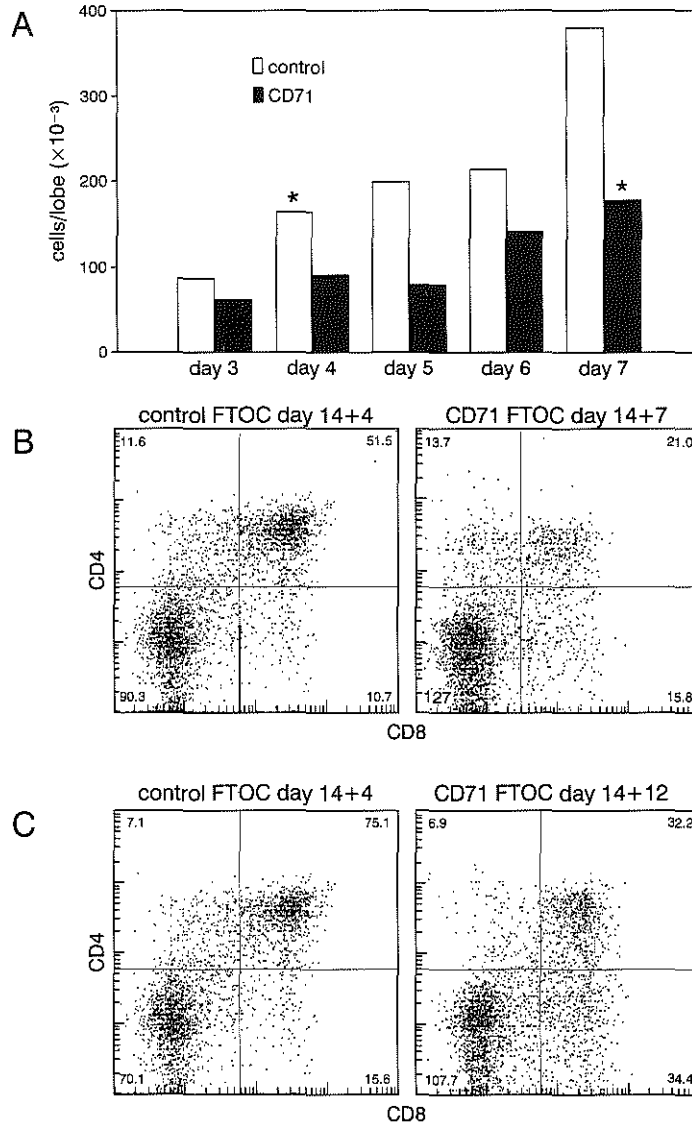

Figure 6. Effect of CD71 treatment on proliferation and differentiation at different days of the organ culture. In figure A, the cell recoveries at days 3 to 7 of the organ culture are shown for control and CD71 treated cultures (indicated by bars with different bar patterns). Asterisks indicate the samples that were used for two-color staining with CD4 and CD8 mAb, presented in figure B. Cells from the control FTOC at day 4 and from the CD71 treated FTOC at day 7 were stained with CD4-biotin and CD8-FITC, followed by streptavidin-R-Phycoerythrin. Subpopulations were quantified by quadrant analysis, with the numbers indicating thousands of cells. In a second experiment, a control FTOC at day 4 was compared to an anti-CD71 treated FTOC at day 12 (C). Cells were staind and analyzed as indicated for figure B.

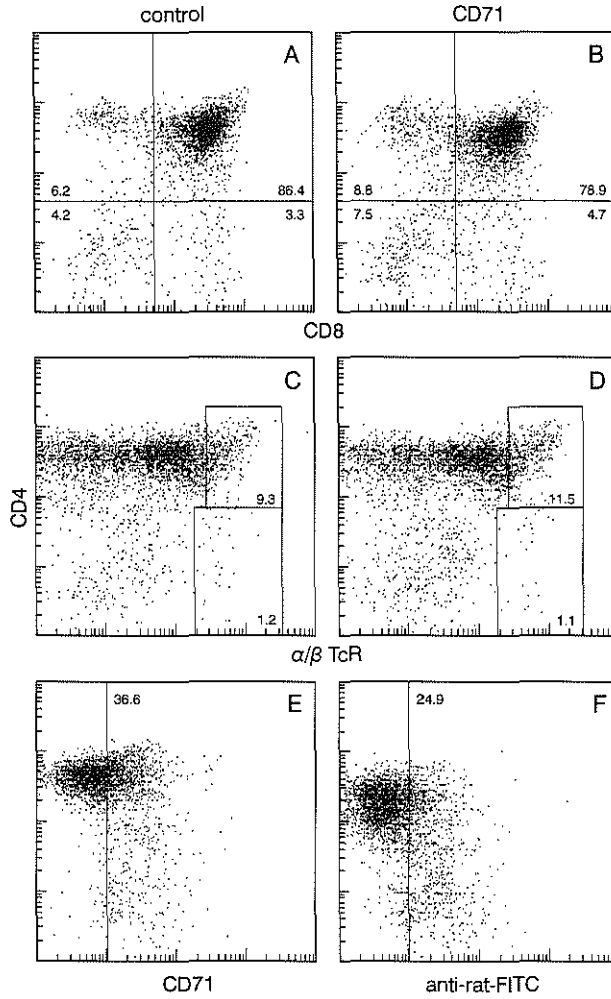

Figure 7. Effect of CD71 treatment on T cell differentiation in FTOC day 16+5. Thymocytes from control cultures (A,C,E) and CD71 treated cultures (B,D,F) were used for two-color analysis. For CD4/CD8 marker analysis (A,B), cells were stained with CD4-R-Phycoerythrin and CD8-FITC. Subpopulations were quantified by quadrant analysis. For CD4/$\alpha\beta$TcR phenotyping (C,D), cells were stained with CD4-R-Phycoerythrin and $\alpha\beta$TcR-biotin, followed by streptavidin-Tricolor. CD71 expression on thymocytes from the control culture was analysed by staining with CD4-R-Phycoerythrin and CD71-FITC (E). Surface expression of the CD71 mAb in the culture was detected by successively staining cells from the CD71 treated culture with anti-rat-FITC, normal rat serum, and CD4-R-Phycoerythrin (F). Marker lines (E,F) separate $CD71^-$ from $CD71^+$ cells.

experiment, the CD71 treated culture at day 12 could be compared to a control culture at day 4, with recoveries of 181,000 resp. 168,000 cells per thymus lobe. Under these conditions, more $CD4^-8^-$ and $CD4^-8^+$ cells, but less $CD4^+8^+$ cells developed after CD71 treatment (Fig. 6C) and all cells were large in size (data not shown).

Together, these data suggest that immature thymocytes also require iron for their differentiation at developmental stages where proliferation is not occurring.

Proliferation and differentiation of thymocytes in FTOC day 16+5 are not inhibited by CD71 treatment

As a consequence of CD71 treatment, T cell development in the $\alpha\beta$ T cell lineage is completely inhibited in FTOC day 14+7 (Fig. 4A). Such a complete inhibition in the development of $\alpha\beta$TcR thymocytes was also reported for FTOC day 14+7, cultured in the presence of IL-2 (27). However, in the same study, no inhibition of T cell development was observed when fetal thymuses of day 16 or older were cultured with IL-2, indicating that only the immature $CD4^-8^-$ thymocytes were the target of the IL-2 treatment. To determine, whether CD71 treatment also specifically inhibits the development of $CD4^-8^-$ cells, we cultured fetal thymuses of gestational day 16 in the presence of the CD71 mAb or a control mAb. *In vivo*, the fetal thymus of gestational day 16 already contained $CD4^-8^-$ and $CD4^-8^+$ cells, whereas $CD4^+8^+$ cells were at the onset of their development (13). The latter cell type could be first detected at gestational day 17. Under these experimental conditions, we observed no effect of CD71 treatment. Equal numbers of thymocytes were recovered at the end of the culture period of five days, i.e. 276,000 resp. 250,000 cells per lobe in control and CD71 treated cultures. Also, no differences could be observed in the composition of thymocyte subpopulations, as defined by CD4/CD8 or CD4/$\alpha\beta$TcR expression (Fig. 7A-D). In both cultures, non-mature cells, with low level $\alpha\beta$TcR expression, as well as mature cells, with high level $\alpha\beta$TcR expression, developed normally. Staining with a FITC-conjugated anti-rat antiserum showed the presence of CD71 mAb on the surface of a minority of the thymocytes; most thymocytes had a $CD4^+CD71^-$ phenotype (Fig. 7F). However, this staining profile was identical to the profile of thymocytes from the control culture, stained with mAb against CD4 and CD71 (Fig. 7E). This indicates that all $CD71^+$ thymocytes, possibly present in the culture, were all saturated with the CD71 mAb.

These results indicate, therefore, that CD71 treatment affects the immature $CD4^-8^-3^-$ and/or $CD4^-8^+3^-$ cells.

Discussion

Iron is essential to proliferation of the cell. Its uptake by cells requires specific binding of the major iron-transport protein, transferrin, to cell surface transferrin receptors, followed by endocytosis of the receptor-ligand complexes and the subsequent release of iron to the cytoplasm (28). The intracellular iron either serves as a substrate for the biosynthesis of iron-containing proteins or is stored in ferritin deposits. Iron uptake by the proliferating cell is essential for the iron-containing enzyme ribonucleotide reductase involved with DNA synthesis (29). In the previous paper, we have shown that cycling immature thymocytes are characterized by expression of the transferrin receptor. In this study, we analyzed the need for iron in proliferation and maturation of thymocytes in organ culture.

Organ cultures of day 14 fetal thymus were treated with the CD71 mAb ER-MP21 (15). This mAb precipitates a homodimer of 200 kDa and, like other CD71 mAb of the IgG isotype, binds to a epitope on the receptor different from the transferrin- binding site (15,30-32). In general, growth inhibition by CD71 mAb is caused by iron starvation of the cells (33-35). The underlying mechanisms (proposed for IgG CD71 mAb) are that mAb binding decreases CD71 recycling between the cell surface and intracellular compartments and enhances CD71 degradation (26,34-37). Both decreased recycling and enhanced degradation cause, in most studies, a reduction of CD71 cell surface expression, leading to reduced iron uptake and concomitant growth inhibition in the presence of CD71 mAb.

As expected we found that proliferation of thymocytes in FTOC day 14+7 was inhibited by CD71 treatment. However, proliferation was only partially inhibited and the number of cells still expanded at least ten-fold from the start of the culture. This was not caused by suboptimal mAb treatment, because all TfR molecules were saturated with the CD71 mAb, even after seven days of organ culture. Also, CD71 treatment did not cause a reduced surface expression of CD71. Our results suggest that for thymocytes a decrease in the recycling rate of CD71 is a major cause for a reduced iron uptake. However, when we compared CD71 expression on *large* thymocytes from control and CD71 treated cultures, a reduction in CD71 expression could be observed. Therefore, it seems likely that both CD71 degradation and decreased recycling cause reduced iron uptake in thymocytes leading to inhibition of the proliferation.

T cell differentiation was also inhibited by CD71 treatment. Thymocytes expressing the CD3/$\alpha\beta$TcR complex, whether at low or high level, did not develop in the CD71 treated cultures. These results suggest that the transition of $CD3^-$ to $CD3^{low}$ cells was blocked by the CD71 treatment. At this transition cell division stops, cells enter the G_0 phase of the cell cycle and become small in size (6,8,38). In the previous paper (13), we have shown that downregulation of CD71 also marks the transition of $CD3^-$ to

$CD3^{low}$ cells. The few $CD4^+8^+3^-$ thymocytes that developed in the CD71 treated cultures already had reduced CD71 expression (data not shown), indicating that cells stopped cycling. Thus, similar to proliferation, differentiation was only partially inhibited; $CD4^-8^-$ cells, present in day 14 fetal thymus, could still develop into $CD4^+8^+3^-$ cells.

After CD71 treatment, only large immature $CD4^-8^-$, $CD4^-8^+$, and $CD4^+8^+$ thymocytes were present in the culture. These three thymocyte subpopulations contain all cycling cells (3,5,8,9,14) and all CD71 expressing cells in fetal thymus, as we showed previously (13). Does CD71 treatment, therefore, affect all CD71 expressing thymocyte subpopulations to the same extent or is one $CD71^+$ thymocyte subpopulation more sensitive? Our results indicate that the $CD4^-8^-$ subpopulation contains the target cells for CD71 treatment. We observed that, in FTOC day 14+7, only $CD4^-8^-$ cells were not reduced in cell number, whereas, in FTOC day 16+5, proliferation and differentiation could not be inhibited by treatment with CD71 mAb. As day 16 fetal thymus, like day 14 fetal thymus, mainly contained $CD4^-8^-$ thymocytes (13), it seems likely that cells sensitive for CD71 treatment are present within the $CD4^-8^-$ thymocyte subpopulation. In FTOC day 14+7, CD71 treatment started before the sensitive cells had developed, whereas in FTOC day 16+5 cells apparently already developed beyond the sensitive stage.

Both day 14 and 16 fetal thymus contain mainly $CD4^-8^-$ thymocytes, but differ from each other in the subpopulations defined by expression of CD44 and CD25 (39; unpublished observations). In the day 14 fetal thymus, only $CD44^+25^-$ cells and, its progeny, the $CD44^+25^+$ cells were present. The day 16 fetal thymus still contained cells with these phenotypes, but, at this stage, the $CD44^-25^+$ and $CD44^-25^-$ cells were the major thymic cell populations. Since T cell differentiation in FTOC day 16+5 was not inhibited by CD71 treatment, $CD44^-25^-$ cells alone or both $CD44^-25^+$ and $CD44^-25^-$ cells were beyond the sensitive stage of CD71 treatment. Thus, $CD44^+25^-$ and $CD44^+25^+$ cells, present in day 14 fetal thymus, are sensitive to CD71 treatment. However, normal numbers of $CD44^-25^+$ cells developed in CD71 treated FTOC day 14+7, which suggests that thymocytes develop normal up to this stage. Combining these results, it seems likely that a subpopulation of the $CD44^-25^+$ cells or the transitition of $CD44^-25^+$ to $CD44^-25^-$ cells is sensitive for the treatment. Recently, the $CD25^+$ $CD4^-8^-3^-$ stage of adult thymocyte differentiation was identified as a possible major branching point for the development of T cells into different lineages (40). Suda and Zlotnik (40) showed that either the $\alpha\beta$TcR expressing $CD4^+8^-$ or $CD4^-8^+$ single-positive lineages or the $CD4^-8^-$ $\alpha\beta$ and $\gamma\delta$ T cell lineages developed from the $CD25^+$ $CD4^-8^-3^-$ cells. Authors speculate that this branching point probably also occurs in fetal thymocyte development. We observed that $\gamma\delta TcR^+$ $CD4^-8^-$ cells escaped CD71 mediated growth inhibition and developed in normal numbers. Combined with the assumed presence of a fetal branching point, this indicates that thymocytes in CD71

treated organ culture could develop into the $CD25^+$ $CD4^-8^-3^-$ stage of thymocyte differentiation. Together, these data indicate that the transition of $CD44^-25^+$ to $CD44^-25^-$ $CD4^-8^-3^-$ cells is probably most sensitive to treatment with CD71.

Although our experiments indicate that a specific stage of T cell development is affected by CD71 treatment, we did not observe a complete block of T cell development. As we discussed above, the iron uptake by the cells is only reduced in the presence of CD71 mAb. Thymocytes can still internalize iron and, therefore, cells could still develop beyond the thymocyte stage that we indicated as most sensitive for CD71 treatment. Both, proliferation and differentiation of thymocytes occurred in CD71 treated cultures. However, cells did not develop beyond the $CD4^+8^+3^-$ developmental stage and the question arises whether the inhibition of differentiation is only caused by the inhibition of proliferation. Two models explaining the iron-dependency of proliferation and differentiation can be proposed. In the first model, differentiation completely depends on the iron-dependent process of proliferation, indicating that control and CD71 treated cultures with equal cell yields have the same developmental stage of differentiation. In the second model it is assumed that differentiation, to a minor extent, also occurs independent of proliferation, but is iron-dependent. Under conditions of reduced iron availability, as caused by CD71 treatment, iron is probably only available for proliferation driven differentiation. According to the second model, control and CD71 treated cultures with equal cell yields will have different developmental stages of differentiation. Our observations support the second model. Comparing control and CD71 treated cultures with equal cell yields, we found that CD71 treated thymocytes were behind in their development, compared to control thymocytes. These results indicate that iron is taken up by the thymocyte not only for proliferation, but also for differentiation-dependent cell functions.

In the accompanying paper, we showed that large cycling thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ can be characterized by the expression of a transferrin receptor (13). Iron-uptake through the transferrin receptor is a major mechanism for cells to obtain iron. This study shows that iron is an essential nutrient for T cell development. Both proliferation and differentiation of thymocytes were inhibited by CD71 treatment and our data suggest that the most sensitive cells can be found within the $CD4^-8^-3^-$ thymocyte subpopulation. In addition, we found evidence that differentiation occurs independent of proliferation and requires iron. Our study supports observations that iron deficiency, one of the most common health problems caused by malnutrition, leads to impaired cell mediated immunity (41,42). As our data indicate, this may be caused by the inhibition of T cell development within the thymus (43).

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CHAPTER 5

Selective blockade of MHC class II antigens on thymic stromal cells interferes with T cell differentiation and coreceptor expression*

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Summary

The interactions between CD4 and the $\alpha\beta$TcR on the thymocyte and MHC class II molecules on the thymic stromal cells are essential to the development of mature $CD4^+8^-$ thymocytes. To identify the stage of T cell differentiation where these interactions become important, we cultured BALB/c day 14 and day 16 fetal thymuses in the presence of the rat anti-I-A^d/E^d mAb M5/114. We observed that anti-Ia treatment interfered with the development of mature $CD4^+8^-3^{high}$ thymocytes. However, the other CD4 expressing thymocyte subpopulations, with a $CD4^+8^+3^{low}$, a $CD4^+8^+3^-$, and a $CD4^+8^-3^-$ phenotype, were also affected by the treatment. Intriguingly, we observed elevated levels of CD4 expression on the surface of these thymocytes. We calculated an 1.4-1.5 times upregulation of CD4 on the surface of $CD4^+8^+$ thymocytes, a level of expression normally observed on mature $CD4^+8^-$ thymocytes. Our data suggest that the blockade lies early in T cell differentiation, at the $CD4^+8^+3^-$, or even the $CD4^+8^-3^-$ stage of development. Furthermore, our data indicate that CD4-MHC interactions are already apparent before a CD3/$\alpha\beta$TcR complex is expressed. We suggest that under the present experimental conditions CD4 upregulation is an attempt of the thymocyte to become positively selected. However, it fails to do so because anti-Ia treatment abrogates physical contact between the developing thymocyte and the thymic stroma.

Introduction

The thymus provides an optimal environment for the differentiation of T lymphocytes (1). This process can be monitored by analysis of the expression of the CD3/$\alpha\beta$TcR complex and the expression of the coreceptor molecules CD4 and CD8 on the surface of the developing T cells. Precursor T cells derived from the fetal liver or the adult bone marrow home to the thymus and enter a major precursor population presently characterized by the absence of CD4 and CD8, so called the 'double negatives'. From these precursors the mature $CD4^+8^-$ and $CD4^-8^+$ 'single positive' thymocytes develop, expressing the CD3/$\alpha\beta$TcR complex at a high concentration at the cell surface (2). The major intermediate population in this T cell differentiation pathway is the 'double positive' thymocyte, expressing both CD4 and CD8. The majority of the cells in this particular population expresses low levels of CD3 and $\alpha\beta$TcR molecules. It is at this particular level in differentiation that "selection" of the maturing T cells occurs (3-5).

During this still poorly understood process, the $\alpha\beta$TcR repertoire, as expressed by mature peripheral T lymphocytes, is shaped under the influence of two selective

forces. *Positive selection* or *'MHC-restriction'* rescues those thymocytes that are able, as mature T cells, to recognize self-MHC molecules that have bound foreign antigenic peptides (3,4,6,7). *Negative selection* either eliminates or inactivates thymocytes bearing autoreactive $\alpha\beta$TcRs (3-5). Driven by these forces thymocytes develop that are either $CD4^+8^-$ and MHC class II restricted or $CD4^-8^+$ and MHC class I restricted. In studies using $\alpha\beta$TcR transgenic mice, it was shown that the restriction preference of the $\alpha\beta$TcR determines the CD4/CD8 phenotype (4). Thus, a class I MHC restricted transgenic TcR was expressed only on $CD4^-8^+$ T cells (8), whereas a class II MHC-restricted transgenic TcR was expressed only on $CD4^+8^-$ T cells (9,10).

It is generally accepted that stromal cells in the thymus are involved in this selection process (9,11-14). Moreover, the T cell differentiation process can be influenced by monoclonal antibodies against epitopes on stromal cells. Thus, for the selection and development of $CD4^+8^-$ helper T cells, the interaction of the $\alpha\beta$TcR and CD4 on thymocytes with MHC class II molecules on thymic stromal cells appears crucial. Such interactions can be blocked with anti-Ia or CD4 antibodies. Anti-Ia treatment of neonatal thymus *in vivo* as well as fetal thymus *in vitro* completely abrogated helper T cell function (15,16). This defect was caused by the absence of $CD4^+8^-$ thymocytes and peripheral helper T cells (17,18). However, anti-Ia treatment did not affect other CD4/CD8 defined thymic subpopulations, hence, normal CTL function was detected. Similar results were observed with CD4 treatment (19-21) and in gene 'knock-out' mice, i.e. mice missing the genomic information for MHC class II antigens (22) or CD4 antigens (23). Again, $CD4^+8^-$ thymocytes and helper T cell function did not develop, whereas the other cells of the $\alpha\beta$ T cell lineage developed normally. Together, these studies indicate that specific interactions of TcR and CD4 on thymocytes with MHC class II molecules on thymic stromal cells are instrumental to positive selection of $CD4^+8^-$ helper T cells.

In this study we tried to identify at what particular stage of T cell differentiation, interaction between MHC class II molecules and the $\alpha\beta$TcR and CD4 becomes important for further T helper cell development. To that purpose we used the fetal thymus organ culture (FTOC) as a model system, because it supports complete differentiation of cells of the $\alpha\beta$ T cell lineage (24). Also, the gradual appearance of all different thymocyte subpopulations, as identified by CD3, CD4 and CD8 expression, allows accurate detection of effects caused by mAb treatment. Using anti-Ia treatment of FTOC we observed not only the absence of $CD4^+8^-$ thymocytes, but also an upregulation of CD4 on $CD4^+8^+$ thymocytes. Our data suggest that blocking of MHC class II molecules on the surface of thymic stromal cells interferes with T cell development at or before the $CD4^+8^+$ differentiation stage.

Materials and methods

Mice

Male and female BALB/c (H-2^d) mice were bred and maintained in the animal facilities of our department. For timed pregnancies, the appearance of a vaginal plug was considered to be day 0 of gestation. Pregnant mice were killed by cervical dislocation and fetuses were dissected from the uterus on different days of gestation. Fetuses of day 14 and day 16 of gestation were used for fetal thymus organ culture.

Antibodies

The antibodies used in this study were purified from hybridoma culture supernatant by affinity chromatography, which yielded always pure antibodies, i.e. only heavy and light chains were detected by PAGE. For flowcytometric analyses of organ cultured thymocytes mAb conjugated with FITC, biotin or R-Phycoeryhtrin were used. Most of the FITC and biotin conjugates were prepared in our own laboratory, conjugated using standard procedures. The properties of the monoclonal antibodies used are described in detail in the indicated references. The following antibodies were used: anti-L3T4 (CD4) from the rat hybridoma GK1.5 (25), conjugated to R-Phycoerythrin (Becton Dickinson, Mountain View, CA, USA) or anti-MT4 (CD4) from the rat hybridoma H129.19 (26), conjugated to biotin; anti-Lyt-2 (CD8) from the rat hybridoma 53-6.7 (27), was used as a FITC-conjugate; a CD3 mAb from the hamster hybridoma 500-A2 (28), was conjugated to biotin; anti-$\alpha\beta$TcR (anti-TcR2) from the hamster hybridoma H57-597 (29), used either biotinylated or FITC-conjugated. To avoid aspecific staining, all conjugates were carefully titrated.

For anti-Ia treatment of the fetal thymus organ culture, purified mAb from the rat hybridoma M5/114 (IgG2b) was used (30). This antibody recognizes an allodeterminant present on both the I-A and I-E molecule. The mAb NLDC-145 was used as an isotype control antibody (31). This mAb stains thymic cortical epithelial cells and medullary interdigitating cells, but does not interfere with T cell differentiation in vivo (32), as well as in vitro (unpublished observations).

Fetal thymus organ culture

Fetal thymus lobes were removed from fetuses of days 14 and 16 of gestation and placed in organ culture (33,34). Briefly, four to five single thymus lobes were placed on the surface of polycarbonate filters (0.8 μm pore size; Poretics, Livermore, USA) supported on 1 mm thick squares of gelatin foam sponge (Sterispon no.2; Allen and Hanbury, London, UK) in 2 ml of medium in 60 mm plastic petri dishes (Falcon; Becton Dickinson). The culture medium consisted of Iscove's modified Dulbecco's medium containing 25 mM HEPES (GIBCO Life Technologies Ltd, Paisley, UK) and supplemented with penicillin, streptomycin, L-Glutamin and 10% FCS (Hyclone, Logan, Utah, USA), heat inactivated (30 min, 56°C). The cultures were grown in a humidified incubator with 8.5% CO_2 in air at 37°C. Day 14 fetal thymuses were cultured for seven days (FTOC day 14+7), while day 16 fetal thymuses were cultured for five days (FTOC day 16+5). At the end of the culture period the lobes were harvested and viable thymocyte suspensions were prepared, by putting four to five lobes in a small volume of PBS containing 0.5% BSA and 2mM sodium azide and disrupting the tissue using a small potter homogenizer. Cell counts were made using a Coulter counter (model ZB1, Coulter Electronics Ltd, Luton , England).

Antibody blocking

In vitro anti-Ia treatment was performed with purified M5/114 mAb (30). This antibody was added either at day 0 of the FTOC day 16+5 or at day 2 of the FTOC day 14+7. Together with the mAb, the culture medium was replenished. In most experiments doses of 50, 100 and 200 μg/ml of purified mAb were used. Control cultures received PBS only, or an isotype control antibody (NLDC-145) at a dose of 200 μg/ml. All cultures received the same amount of PBS and the contribution of PBS to the cultures

never exceeded 10%.

Immunofluorescence and flow cytometric analysis

For flow cytometric analysis, cell suspensions were prepared in PBS containing 0.5% BSA and 2 mM sodium azide. 50,000-100,000 cells (in a volume of 10-20 μl) were incubated on ice for 30 min with 25 μl of the appropriate mAb or mixture of mAb, FITC-, biotin-, or R-Phycoerythrin conjugated. After three washes with the PBS-BSA-NaN3 buffer, cells were further incubated for 30 min on ice, with appropriate dilutions of R-Phycoerythrin conjugated Streptavidin (Caltag, San Francisco, CA, USA). Finally, cells were again washed three times and collected in a small volume for flowcytometric analysis. Two-color staining with CD3 and CD4 mAb was done using supernatant of the hamster hybridoma 500-A2, producing a CD3 mAb (28). In this procedure, cells were successively incubated with CD3 supernatant, anti-hamster-FITC, normal rat serum, and CD4-R-Phycoerythrin. For two-color staining, FITC (green) and R-Phycoeryhtrin (orange) were used as fluorochromes. To identify dead cells in the samples, Propidium Iodide (PI) was added just before acquisition at a final concentration of 5 μg/ml. Background fluorescence was determined by staining cells with second step antibodies only.

Samples were analyzed for light scatter and fluorescence on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with a 488 nm argon laser and interfaced to a Hewlett-Packard computer running the FACScan software. Calibration of the cytometer was performed by eye, using thymocytes that were unlabeled, labeled with CD8-FITC or labeled with CD4-R-Phycoerythrin. Forward light scatter and perpendicular light scatter were analyzed with linear amplification, while all three fluorescence channels were subject to logarithmic amplification. In most cases 10,000 cells were analyzed. Dead cells were excluded during data analysis on the basis of forward light scatter and PI-staining. All data were analyzed using the FACSCAN or Paint-a-Gate software (Becton Dickinson, Mountain View, CA, USA). Data are presented in this paper as one-parameter histograms or as two-parameter dotplots, with a four log-decade fluorescence scale when appropriate. Specific subpopulations of cells in dotplots were quantified by quadrant or window analysis as indicated in the figure legends.

Immunohistology

Immunohistology was performed on cryostat tissue sections of organ cultured fetal thymus lobes, essentially as described before (13). In this study, frozen sections of organ cultured fetal thymus were incubated with supernatant of the rat hybridoma M5/114 (MHC class II antigens) or PBS and developed with a peroxidase conjugate of rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark).

Results

Fetal thymus organ culture

The effect of anti-Ia treatment on T cell differentiation was studied in two organ culture systems, where the onset of culture was either on day 14 or day 16 of gestation. At both time points, mainly $CD4^-8^-$ thymocytes were present in the fetal thymus (data not shown). Under normal tissue culture conditions, both systems supported the development of $CD4^+8^+$, $CD4^+8^-$ and $CD4^-8^+$ thymocytes (Fig. 1A, 1C and 2A). However, two differences were observed between both culture systems. First, the cell recovery of FTOC day 16+5 was higher than the recovery of FTOC day 14+7, reflecting the difference in the initial cell number of day 14 and day 16 fetal thymi (Fig. 2A). Secondly, in FTOC day 16+5 more $CD4^+8^+$ cells developed, whereas less

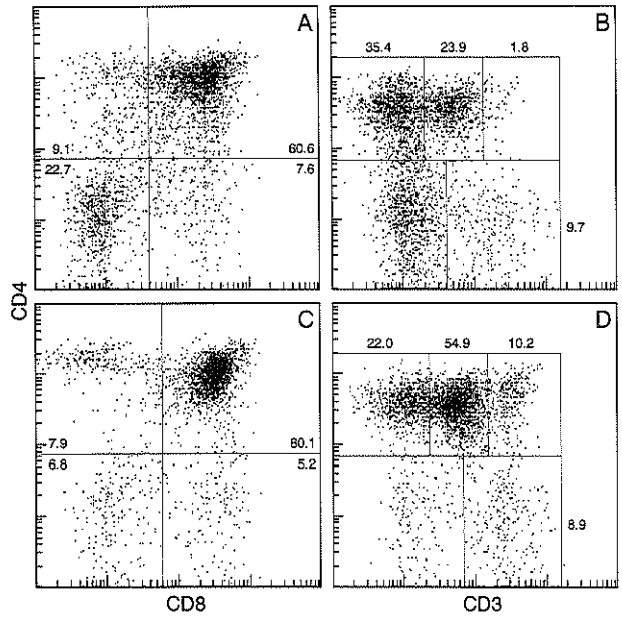

Figure 1. T cell development in FTOC day 14+7 and FTOC day 16+5. Thymocytes from FTOC day 14+7 (A,B) and FTOC day 16+5 (C,D) were used for two-color analysis. For CD4/CD8 marker analysis (A,C), cells were stained with CD4-biotin and CD8-FITC, followed by streptavidin-R-Phycoerythrin. For CD3/CD4 phenotyping (B,D), cells were stained by successive incubations with CD3 supernatant, anti-hamster-FITC, normal rat serum, and CD4-R-Phycoerythrin. Subpopulations were quantified by quadrant analysis for CD4/CD8 dotplots (A,C) and by window analysis for CD4/CD3 dotplots (B,D).

$CD4^-8^-$ cells remained (Fig. 2A). This suggests that a slower progression of T cell development occurs in FTOC day 14+7. Cells with a $CD4^+8^-$ or a $CD4^-8^+$ phenotype developed in equal numbers in both systems (Fig. 2A).

Recently, immature $CD4^+8^-$ thymocytes with no detectable CD3 expression have been identified as an intermediate phenotype between $CD4^-8^-$ and $CD4^+8^+$ cells, in vivo as well as in vitro (35-37). To identify the nature of the $CD4^+8^-$ thymocytes in our two culture systems, we analyzed the expression of CD4 and CD3 on organ cultured thymocytes. A well defined population of $CD4^+3^{high}$ cells, representing mature $CD4^+8^-$ thymocytes, was found in FTOC day 16+5, whereas this population only marginally developed in FTOC day 14+7 (Fig. 1B, 1D, and 2B). $CD4^+3^{low}$ thymocytes, representing mainly the $CD4^+8^+$ thymocyte subpopulation with a low CD3 expression, developed in both systems, although in different numbers. Because of the these differences in T cell development, both culture systems proved useful for the present study. With FTOC day 14+7, the effect of anti-Ia treatment on the development of double positive thymocytes with low expression of the CD3/$\alpha\beta$TcR complex could be

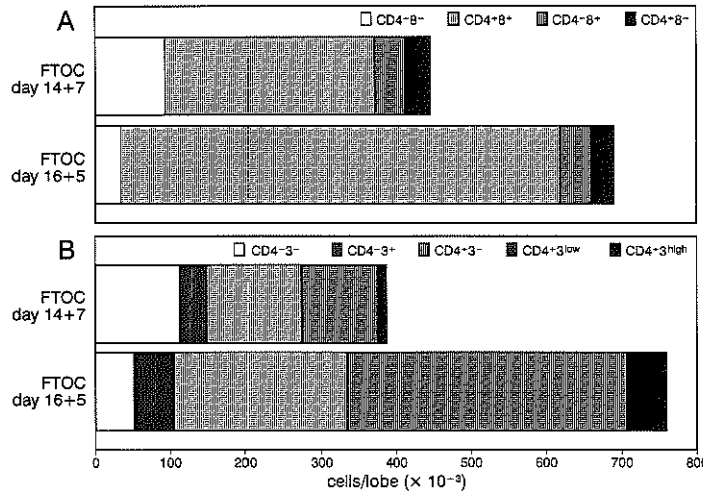

Figure 2. T cell development in FTOC day 14+7 (A) and FTOC day 16+5 (B). Cells were stained and analyzed as indicated in the *legend of Figure 1.* Data represent the mean of 3-5 experiments (CD4/CD8) or 2-4 experiments (CD4/CD3), expressed in absolute cell numbers. The contribution of different subpopulations to the total recovery is shown by different bar patterns in a stacked bar form.

studied, whereas in FTOC day 16+5 the effect on the development of mature CD4 single positive thymocytes with a high expression of the TcR complex could be analyzed (see also figure 10).

Anti-Ia treatment of fetal thymus organ culture

For anti-Ia treatment of the organ culture, we used the rat IgG2b mAb M5/114, which recognizes an allodeterminant present on both the I-A and I-E molecule (30). Since both CD4 and $\alpha\beta$TcR can interact with MHC class II molecules, the anti-I-A/E mAb was added to the organ cultures before $CD4^+8^+$ thymocytes developed. For FTOC day 16+5 this was at the start of the culture (day 0), whereas in day 14 FTOC, mAb was added at day 2 of the culture.

As a first indication of an effect of the mAb treatment, we established the cell recovery at the end of the culture period. Untreated or saline treated cultures produced comparable cell numbers (Fig. 3). Treatment with an isotype control antibody NLDC-145, in a dose of 200 μg/ml, resulted in a cell recovery comparable with saline treated cultures (data not shown). However, anti-Ia treatment of both culture systems caused reductions in cell number. In FTOC day 14+7 these reductions were most pronounced at doses of 100 and 200 μg/ml, whereas the low dose of 50 μg/ml

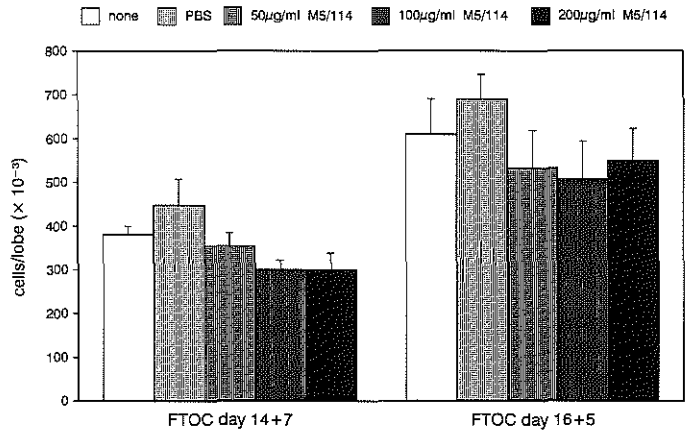

Figure 3. Effect of anti-Ia treatment on cell recovery in FTOC day 14+7 and FTOC day 16+5. Organ cultures were non-treated or treated with saline or different doses of M5/114 (50, 100 and 200 µg/ml), indicated by bars with different bar patterns. Bars represent the mean ± SEM of 3-5 experiments. Differences in cell recovery between anti-Ia treated and control cultures were not statistically significant.

caused only a mild reduction (Fig. 3). In FTOC day 16+5, all three doses gave equal reductions in cell number (Fig. 3).

The mAb enters the thymus and saturates the MHC class II molecules

To adress the question whether the anti-I-A/E antibody entered the thymic lobe in culture and completely saturated the MHC class II molecules on the stromal cells we performed immunohistology. When frozen sections of saline treated cultures were stained with a peroxydase-conjugated antiserum, no staining of cells in the thymus could be observed (Fig. 4A). When control sections were stained with the anti-I-A/E antibody M5/114, a fine reticular and a confluent staining pattern, was observed (Fig. 4B). A similar staining pattern was also observed on sections of the anti-I-A/E treated thymus, incubated with the peroxydase conjugated antiserum alone (Fig. 4C). This staining pattern indicates that (a) the antibody has penetrated the thymus lobe, (b) the antibody localizes to thymic stromal cells, and (c) a normal thymic architecture as present in the control cultures is maintained, indicating that anti-I-A/E treatment does not disturb the morphology of thymic microenvironments. The thymic stromal cells were completely saturated with the antibody, because additional staining of an adjacent section with M5/114 showed an identical staining pattern and intensity (Fig. 4D). The level of MHC class II antigen expression in the the anti-I-A/E treated thymus is the same as in saline treated control cultures (compare Fig. 4B, 4C, and 4D).

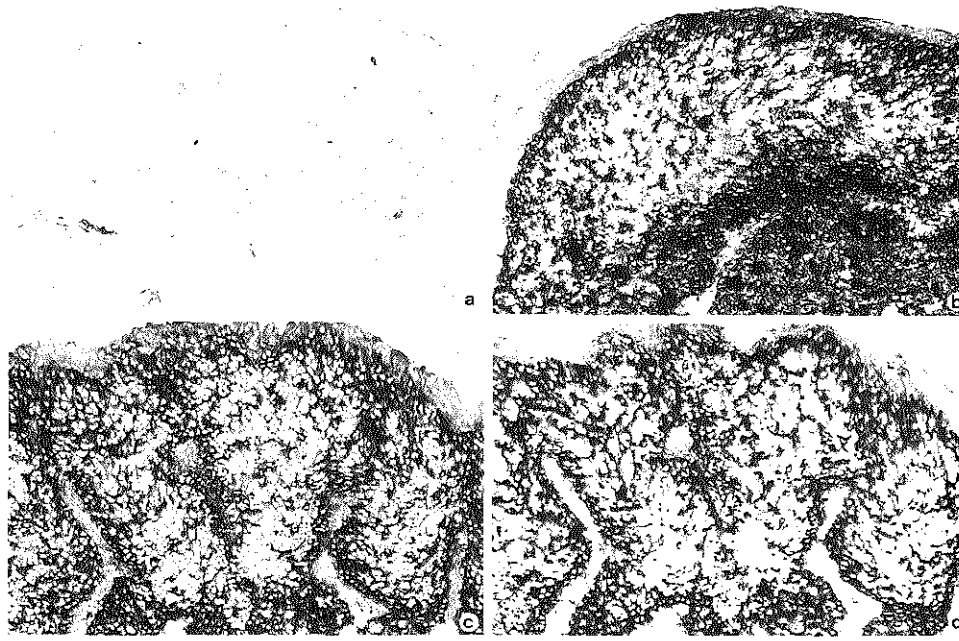

Figure 4. Penetration of anti-Ia mAb in organ cultured fetal thymus (FTOC day 16+5), detected with immunohistology. Adjacent pairs of frozen sections of saline treated (A,B) and anti-I-A/E treated FTOC (C,D), incubated with PBS (A,C) or an anti-I-A/E mAb (200 μg/ml M5/114; B,D). The mAb enters the thymus (C) and saturates the MHC class II molecules (B,C,D). Magnification: x 110.

These results were obtained with a dose of 200 μg/ml of purified antibody in both FTOC day 14+7 and FTOC day 16+5. With lower doses of 50 and 100 μg/ml we did not observe complete saturation, as judged with immunohistology (data not shown). Therefore, we will only report data obtained with the highest dose.

Anti-Ia treatment inhibits development of $CD4^+8^-$ thymocytes

It was reported that both in vivo as well as in vitro anti-Ia treatment of the thymus results in a failure to develop $CD4^+8^-$ thymocytes (15-20). The development of $CD4^+8^-$ thymocytes was also inhibited in our two culture systems, however, in each system to a different extent (Fig. 5). In FTOC day 16+5, anti-I-A/E treatment resulted in an almost complete absence of $CD4^+8^-$ thymocytes (Fig. 5C,D), whereas in FTOC day 14+7 this population was only mildly reduced (Fig. 5A,B).

Since total cell recovery was affected by anti-I-A/E treatment we determined the development of the different CD4/CD8 subpopulations in absolute numbers (Fig. 6). Again, we found in FTOC day 14+7 a partially reduced number of $CD4^+8^-$ thymocytes

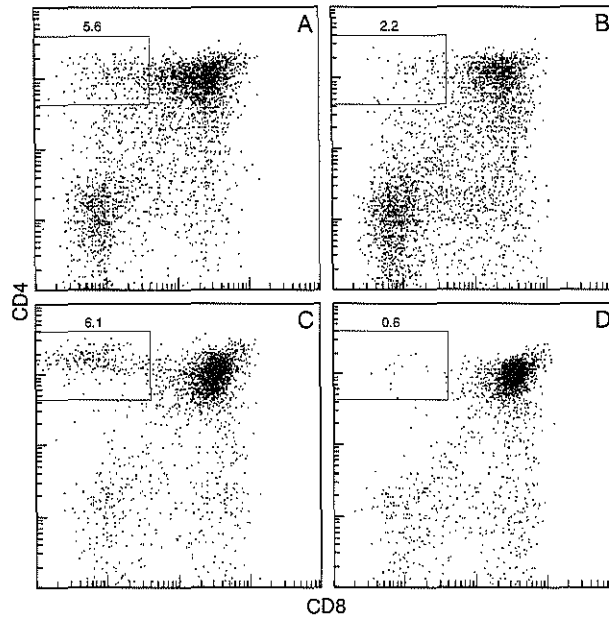

Figure 5. Effect of anti-Ia treatment on the development of CD4/CD8 defined subpopulations. Thymocytes from saline treated (A,C) and anti-I-A/E treated (200 μg/ml M5/114; B,D) FTOC day 14+7 (A,B) and FTOC day 16+5 (C,D) were used for two-color analysis. Cells were stained with CD4-biotin and CD8-FITC, followed by streptavidin-R-Phycoerythrin. The box in each figure indicates $CD4^+8^-$ thymocytes (as a percentage of the total).

(max. 50%; Fig. 6A), whereas in FTOC day 16+5 cultures the reduction was up to 90% (Fig. 6B). Also, $CD4^+8^+$ thymocytes were reduced in number in both systems. A reduction to 60-70% was observed in FTOC day 14+7 (Fig. 6A), whereas in FTOC day 16+5 the effect was less pronounced (Fig. 6B). In contrast, the total number of $CD4^-8^-$ and $CD4^-8^+$ thymocytes remained constant during anti-I-A/E treatment. Thus, the reduction in total cell recovery, as illustrated in Figure 3, is caused by a reduction in the number of both $CD4^+8^+$ and $CD4^+8^-$ thymocytes (Fig. 6A,B).

Anti-Ia treatment inhibits development of thymocytes with a $CD4^+3^{high}$ phenotype

We studied the expression of CD3 on developing thymocytes in FTOC by staining the cells with CD4 and CD3 mAb. $CD4^+8^-$ thymocytes are characterized by expression of CD4 and a high level of CD3 (indicated by a box in Fig. 7). A well defined population of $CD4^+3^{high}$ cells, present in saline treated FTOC day 16+5 was strongly reduced by anti-Ia treatment (Fig. 7C, 7D, and 8A). The reduction in the number of

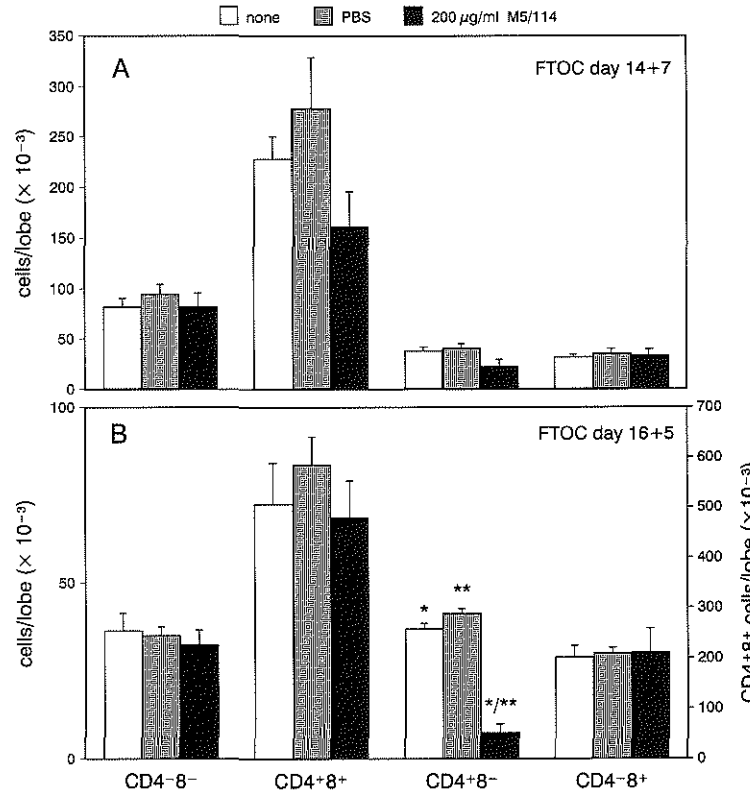

Figure 6. Development of CD4 and CD8 defined subpopulations in anti-Ia treated FTOC. For FTOC day 14+7 (A), and FTOC day 16+5 (B), a non-treated, a saline treated and an anti-I-A/E (200 µg/ml) treated culture are shown, indicated by bars with different bar patterns. Cells were stained and analyzed as indicated in *legends of Figure 1 and 5.* Bars represent the mean ± SEM of 3-5 experiments. Asterisks (*/**) indicate differences of statistical significance, of the anti-I-A/E treated culture with a non-treated (*) respectively a saline treated culture (**).

$CD4^+3^{high}$ cells matched the reduction in the number of $CD4^+8^-$ cells (data not shown). Thus, the development of mature $CD4^+8^-$ thymocytes in FTOC day 16+5 is inhibited when anti-Ia mAb block MHC class II antigens. In addition, we also observed reductions in the number of $CD4^+3^{low}$ and $CD4^+3^-$ thymocytes (Fig. 8A). The CD4-negative populations were not affected by anti-Ia treatment (Fig. 8A).

In FTOC day 14+7, we already noticed that mature $CD4^+8^-$ thymocytes with a high CD3 level only marginally developed (Fig.2). In this culture system, we were unable to observe an effect of the anti-Ia treatment on this mature $CD4^+3^-$ cell population (Fig. 7A,B, 8B). In addition, only the $CD4^+3^-$ thymocytes were reduced in cell number (Fig. 8B). The other CD4 and CD3 defined thymocyte subpopulations were unaffected

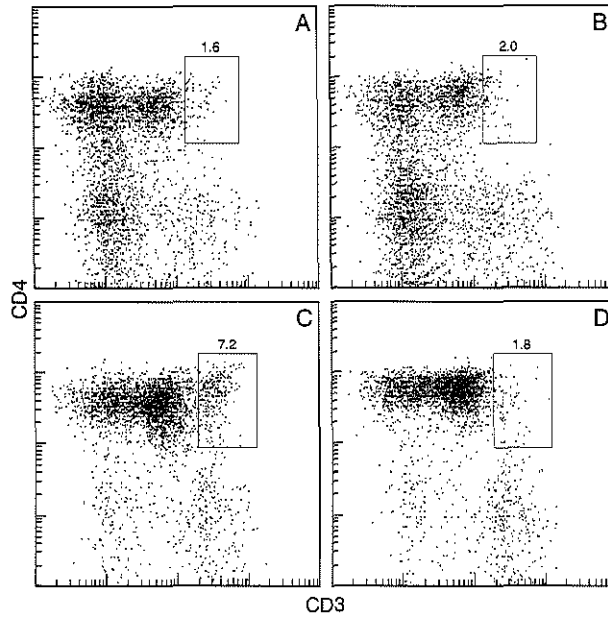

Figure 7. Effect of anti-Ia treatment on the development of CD4/CD3 defined subpopulations in FTOC. Thymocytes from saline treated (A,C) and anti-I-A/E treated (200 μg/ml M5/114; B,D) FTOC day 14+7 (A,B) and FTOC day 16+5 (C,D) were used for two-color analysis. Cells were stained by successive incubations with CD3 supernatant, anti-hamster-FITC, normal rat serum, and CD4-R-Phycoerythrin. The box in each figure indicates $CD4^+3^{high}$ thymocytes (as a percentage of the total).

by anti-Ia treatment (Fig. 8B).

Anti-Ia treatment upregulates CD4 but not CD8 on $CD4^+8^+$ thymocytes

Treatment of FTOC day 14+7 and FTOC day 16+5 with the anti-I-A/E mAb M5/114 not only inhibited the development of $CD4^+8^-$ and $CD4^+8^+$ thymocytes, but also caused upregulation of CD4 on the surface of $CD4^+8^+$ thymocytes (Fig. 9A and 9D). This increase in cell surface expression of CD4 was not caused by an increase in overall cell size, as forward light scatter of $CD4^+8^+$ cells from saline treated and anti-Ia treated cultures were virtually identical (Fig. 9C and 9F). In contrast, cell surface CD8 was not upregulated on $CD4^+8^+$ thymocytes (Fig. 9B and 9E). Also, no increase in the expression of CD3 and the $\alpha\beta$TcR could be observed after anti-Ia treatment (data not shown).

To quantify the difference in the level of CD4 expression, we stained cells from one control mAb treated culture and one anti-I-A/E treated culture with a FITC-conjugated

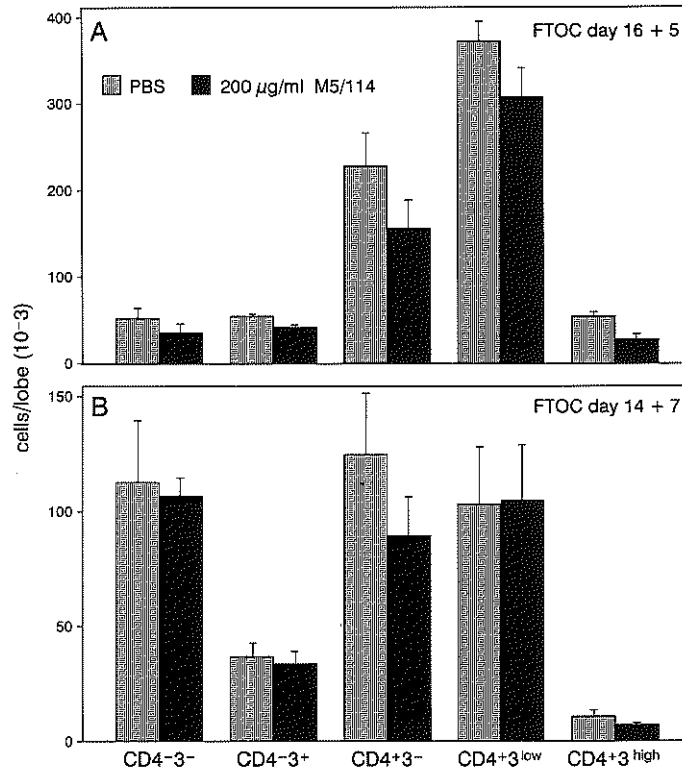

Figure 8. Development of CD4 and CD3 defined subpopulations in anti-Ia treated FTOC. For FTOC day 14+7 (A), and FTOC day 16+5 (B), a saline treated and an anti-I-A/E (200 µg/ml) treated culture are shown, indicated by bars with different bar patterns. Cells were stained and analyzed as indicated in *legends of Figure 1 and 7.* Bars represent the mean ± SEM of two to four experiments.

CD4 mAb. By comparing the median fluorescence intensity of the stained cells with that of standard FITC-coated beads, we were able to establish the amount of FITC that was bound to the cells. We found that CD4 expression on anti-I-A/E treated thymocytes was 1.4-1.5 times upregulated (data not shown).

Discussion

In the present study, we attempted to identify at what particular stage of T cell differentiation the interactions of MHC class II molecules with the $\alpha\beta$TcR and CD4 become important for the development of the helper T cell lineage. To that purpose,

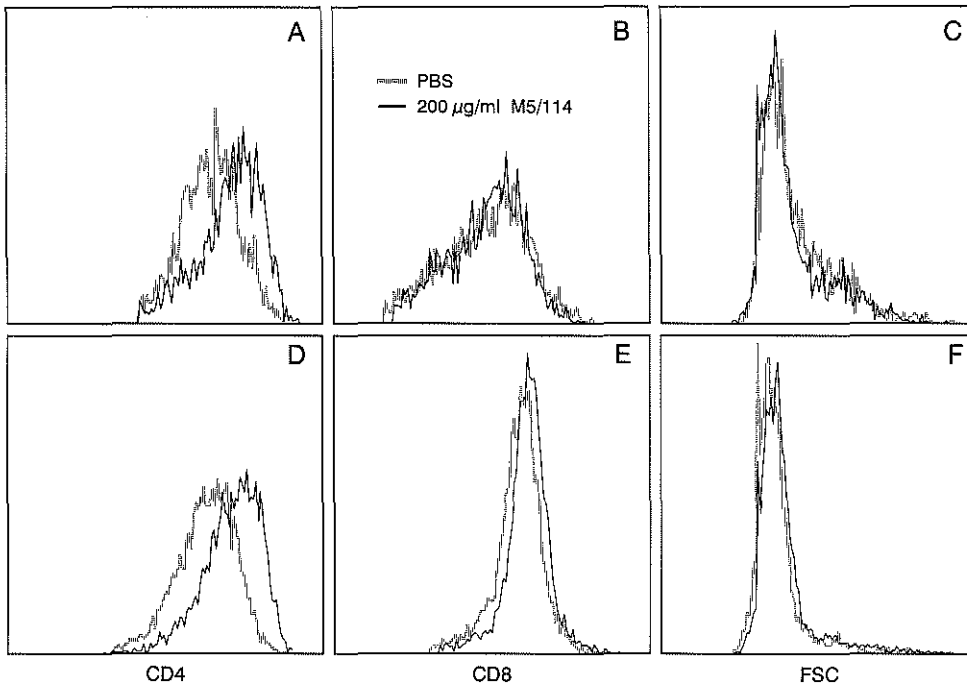

Figure 9. Effect of anti-Ia treatment on the expression of CD4 by $CD4^+8^+$ thymocytes in FTOC. Thymocytes from saline treated and anti-I-A/E treated (200 μg/ml M5/114) FTOC day 14+7 and FTOC day 16+5 were used for two-color analysis. Cells were stained as indicated in the *legend of Figure 1.* $CD4^+8^+$ thymocytes were gated and the expression of CD4 (A,D) and CD8 (B,E), and cell size (measured as forward light scatter; C,F) were depicted in histogram form, for both FTOC day 14+7 (A-C) and day 16+5 (D-F). Each picture shows histogram overlays of a saline treated culture (dotted line) and an anti-I-A/E treated culture (solid line). On the X-axis only 128 of a total of 256 fluorescence channels are presented.

fetal thymuses in organ culture were treated with an anti-Ia monoclonal antibody interfering with these particular interactions. We used organ cultures of day 14 and day 16 fetal thymuses. Both organ culture systems supported the development of mature $CD4^+8^-$ thymocytes. In FTOC day 14+7, however, T cell differentiation is much slower and substantial numbers of $CD4^+8^-$ thymocytes will only develop in prolonged cultures (data not shown). Nevertheless, FTOC day 14+7 proved useful, because here we were able to study the effect of anti-Ia treatment on the development of $CD4^+8^+3^-$ and $CD4^+8^+3^{low}$ thymocytes (Fig. 10). In FTOC day 16+5, on the other hand, we could especially observe the effect of anti-Ia treatment on the development of mature $CD4^+8^-3^{high}$ thymocytes (see also Fig. 10).

As expected, the development of $CD4^+8^-$ thymocytes with a high level of CD3

expression, in FTOC day 16+5, was almost completely inhibited by anti-Ia treatment. This result confirms earlier published studies showing that anti-Ia treatment in vivo as well as in vitro inhibits the development of thymocytes with a helper cell function and a $CD4^+8^-$ phenotype (15-20,38,39). We used fetal thymuses of the BALB/c mouse, which expresses H-2I-A as well as H-2I-E molecules, in combination with a mAb that recognizes both these MHC molecules in the BALB/c mouse (30). This rat mAb M5/114 is functionally active; it strongly inhibits the proliferative response of BALB/c T cells against the antigens $G^{60}A^{30}T^{10}$ and $G^{58}L^{38}\varnothing^4$, responses that are I-A respectively I-E restricted (30,40). This suggested that M5/114 was also able to inhibit both the I-A and the I-E restricted helper T cell development in our organ cultures. Our study required such an antibody, because anti-I-A treatment alone does not interfere with thymic I-E expression and leaves the development of I-E restricted mature $CD4^+8^-$ thymocytes unaffected (41). The absence of mature $CD4^+8^-3^{high}$ thymocytes in our day 16 fetal thymus organ culture system indicates that I-A as well as I-E restricted helper T cell development was totally abrogated by this antibody. Recently, it was shown that the mAb M5/114 recognizes a part of the β_1 domain of the I-A molecule, involved with binding of the TcR and/or the antigenic peptide (42). This suggests that the absence of $CD4^+8^-$ cells in the organ culture is primarily caused by inhibition of TcR-MHC interactions.

Some $CD4^+8^-$ thymocytes do still develop in FTOC day 16+5 after anti-Ia treatment. These cells probably do not express CD3 and are the immature intermediates, which have been identified in T cell differentiation between $CD4^-8^-$ and $CD4^+8^+$ thymocytes (35-37). In FTOC day 14+7, almost all $CD4^+8^-$ thymocytes are of this immature CD3 negative phenotype. Surprisingly, this immature, $CD4^+8^-3^-$ thymocyte subpopulation in FTOC day 14+7 is reduced in cell number after anti-Ia treatment. This indicates that cells expressing CD4, but not a TcR, can bind to MHC class II molecules on thymic stromal cells. Can CD4 be used as an adhesion molecule? It has been shown that human B-lymphoblastoid cell lines bearing MHC class II molecules can strongly bind to a fibroblast line transfected with the CD4 glycoprotein (43). CD4 and anti-Ia mAb completely inhibited the binding, indicating a direct interaction of the two molecules. However, authors comment that high levels of CD4 were probably crucial to demonstrate the high affinity, stable interaction (43). They speculate, that at more physiological expression levels, it is likely that CD4 and MHC class II molecules can mediate low-affinity transient interactions. In the thymus, it is conceivable that CD4 helps anchoring the thymocyte to the stromal cell, just before the onset of TcR expression and the start of selection. Our results indicate that our anti-Ia mAb already interferes with these interactions on immature CD4 positive cells that do not yet express an $\alpha\beta$TcR.

Anti-Ia treatment also caused a reduction in the number of $CD4^+8^+$ thymocytes and

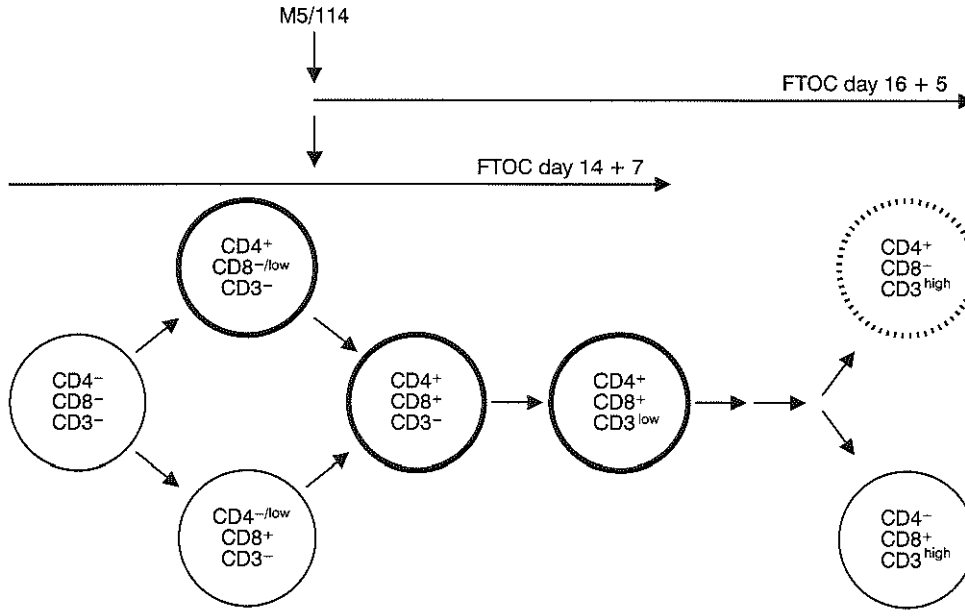

Figure 10. A model of T cell differentiation in the $\alpha\beta$TcR lineage. The major cell types, contributing to this model, are also present in fetal thymus organ cultures. The horizontal lines (in the top of the figure) indicate the major cell types present in FTOC day 14+7 and FTOC day 16+5 at the end of the culture period. After anti-Ia treatment mature $CD4^+8^-3^{high}$ thymocytes are absent in the organ cultures (cell with dotted contour), whereas immature CD4 expressing thymocytes show upregulation of CD4 and/or a reduction in cell number (cells with thick line contour).

this effect was again most pronounced in FTOC day 14+7. Simultaneously, we observed that the number of $CD4^+3^{low}$ and $CD4^+3^-$ thymocytes was reduced. The $CD4^+3^{low}$ subset represents mainly small $CD4^+8^+3^{low}$ thymocytes, whereas the $CD4^+3^-$ population is very heterogeneous, containing immature $CD4^+8^-3^-$ cells as well as immature $CD4^+8^+3^-$ blast or small cells. Together, this seems to indicate that not only $CD4^+8^+3^{low}$ thymocytes, but also $CD4^+8^+3^-$ and $CD4^+8^-3^-$ thymocytes are affected by anti-Ia treatment. However, anti-Ia treatment does not cause an absolute block, since these populations are only reduced in number and not completely absent.

In addition to the reduction in cell number, we observed an increase of surface CD4 expression on $CD4^+8^+$ thymocytes, in both FTOC day 14+7 and FTOC day 16+5. The CD4 concentration per cell was 1.4-1.5 times upregulated, reaching a level of CD4 expression normally present on mature $CD4^+8^-$ thymocytes. The upregulation is a specific effect of the anti-Ia treatment, because the expression of CD8, CD3 and the $\alpha\beta$TcR on $CD4^+8^+$ thymocytes remained normal and the double positive cells did not increase in size. Surprisingly, close examination of the figures in most published

studies using anti-Ia treatment revealed that upregulation of CD4 was unknowingly reported (17-20). A similar upregulation of CD4 on $CD4^+8^+$ thymocytes has now been described in mutant mice lacking expression of MHC class II antigens in the thymus (22). Our results and the results by Cosgrove *et al.* (22) indicate that thymocytes upregulate CD4 when CD4-MHC and TcR-MHC interactions are disturbed. When we analyzed CD4 expression in a CD4/CD3 double staining, we observed upregulation of CD4 on both $CD4^+3^{low}$ and $CD4^+3^-$ thymocytes after anti-Ia treatment (see figure 7). This means that not only $CD4^+8^+3^{low}$ thymocytes, but also $CD4^+8^+3^-$ and/or $CD4^+8^-3^-$ thymocytes upregulated CD4 on their surface. This phenomenon confirms our observations that CD4-MHC interactions occur in the absence of TcR-MHC interactions. And again, it shows that our anti-I-A/E mAb inhibits these CD4-MHC interactions at an immature developmental stage, when CD4, but not the $\alpha\beta$TcR is expressed (Fig. 10).

The question arises how upregulation of CD4 on the surface of $CD4^+8^+$ thymocytes can be explained. Recently, it was reported that transgenic mice expressing MHC class II molecules at a level, five times higher than normal showed an almost normal development of $CD4^+8^-$ thymocytes (44). However, surface expression of CD4 was downregulated on $CD4^+8^+$ and to a lesser extent also on $CD4^+8^-$ thymocytes in these mice. Together, our data and these data (published by Fehling et al; ref. 44), indicate that the level of CD4 expression on developing thymocytes can be regulated by the level of MHC expression at the stromal cell. In terms of T cell differentiation, this means that a minimal level/amount of CD4-MHC and TcR-MHC interactions is required to reach a certain threshold, allowing cells to be positively selected. We suggest that in our study, under the conditions of reduced TcR-MHC and CD4-MHC interactions, thymocytes upregulate CD4 in an attempt to reach this selection threshold.

How elevated levels of coreceptor, i.e. CD4 or CD8, affect positive and negative selection has been studied in CD4 respectively CD8 transgenic mice. The positive selection of $CD8^+$ thymocytes in female mice, expressing a transgenic $\alpha\beta$TcR specific for the H-Y antigen presented by $H\text{-}2D^b$ molecules, was dramatically reduced by over-expression of a CD4 transgene (15-30 times normal; ref. 45). Similarly, high expression of a CD8 transgene in $\alpha\beta$TcR transgenic mice caused deletion of cells normally positively selected, whereas moderate levels of the CD8 transgene supported or enhanced positive selection (46-48). These data seem to support an affinity model for thymic selection where low affinity interactions of TcR and MHC will lead to positive selection, whereas high affinity interactions will lead to negative selection (49,50). Together, our results and the results from the coreceptor transgenic animals indicate that levels of coreceptor-MHC interactions are important for selection, by contributing to the affinity of the TcR-MHC interaction or to the overall avidity of a thymocyte for a selecting stromal cell (47).

The role of CD4-MHC interactions has also been studied by treating the thymus, in vivo as well as in vitro, with CD4 mAb (19-21,51). Again, thymocytes with helper T cell function and a $CD4^+8^-$ phenotype did not develop. However, in these particular studies they did not observe upregulation of the surface expression of CD4 on $CD4^+8^+$ thymocytes, but of the $\alpha\beta$TcR (19,20). Upregulation of this molecule was also observed when thymocytes were brought into single cell cultures at 37°C (51). Interestingly, Ia^+ cells added to the culture inhibited the upregulation of the $\alpha\beta$TcR, suggesting that, during normal T cell differentiation, Ia engagement of CD4 molecules causes the inhibitory signal to maintain a low level of TcR expression on $CD4^+8^+$ thymocytes. CD4 mAb treatment inhibits this CD4-MHC interaction and thus causes the upregulation of the $\alpha\beta$TcR (51). On the other hand, inhibition of CD4-MHC interaction could have induced the cell to raise the expression of the TcR in an attempt to reach a selection threshold, as we discussed above.

Interestingly, only when CD4-MHC and TcR-MHC interactions were completely absent, as in knockout mice lacking MHC class II molecules, upregulation of both CD4 and $\alpha\beta$TcR was observed (22). This suggests that mAb treatment, with either CD4 or anti-Ia mAb, does not result in complete inhibition of both CD4-MHC and TcR-MHC interactions. However, blocking either of these interactions by the mAb is in itself enough to inhibit the development of mature $CD4^+8^-$ thymocytes. Together, these data indicate that upregulation of CD4 and/or the $\alpha\beta$TcR can not compensate for a loss of physical contact between the thymocyte and the thymic stromal cells.

In summary, we have shown that anti-Ia treatment interferes with the development of mature $CD4^+8^-$ thymocytes. However, it was our initial goal to identify the stage of T cell differentiation where interactions of MHC class II with CD4 and $\alpha\beta$TcR become important for the development of helper T cells. Our results sofar indicate that the $CD4^+8^+3^{low}$ thymocyte is a proper candidate, because CD4 expression was upregulated in this population. However, it cannot be excluded that the blockade lies even earlier, since CD4 upregulation was observed on cells with a $CD4^+3^-$ phenotype. This indicates that $CD4^+3^-$ cells, i.e $CD4^+8^+3^-$ and/or $CD4^+8^-3^-$ cells, use CD4 to "anchor" onto MHC class II expressing stromal cells. Our study shows that positive selection cannot occur, if the physical contact between one of these immature thymocyte populations and the thymic stroma is abrogated.

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CHAPTER 6

Cytokines induce phenotypic changes in murine thymic microenvironments*

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Summary

Lymphostromal interaction is of prime importance during T cell development. Cell-cell interaction but also locally secreted cytokines have major effects on proliferation and selection of maturing T cells. Recently, it was observed that lymphostromal interaction is a bi-directional phenomenon: under experimental conditions where mature T cells cannot develop in the thymus, the medullary epithelial compartment was severely decreased in size and composition. This indicates that mature T cells influence the integrity of this compartment. Because cytokine treatment of the thymus (especially with IL-2 and IL-4) results in a maturation arrest of immature $CD4^-8^-$ thymocytes, we reasoned that the development of thymic microenvironments would also be affected. To test this hypothesis we analyzed the development of cortical and medullary epithelial cells in 12-day organ cultures of day 14 fetal thymus, either untreated or treated with IL-2 or IL-4.

In control organ culture, we found a normal development of the cortical microenvironment, but medullary epithelial cells were reduced in cell number. Since the development of mature $\alpha\beta$ and $\gamma\delta$ T cells was not inhibited in FTOC, our findings suggest that the fetal thymic medulla is not essential to the development of these mature T cells.

In FTOC, treated with IL-4, thymic epithelial cells developed like in control FTOC. In IL-2 treated organ cultures, however, cortical epithelial cells were almost completely absent, whereas medullary epithelial cells, not organized as a clearcut medulla, increased in numbers. It appears that T cell derived cytokines play a key role in the regulation of the composition of thymic microenvironments.

Introduction

Thymic stromal cells play a crucial role in the differentiation of immunocompetent T lymphocytes. The anatomical distribution of lymphoid and stromal cells indicates that different thymic stromal cells promote different stages of T cell differentiation (1-5). Both physical interactions as well as cytokines seem to mediate the essential intercellular signalling. The role of several cytokines, in particular IL-1, IL-2, IL-4, and IL-7, in the development of immature thymocytes is well established (6). For example, IL-2 or anti-IL-2R treatment of thymus in both *in vitro* as well as *in vivo* modelsystems completely abrogates the development of $\alpha\beta$ T cells (7-15). In addition to these results, one of us recently noticed that IL-2 treatment of fetal thymus organ cultures (FTOC) resulted in the expansion of $Fc\gamma RII^+$ $CD4^-8^-3^-$ cells. Such cells showed a morphology of large granular lymphocytes and displayed broad cytotoxic activity (16). $\gamma\delta TcR^+$

thymocytes developed normally in IL-2 treated cultures. Likewise, we previously reported that IL-4 treatment of the FTOC resulted in inhibition of T cell development in the $\alpha\beta$ T cell lineage, but not the $\gamma\delta$ T cell lineage (17,18). Cytokines, like IL-2 and IL-4 have a direct effect on developing T cells, as receptors for these cytokines are only expressed by immature thymocytes (6). Whether these cytokines also effect the development of thymic stromal cells has not been reported. IL-2 and IL-4 probably do not influence thymic stromal cells directly, since receptors for both cytokines on the surface of thymic stromal cells have sofar not been described. Altered or inhibited T cell development, however, could influence the development of thymic stromal cells: various animal models demonstrate that normal development of the medulla in adult thymus depends on the presence of TcR^+ cells (19-21). This dependency was most clearly demonstrated in SCID mice and cyclosporin A treated mice.

Mature $\alpha\beta$ T cells do not develop in the thymus of SCID mice, due to a genetic defect which prevents the productive rearrangement of T and B cell receptor genes (22,23). Recently it was shown that thymic medullary epithelium failed to mature in these SCID mice, but not in SCID mice containing either endogenously arising TcR^+ cells ("leaky" SCID mice; ref. 24) or exogenously transferred BM cells giving rise to TcR^+ cells (19,25). Similar observations have also been reported in Cyclosporin A (CsA) treated mice: mature $\alpha\beta TcR^+$ $CD4^+8^-$ and $CD4^-8^+$ thymocytes do not develop in the thymus of these mice. Concomitantly, the thymic medulla appeared almost completely absent (20,21,26-28). After cessation of CsA treatment, the various cellular components of the medulla reappeared and the stromal architecture returned to normal (20,21).

These studies indicate that intact thymic microenvironments are dependent on the presence of functional T cells, suggesting that *in vivo* or *in vitro* cytokine treatment not only affects T cell development but also affects the development of thymic epithelial cells (TEC). To investigate this possibility, we analyzed the effect of IL-2 and IL-4 on the development of medullary and cortical TEC in FTOC.

Materials and methods

Mice

BALB/c mice were purchased from the Experimental Animal Breeding Facility (University of Leuven, Belgium) and maintained in the animal facilities of our department. For timed pregnancies, mice were mated for 16 h and the appearance of a vaginal plug was considered to be day 0 of gestation. Pregnant mice were killed by cervical dislocation and fetuses were dissected from the uterus. Fetuses of day 14 of gestation were used for fetal thymus organ culture.

Cytokines

Purified human rIL-2 was produced by recombinant DNA technology in *Escherichia coli* (a generous

gift from R. Devos, Roche Research, Gent, Belgium; ref. 29). The biological activity was measured in a 24-h assay using IL-2-dependent human splenocytes and 1 U was defined as the amount inducing 50% of maximal proliferation.

Purified murine rIL-4 was produced by recombinant DNA technology in Baculovirus. The activity of IL-4 was evaluated by means of a proliferation assay using the IL-4-dependent cell line FDCp-1. To this purpose, the Il-4 containing sample was serially diluted and added to the FDCp-1 cells (2×10^3/well). After 4 days, the number of FDCp-1 cells was estimated by measuring the level of the endogenous enzym hexosaminidase (30). One unit was defined as the amount inducing 50% of maximal proliferation.

Recombinant murine IFN-γ was derived from the supernatant fluid of Mick cells, a CHO cell line that carries and expresses an amplified murine IFN-γ cDNA (31). This IFN was purified by affinity chromatography on the F3 mAb to a specific activity of $\approx 10^6$ IU/mg protein. Human rIFN-γ produced in *Escherichia coli* was obtained from Bioferon (Laupheim, Federal Republic of Germany) by the courtesy of Dr. W. Wolf. It contained 0.7 mg/ml pure protein.

Fetal thymus organ culture (FTOC)

Fetal thymus lobes were removed from fetal mice at day 14 of gestation and placed in organ culture, according to the methods of Mandel (32) and Jenkinson (33), as reported previously (9). The organ cultures were treated with IL-2 (1000 U/ml), IL-4 (100 U/ml), IFN-γ (500 U/ml), or a mixture of IL-4 (100 U/ml) and IFN-γ (500 U/ml).

Immunohistology

Immunohistology was performed on cryostat tissue sections of organ cultured fetal thymus lobes, essentially as described before (34). Briefly, sections were incubated with culture supernatant of the hybridomas H129.19 (anti-MT4; ref. 35), ER-TR4 (36), ER-TR5 (36), and M5/114 (anti-MHC class II; ref. 37), and developed with a horseradish peroxydase conjugate of rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark).

Results

Immunohistological analysis of organ-cultured fetal thymus

The aim of this study was to analyze the architecture of the thymic stroma in organ-cultured fetal thymuses, treated with IL-2 and IL-4. Changes in thymic microenvironments were identified with monoclonal antibodies directed against cortical or medullary epithelial cells (ER-TR4 resp. ER-TR5; ref. 36), and a monoclonal antibody against MHC class II molecules (M5/114; ref. 37). The characteristic staining patterns of the mAb ER-TR4 and ER-TR5 on 6 days old neonatal thymus, are shown in Figure 1. Like in adult thymus (data not shown), ER-TR4 stains all cortical epithelial cells with a fine reticular staining pattern (Fig. 1a), whereas ER-TR5 stains all medullary epithelial cells, with their typical spindle shaped morphology (Fig. 1b). The six days old neonatal thymus is the *in vivo* equivalent of the (day 14) fetal thymus cultured for 12 days. Both mAb already distinguish cortical and medullary regions in the day 14 fetal thymus, at the start of the culture (38,39).

Thymic lobes used for immunohistological analysis can not be used for flow

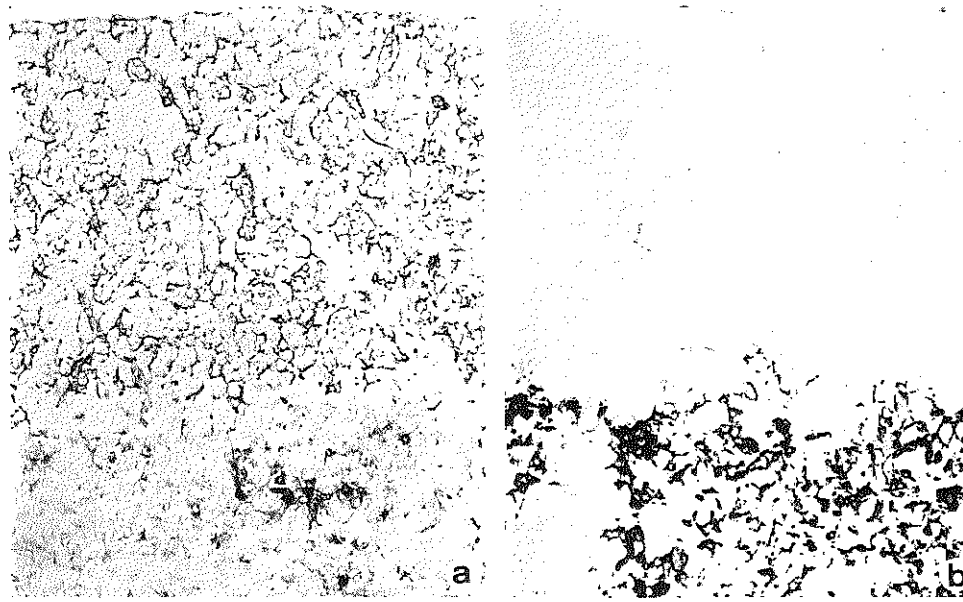

Figure 1. Thymic microenvironments in neonatal BALB/c thymus (6 days old). Frozen sections were stained with the mAb ER-TR4 (1a), against cortical epithelial cells, and ER-TR5 (1b), against medullary epithelial cells. Magnification: x 140.

cytometric analysis of T cell development. To ascertain that T cell development in the lobes used for immunohistology agreed with T cell development in lobes used for flow cytometric analysis (9,16-18), frozen sections of organ-cultured fetal thymus were also stained with a CD4 mAb. In organ culture, CD4 is mainly expressed by maturing thymocytes with $CD4^+8^+3^{-/low}$ and $CD4^+8^-3^{high}$ phenotype. Absence of $CD4^+$ cells on sections will therefore indicate an absence of $\alpha\beta$ T cell development.

We will now first present the development of cortical and medullary microenvironments in untreated organ cultures. Subsequently, we will report on the effects of the cytokines IL-2 and IL-4 on the development of these thymic microenvironments.

Thymic microenvironments in untreated control FTOC

The presence of $CD4^+$ thymocytes indicates normal development of thymocytes in the control FTOC (Fig. 2a). Cortical epithelial cells, recognized by ER-TR4, were found throughout the cultured thymus. However, the staining pattern did not show a fine reticular network, as in the intact neonatal thymus, but the cortical epithelial cells were more closely packed (Fig. 2b). $CD4^+$ cells appeared to be present in these cortical areas (compare Fig. 2a and 2b). To our surprise, we could not clearly identify the

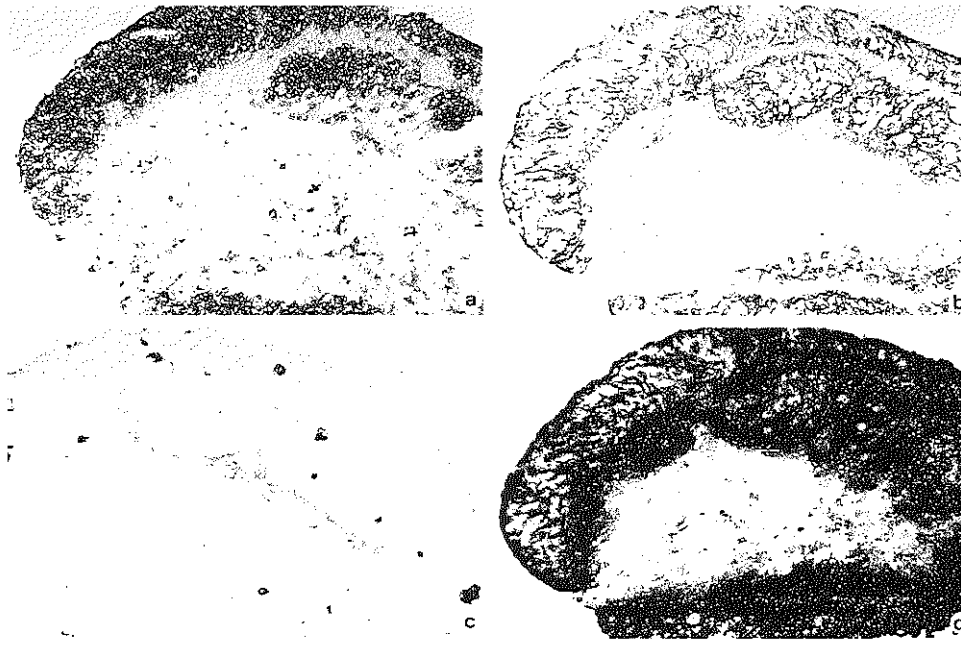

Figure 2. Thymic microenvironments in control FTOC. Frozen sections of organ cultured fetal thymus were stained with mAb against thymocytes (2a) and stromal cells (2b-d). 2a, CD4 expression; 2b, ER-TR4$^+$ cortical epithelium; 2c, ER-TR5$^+$ medullary epithelium; 2d, MHC class II expression. Magnification: x 65.

thymic medulla in FTOC and ER-TR5$^+$ cells, when present, resembled the small Hassall's corpuscles found in the mouse thymus (Fig. 2c). We observed strong staining for MHC class II molecules, in, predominantly, a confluent staining pattern (Fig. 2d).

Thus, in normal FTOC, as compared to neonatal thymus, there is an abnormal development of the medullary thymic microenvironment. Moreover, the frequency of ER-TR5$^+$ cells is even lower than at the start of the organ culture (data not shown). It has to be mentioned that this perturbed microenvironment apparently does not affect the development of mature $\alpha\beta$ T cells (9,16,17,40-42).

Thymic microenvironments in IL-2 treated FTOC

Recently, we showed that IL-2 treatment of fetal thymus resulted in the expansion of CD4$^-$8$^-$3$^-$ cells with a morphology of large granular lymphocytes and with broad cytotoxic activity (16). $\gamma\delta$ T cells were not affected by IL-2 treatment, whereas the development of $\alpha\beta$ T cells was almost completely abrogated. As a result, cell yield was

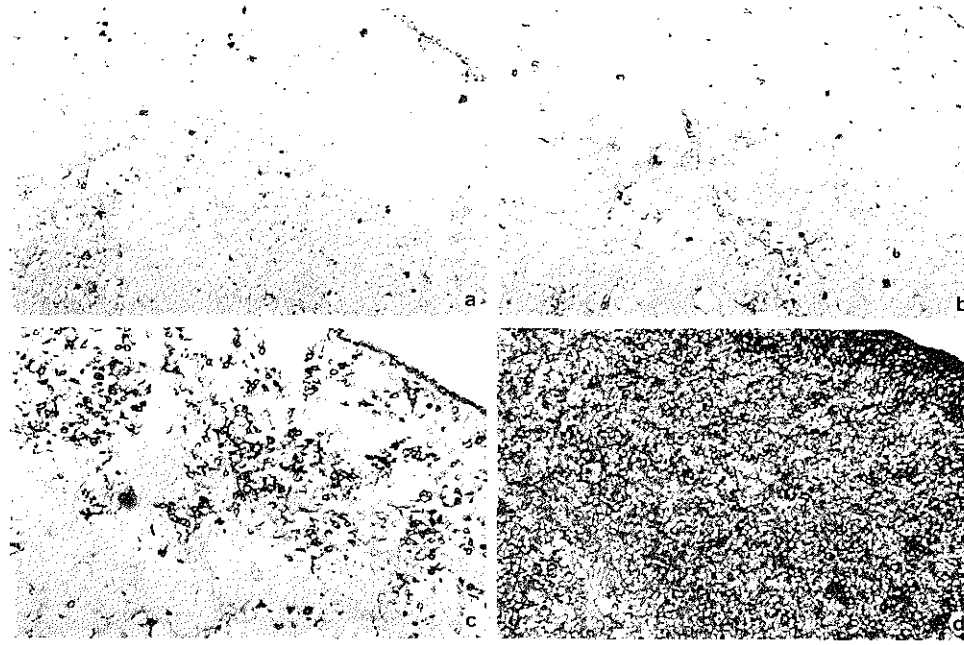

Figure 3. Thymic microenvironments in IL-2 treated FTOC. Frozen sections of organ cultured fetal thymus were stained with mAb against thymocytes (3a) and stromal cells (3b-d). 3a, CD4 expression; 3b, ER-TR4$^+$ cortical epithelium; 3c, ER-TR5$^+$ medullary epithelium; 3d, MHC class II expression. Magnification: x 80.

only reduced by 24% and the size of the IL-2-treated thymic lobes was comparable to control lobes.

Our present immunohistological analysis of IL-2-treated thymus confirms, that CD4$^+$ thymocytes do not develop (Fig. 3a). Interestingly, the organization of the thymic microenvironments was completely different from what we observed in the neonatal thymus (Fig. 1) or in the control FTOC (Fig. 2). After IL-2 treatment, ER-TR4$^+$ cortical epithelial cells were only scarcely present (Fig. 3b), whereas medullary epithelial cells were now the major stromal cell population (Fig. 3c). All stromal cells expressed MHC class II molecules at high levels, indicated by an intense staining pattern (Fig. 3d).

These results demonstrate that the IL-2 induced changes in T cell development are accompanied by changes in development of thymic microenvironments. Our data indicate that the expansion of CD4$^-$8$^-$3$^-$ large granular lymphocytes, observed by us in IL-2-driven FTOC (16), goes together with expansion of the medullary type of thymic epithelial cells.

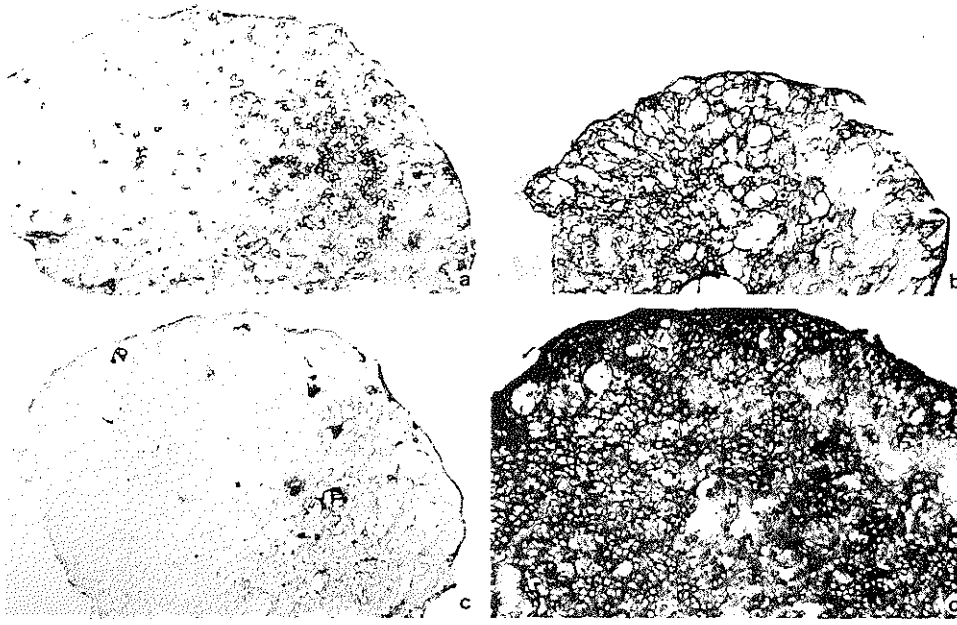

Figure 4. Thymic microenvironments in IL-4 treated FTOC. Frozen sections of organ cultured fetal thymus were stained with mAb against thymocytes (4a) and stromal cells (4b-d). 4a, CD4 expression; 4b, ER-TR4$^+$ cortical epithelium; 4c, ER-TR5$^+$ medullary epithelium; 4d, MHC class II expression. Magnification: x 80.

Thymic microenvironments in IL-4 treated FTOC

IL-4 treatment of day 14 fetal thymus resulted in a decreased cell yield (<15% as compared to the control culture) and a smaller size of the lobes at day 12 of the organ culture, as compared to control FTOC or IL-2 treated FTOC (17). CD4$^-$8$^-$ thymocytes constituted the major thymocyte subpopulation after 12 days of culture. Surprisingly, at this point of time mature CD4$^+$8$^-$ and CD4$^-$8$^+$ cells were present in the apparent absence of their CD4$^+$8$^+$ precursors (17). However, at day 6 of the culture, all CD4/CD8 defined thymocyte subpopulations developed in the same numbers as in control FTOC (17). These findings indicate that development of CD4$^-$8$^-$ through CD4$^+$8$^+$ into CD4$^+$8$^-$/CD4$^-$8$^+$ cells occurs only transiently. The presence of mature T cells in the IL-4 treated thymus can be explained by their longer lifespan as compared to CD4$^+$8$^+$ cells. Thus, the normal day 14 fetal thymus contains prothymocytes that had developed beyond the IL-4 sensitive stage. And indeed, IL-4 treatment of day 13 fetal thymus completely blocked $\alpha\beta$ T cell development, but not $\gamma\delta$ T cell development (17).

Staining thymus sections with a CD4 mAb confirmed the presence of mature $CD4^+$ cells in the IL-4-treated thymus (Fig. 4a). In contrast to the IL-2 treated cultures, the organization of thymic microenvironments was comparable to what we observed in control FTOC. Closely packed cortical epithelial cells were observed throughout the entire thymic lobe (Fig. 4b), whereas only a few scattered medullary, Hassall-like, epithelial cells were present (Fig. 4c). MHC class II molecules were strongly expressed by all stromal cells (Fig. 4d). The 'normal' organization of the thymic microenvironments, as compared to the control, seems to be in agreement with a wave of normal T cell differentiation in the IL-4 treated thymus, as observed by flow cytometric analysis (17). These results indicate that, in contrast to IL-2, IL-4 does not affect the development of thymic microenvironments.

The IL-4 mediated inhibition of T cell development in FTOC could be reversed by addition of IFN-γ to the IL-4 treated organ culture (18). In such experiments, we also observed a normal development of thymic microenvironments, comparable to control FTOC (data not shown).

Discussion

In this study we analyzed the development of thymic microenvironments in fetal thymus organ culture. This *in vitro* model system supports the development of $\alpha\beta$ and $\gamma\delta$ T lymphocytes (16,17,40-42). The development of mature T cells in the adult thymus requires intact cortical and medullary microenvironments. However, our data indicate otherwise in experimental conditions where fetal thymus organ culture is applied. Only a small number of medullary TEC developed after 12 days of organ culture and these cells were not organized in defined medullary areas. In addition, the medullary TEC did not show a normal morphology, but rather displayed a morphology comparable to Hassall's corpuscles. The assumed function of Hassall's corpuscles as thymic graveyards (43) suggests they are not involved in promoting T cell differentiation. Our findings are supported by recently published observations that only MHC class II expressing cortical TEC and fetal mesenchymal cells, in FTOC, are required for the development of immature $CD4^-8^-$ cells into mature CD4 or CD8 expressing T cells (44). Together, the data suggest that medullary TEC in fetal thymus are not involved in the development of mature $\alpha\beta$ T cells.

In the adult thymus of SCID mice, of CsA treated mice, or of mice lacking the TcR-α gene a medulla, as identified with mAb against medullary TEC, was reported absent (19-21,28,45). Surprisingly, the thymic medulla was restored as soon as TcR^+ cells were introduced. In CsA treated mice, this occurred after cessation of the treatment (20,21). In SCID mice, the medulla was only restored after the mice were reconstituted

with either allogeneic bone marrow cells or lymph node cells, as reported by Shores et al. (19), respectively Surh et al. (46). These studies indicate that intrathymically developing T cells as well as peripheral T cells have the capacity to induce the development of the thymic medulla. This finding implicates that lymphostromal interaction in the thymus is, in general, symbiotic: not only stromal cells can signal developing lymphoid cells, but also lymphoid cells signal the development of thymic stromal cells. In our control FTOC, this latter signal did not occur as the presence of TcR^+ T cells did not induce/support normal development of medullary TEC. Together these *in vitro* data indicate that (1) TcR^+ T cells develop in FTOC in the absence of a normal thymic medulla, and (2) developing TcR^+ T cells in FTOC cannot induce normal development of the thymic medulla in FTOC. If our results with FTOC reflect the *in vivo* situation, these data seem to indicate different functions for the medulla in fetal and adult thymus.

In this context, it was shown that the development of fetal $\gamma\delta$ T cells, expressing the TcR-Vγ3 segment, only occurred in fetal thymus (47). Moreover, Farr et al. (48) proposed that fetal thymic medullary TEC play a role in the development of $\gamma\delta$ T cells. He observed that $\gamma\delta TcR^+$ thymocytes colocalize with medullary TEC in late fetal and neonatal thymus, whereas in adult thymus these cells were found scattered throughout cortex and medulla and most concentrated in the subcapsular areas of the thymus (48). Our data, however, seem to be contradictory to these observations. Here we show that $\gamma\delta$ T cells develop in normal numbers, both in control FTOC as well as in IL-4 treated FTOC (18); both types of culture show an aberrant development of medullary TEC. Our results, therefore, suggest that $\gamma\delta$ T cells do not depend on the fetal thymic medulla for their development. Comparatively, in adult thymus $\gamma\delta$ T cells develop in the apparent absence of a medulla, since in 'knock-out' mice, homozygous for a disrupted TcR-α gene, both $\alpha\beta$ T cells as well as the thymic medullary TEC are absent, whereas $\gamma\delta$ T cells develop in normal numbers (45).

Based on these observations we conclude that the thymic medulla in fetal thymus is not involved in the development of $\alpha\beta$ T cells; a role of this compartment in the development of $\gamma\delta$ T cells remains to be established. Likewise, a role for the adult thymic medulla in the development of mature $\alpha\beta$ T cells is also not clear (19,45,46).

In this study we analyzed the development of the cortical and medullary microenvironments in cytokine treated organ cultures, as compared to control FTOC and neonatal thymus. Analysis of the IL-4 treated culture showed that the transient wave of normal T cell development is accompanied by 'normal' development of thymic microenvironments, as in control FTOC. The IL-4 treated culture can therefore be considered as a 'cytokine' control FTOC in our study. At the end of the culture period, when $CD4^+8^+$ cells are not present anymore, the IL-4 treated culture shows, even more explicitly than control culture, that mature T cells present in fetal thymus do not induce or maintain a proper thymic medulla. In addition, our results demonstrate that

IL-4 affects T cell development but not the (development of) thymic stromal cells.

In contrast to control and IL-4 treated FTOC, we observed expansion of medullary TEC in FTOC treated with high dosis of IL-2. Medullary TEC became the major TEC population, but were not organized in clearly defined medullary regions. Cortical TEC on the other hand, were almost completely absent under these culture conditions. The disorganization of the thymic stroma, caused by IL-2 might be explained in different ways. First, IL-2 directly affects the development and/or function of thymic stromal cells. This explanation requires the expression of IL-2R by thymic stromal cells, but its presence on the surface of thymic stromal cells has not yet been reported. Secondly, the effect of IL-2 could be indirect: changes in thymic microenvironments are caused by IL-2 induced changes in T cell development. It has been shown that the IL-2/IL-2R pathway is essential for T cell development, as treatment with IL-2 or anti-IL-2R mAb blocks $\alpha\beta$ T cell development at an $CD4^-8^-3^-$ stage (7-15). However, the absence of $\alpha\beta$ T cell development can not explain why the cortical TEC did not develop, since cortical TEC are normally present in day 14/15 fetal thymus and SCID thymus, that also contain only $CD4^-8^-3^-$ cells. Moreover, the purpose of an expansion of medullary TEC remains elusive, since we stated above that these cells are not essential for $\alpha\beta$ and $\gamma\delta$ T cell development in FTOC. Thus, we could not link the block in T cell development to changes in thymic microenvironments. Therefore, we favor a third explanation that $Fc\gamma RII^+$ $CD4^-8^-3^-$ cells, preferentially expanding within our IL-2 treated organ culture (16), are responsible for the observed stromal disorganization. This cell type also developed in suspension cultures of day 14/15 fetal thymocytes, stimulated with IL-2 (49), indicating that the development of $Fc\gamma RII^+$ $CD4^-8^-3^-$ cells does not depend on interaction with thymic microenvironments. A variety of cytokines, such as IFN-γ, TNF, and several CSFs, are produced by NK cells (50) and could be possible mediators of the observed changes in thymic microenvironments. However, the precise inductive mechanism remains to be eludicated.

The strong reduction of $ER\text{-}TR4^+$ cells, observed in the IL-2 treated cultures, does not necessarily indicate the absence of cortical TEC. It could also mean that only the ER-TR4 antigen was progressively lost, whereas the cortical TEC were still present. In *Ha-Ras* transgenic mice, a cortical and a medullary epithelial marker were lost, whereas other epithelial cell-specific mAb still recognized their respective cell type (51). Therefore, changes in thymic organization and T cell development can also, probably through a disturbed cytokine balance, affect the expression of specific cell markers.

In conclusion our data show the disorganization of the thymic stroma in IL-2 treated fetal thymus is correlated with (and probably caused by) the expansion of $Fc\gamma RII^+$ $CD4^-8^-3^-$ cells. Cytokines released by these cells probably mediate the thymic disorganization by disturbing the delicate cytokine balance within different compartments in the thymus.

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CHAPTER 7

Characterization of murine thymic microenvironments by novel anti-stromal antibodies*

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Summary

Monoclonal antibodies were developed against mouse thymic stromal cells, using the anti-idiotype strategy. Rats were immunized with a rat mAb recognizing an adhesion molecule on the thymocyte surface, for which a ligand on thymic stromal cells (or the extracellular matrix) had not yet been identified. Using this strategy, anti-idiotype mAb can be developed that recognize a functional epitope on the ligand, involved with adhesion of thymocytes. In this study, the immunogen was a mAb against phagocytic glycoprotein 1 (Pgp-1), a molecule expressed by thymus-homing cells and the earliest prothymocytes within the thymus.

After the presence of anti-idiotype activity in the serum of immunized rats was established, hybridomas were obtained producing mAb reactive with thymic stromal cells. Although a true anti-idiotypic nature of these mAb could not be proven, five of these mAb exhibited unique staining patterns of thymic tissue. Four mAb, ER-TR10 to ER-TR13, reacted only with thymic stromal cells in new staining patterns. The fifth mAb, ER-TR14 was not reactive with thymic stromal cells. Instead, it stained medullary thymocytes and peripheral T lymphocytes with a striking focal cytoplasmic staining pattern.

Introduction

The thymus is primarily responsible for the generation of functionally competent T lymphocytes. Cell-cell interactions and locally secreted cytokines mediate the intercellular signalling between thymic non-lymphoid (stromal) cells, i.e. epithelial cells, macrophages and interdigitating cells and the developing thymocytes. The anatomical distribution of lymphoid and stromal cells indicates that different thymic stromal cells promote different stages of T cell differentiation (1-5). Stromal cell functions, like positive and negative selection of developing thymocytes are described to resp. cortical epithelial cells and interdigitating cells (6-9). However, the specific role of each stromal cell type in the promotion of sequential stages of T cell development is still poorly understood. Over the last years, newly developed mAb against thymic stromal cells were developed, revealing a complex phenotypic heterogeneity of thymic microenvironments.

The first published monoclonal antibodies (mAb) against thymic epithelial cells distinguished cortical epithelial cells, recognized by ER-TR4, from medullary epithelial cells, recognized by ER-TR5 (10). Since then, many mAb have been raised against thymic epithelial cells (TEC), revealing a considerable heterogeneity within the epithelial component of the thymus (11-23). The staining patterns of these mAb against TEC

were compared to each other and mAb with identical patterns grouped in Clusters of Thymic Epithelial Staining (CTES; ref. 24). Recently, analysis of TEC mAb focused more on identification of the molecules recognized by these antibodies (19,21-23,25). Some of the TEC molecules were clearly not directly involved with T cell development, because they were cytoplasmically expressed, e.g. cytokeratins (25). Other mAb, able to interfere with T cell development in fetal thymus organ culture (26), recognized not only TEC but also most thymocytes (17,21,22). However, proper functional characterization of most epitopes recognized by the anti-stromal antibodies is still lacking.

We applied a new strategy to the development of mAb against *functional* epitopes on the surface of TEC, namely the *anti-idiotype strategy* (27). The concept of this particular strategy is relatively simple: a monoclonal antibody against a functional molecule on the surface of thymocytes ("Ab1"), for which the ligand has not yet been identified, is used as an immunogen to develop anti-idiotype monoclonal antibodies ("Ab2"). By virtue of the molecular resemblance to the thymocyte molecule, these anti-idiotype mAb should be able to identify the putative ligand on the thymic stromal cells. We used this strategy to identify a putative ligand for phagocytic glycoprotein 1 (Pgp-1 or CD44) within the thymus. Pgp-1 is a cell adhesion molecule, widely distributed on a diverse range of cells, that may have multiple ligands (28,29). Possible ligands include vascular addressins as well as extracellular matrix proteins (collagens, fibronectin). Recently, it was shown that Pgp-1 has a function in binding hyaluronic acid (30-32). The presence of Pgp-1 on the surface of thymus-homing cells and the earliest prothymocytes suggests that interaction between Pgp-1 and its ligand is important for entering the thymus and/or the early phase of T cell development within the thymus (28,29,33-36). Together, these data indicate that the putative ligand for Pgp-1 might be present on endothelial cells, stromal cells, or extracellular matrix proteins within the thymus.

In this manuscript we describe the staining patterns of 5 new monoclonal antibodies. Four mAb define new thymic epithelial subpopulations, further extending the complex phenotypic heterogeneity of thymic stromal cells. One mAb recognizes an antigen, focally expressed in the cytoplasm of medullary thymocytes and peripheral T lymphocytes.

Materials and methods

Animals

Male and female BALB/c (H-2^d) mice were bred and maintained in the animal facilities of our department. Female Louvain (Lou/C) rats were obtained from the Central Institute for Laboratory Animal Breeding, Hannover, Germany.

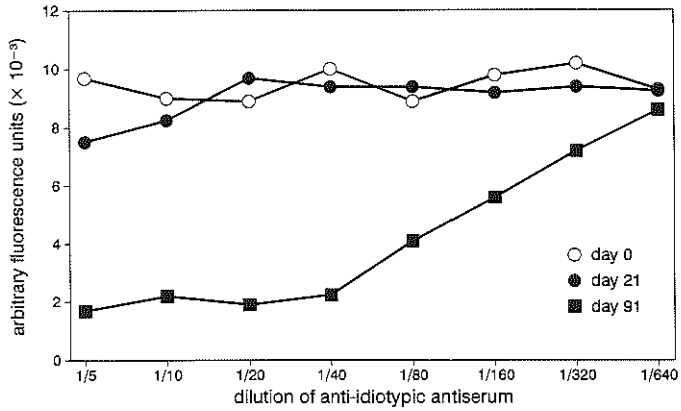

Figure 1. Anti-idiotype activity in rat serum, determined with competetive immunofluorescence assay. Sera from the same rat were used, before immunization (day 0), after the standard protocol of immunization (day 21), or after prolonged weekly immunizations (day 91). Anti-idiotype antibodies, present in day 91 serum, inhibit the binding of anti-Pgp-1 to the BW5147 cell line; serum of day 0 and day 21 does not contain anti-idiotype antibodies.

Immunization strategy

The anti-Pgp-1 mAb was purified from culture supernatant of the rat hybridoma IM7.8.1 (33) by ion exchange chromatography with Bakerbond™ ABx (Baker, Deventer, the Netherlands), using the manufacturers protocol. This method always yielded pure antibodies, i.e. only heavy and light chains were detected by SDS-PAGE. The purified mAb was coupled to Keyhole Limpet Hemocyanin (KLH; Pierce, Rockford, IL, USA) with glutaraldehyde (37).

Lou/C rats were immunized using a 'hyperimmunization' protocol (27; C. Demanet, personal communication). On day 0, rats were i.p. injected with a mixture of anti-Pgp-1-KLH (equivalent to 50 μg Ig) and complete Freund's adjuvant (CFA; Difco, Detroit, MI), on day 7 i.p. with the same amount of anti-Pgp-1-KLH, mixed with incomplete Freund's adjuvant (IFA; Difco) and on day 14 they were given an i.v. boost with anti-Pgp-1-KLH (50 μg) in saline, clarified by centrifugation. This standard protocol was for most cases extended by weekly injections with anti-Pgp-1-KLH/IFA. Fusion was performed three days after the intravenous boost.

Generation of rat x mouse heterohybridomas

Rat spleen cells were fused with the mouse myeloma Sp2/0 (38), according to a protocol recently described by Leenen at al (39). Briefly, spleen cells and Sp2/0 cells were mixed in a ratio of 2 to 1 and washed three times with serum-free RPMI-1640. After the last wash, the cell mixture was carefully resuspended in 0,5 ml of 71% polyethylene glycol (PEG-4000; Merck, Darmstadt, FRG) at 37°C. After 1 min at 37°C the PEG was gradually diluted with 50 ml of pre-warmed serum-free RPMI. Next, the cells were centrifuged (10 min, 70g, room temperature), gently resuspended in DMEM (α-modification) supplemented with 10% FCS, HGF (40 U IL-6/ml), 2-mercaptoethanol (5.10^{-5} M), hypoxanthine (10^{-4} M), azaserine (1 μg/ml) and antibiotics, and plated at 4.10^5 cells per 24-well and/or 8.10^4 cells per 96-well culture plates. After screening for anti-idiotype activity and reactivity with thymus frozen sections, selected hybridomas were subcloned by limiting dilution. The subclasses of the rat immunoglobulins were determined with an agglutination assay from Nordic (Tilburg, The Netherlands) using erythrocytes coated with rat Ig subclass specific antibodies. The monoclonal antibodies were developed to identify

new epitopes on thymic stromal cells, and therefore received the code ER-TR, Erasmus University Rotterdam - Thymic Reticulum. We have already reported on 9 ER-TR mAb (10,40).

Detection of anti-idiotype activity

Two methods were employed to determine whether serum of immunized rats and hybridoma culture supernatants contained anti-idiotype mAb. In the first method, a competetive immunofluorescence assay (CIFA), we analyzed the inhibitory effects of serum and supernatants on the binding of anti-Pgp-1 mAb to the Pgp-1$^+$ cell line BW5147 (41), using an ELISA procedure described by van Soest et al. (42) and modified by Leenen et al. (43). Briefly, Terasaki plates were coated with a monolayer of BW5147 cells, fixed with 0.05% glutaraldehyde (25 min, 4 °C), and stored in PBS/0.02% gelatin/0.02M sodium azide. Hybridoma supernatants and serum were preincubated for 30 min with anti-Pgp-1-biotin (room temperature), using a suboptimal saturating dosis. Next, the fixed BW5147 cells were incubated with this mixture for 30 min, washed, and subsequently incubated with streptavidin-β-galactosidase. The enzyme converted 4-methyl-umbelli-ferryl-β-galactopyranoside (MUF-G) into MUF and its fluorescence, induced by UV irradiation, was measured using a scanning microfluorimeter. The positive and negative control contained only resp. no anti-Pgp-1-biotin in the first incubation step.

In the second method we used a functional assay, based on the induction of homotypic aggregation of BW5147 cells by the anti-Pgp-1 mAb IM7.8.1, according to Belitsos et al. (44). BW5147 cells (2×10^5) in 0.1 ml DMEM, supplemented with 10% FCS, 2-mercaptoethanol (5×10^{-5} M) and penicillin-streptomycin antibiotics at recommended concentrations, were distributed in Falcon 3072 flat-bottomed microtiter plates (Becton-Dickinson, Oxnard, CA) in the presence of mAb IM7.8.1 at titerpoint concentration with or without antiserum (1:100) or hybridoma culture supernatant (undiluted) to be tested for anti-idiotypic activity. After incubation for 16h at 37°C in a 5% CO_2 incubator, the degree of cell aggregation was estimated in a range of 0-5 as described before (45).

Immunohistology

Immunohistology was performed on cryostat tissue sections of adult thymus, spleen and mesenteric lymph node, essentially as described before (46). Briefly, frozen sections of the different organs were incubated with neat culture supernatants of the ER-TR hybridomas or with PBS and developed with a peroxidase conjugate of rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark).

Flow cytometric analysis

For flow cytometric analysis, thymocyte, spleen cell and lymph node cell suspensions were prepared in PBS containing 0.5% BSA and 2 mM sodium azide. 10^5 cells (in a volume of 10-20 μl) were incubated on ice for 30 min with 25 μl supernatant of the Ab2 hybridoma. After three washes with the PBS-BSA-NaN3 buffer, cells were further incubated for 30 min on ice with a FITC conjugate of rabbit anti-rat IgG ($F(ab')_2$-fragments; Cappel/Organon Technika, Oss, the Netherlands). Finally, cells were again washed three times and collected in a small volume for flowcytometric analysis. For cytoplasmic detection of antigens, thymocytes were first fixed with 2% paraformaldehyde (10 min, 4°C) and permeabilised with methanol (20 min, 4°C), before entering the staining protocol.

Samples were analyzed for light scatter and fluorescence on a FACScan (Becton Dickinson, Mountain View, CA, USA), equipped with a 488 nm argon laser and interfaced to a Hewlett-Packard computer running the FACScan software. Calibration of the cytometer was performed by eye, using thymocytes that were unlabeled, labeled with CD8-FITC or labeled with CD4-R-Phycoerythrin. Forward light scatter and perpendicular light scatter were analyzed with linear amplification, while all three fluorescence channels were subject to logarithmic amplification. In most cases 10,000 cells were analyzed. Dead cells were excluded during data analysis on the basis of forward light scatter and PI-staining. All data were analyzed using the FACScan or Paint-a-Gate software (Becton Dickinson, Mountain View, CA, USA). Data are presented in this paper as one-parameter histograms with a four log-decade fluorescence scale.

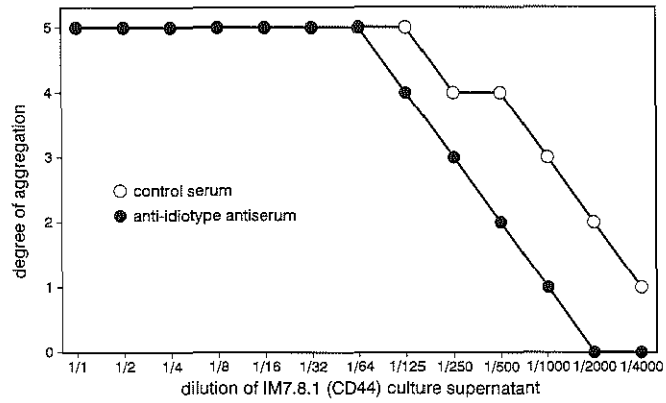

Figure 2. Cellular aggregation of BW5147 cells induced by the CD44 mAb IM7.8.1 and its inhibition with anti-idiotypic serum. The degree of cell aggregation, induced by different concentrations of IM7.8.1, was estimated in a range of 0-5 (45). Cultures were supplemented with either control rat serum (1:100) or an anti-idiotypic rat antiserum (1:100). The latter antiserum inhibits mAb IM7.8.1 induced aggregation of BW5147 cells.

Results

The anti-idiotypic approach

In this study we aimed at preparing monoclonal antibodies against functional molecules on the surface of thymic stromal cells. To that purpose we employed the anti-idiotype strategy, where rats were immunized with the rat mAb IM7.8.1 against Pgp-1 (33). This antibody was originally derived from splenocytes of a rat immunized with the murine M1 myeloid cell line (33). The BW5147 cell line was used as ligand for IM7.8.1 in a competitive immunofluorescence assay (CIFA) in order to detect anti-idiotypic activity in Ab2 antisera and hybridoma culture supernatants. Anti-idiotypic activity was not detected after immunization using the standard protocol (day 21 in Fig. 1), but only after prolonged weekly immunizations (day 91 in Fig. 1). Antisera which thus exhibited anti-idiotypic activity could also inhibit mAb IM7.8.1 induced aggregation of BW5147 cells (Fig. 2). This functional assay should in principle be able to discern Ab2 activity (inhibition) from autologously elicited Ab3 activity (stimulation), that both would have registered as inhibition of fluorescence in CIFA.

One of the rats, developing anti-idiotype activity, was used for preparing hybridomas. Hybridoma supernatants were screened for anti-idiotypic activity using CIFA, cell aggregation and immunohistochemical staining of frozen sections of adult thymus. Although several mAb exhibited immunohistochemical thymic stromal staining activity, none of these behaved as authentic anti-idiotypic Ab2 or Ab3 in CIFA and cell

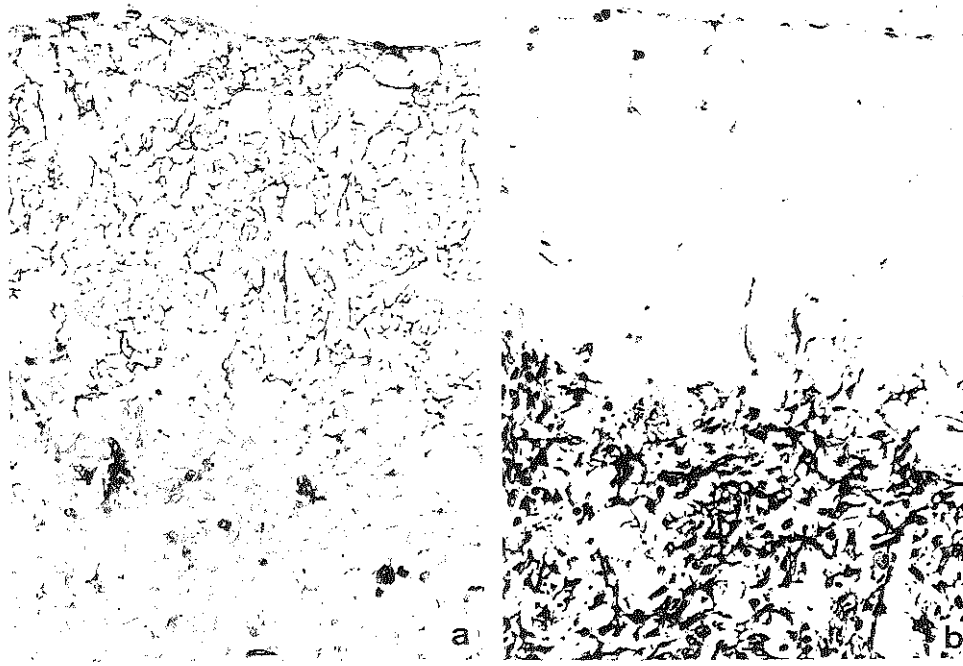

Figure 3. Thymic microenvironments in normal adult BALB/c thymus. Frozen sections were stained with the mAb ER-TR4 (3a), against cortical epithelial cells, and ER-TR5 (3b), against medullary epithelial cells. Magnification: x 140.

aggregation (data not shown). However, five new mAb showing unique thymic staining patterns could be identified and are described in this paper. The staining patterns of the new mAb were compared with the staining patterns of established epithelial cell markers, like ER-TR4 and ER-TR5 (10). Both these mAb recognize the majority of the epithelial cells present resp. in the cortex and the medulla (Fig. 3a and 3b). By comparison, we were able to determine the epithelial nature of the stromal cells and their respective numbers in the sections stained with the new mAb. With this information we could establish the classification of the mAb according to the CTES (Clusters of Thymic Epithelial Staining) nomenclature (3,24). This nomenclature defines five main thymic epithelial staining patterns, numbered I to V. Antibodies that do not fit into the main five staining patterns were temporarily grouped in cluster XX. The thymic epithelial staining pattern, as well as the non-epithelial staining, the CTES classification, and the immunoglobulin isotype of the five mAb are summarized in table 1.

Table 1. Monoclonal antibodies against thymic epithelial cells

clone	isotype	CTES[a]	Staining pattern[b]			
			sc/pv	cort	med	non-epithelial
ER-TR10	IgM	III.B	-	+	s	-
ER-TR11	nd	XX	s	s	+	-
ER-TR12	nd	XX	s	s	+	-
ER-TR13	IgM	III.A	-	+	-	-
ER-TR14	IgM	-	-	-	-	medullary thymocytes endothelial cells

[a] MAb against epithelial cells were classified according to the CTES nomenclature.
[b] Subpopulations of TEC identified in the staining patterns included subcapsular/perivascular TEC (sc/pv), cortical TEC (cort), and medullary TEC (med). -, s, + indicate that no, a subpopulation of, a majority of TEC (≥75%) were stained.

Thymic epithelial markers

ER-TR10 (Fig. 4a) stained the majority of the epithelial cells in the cortex, in a similar reticular staining pattern as ER-TR4. However, ER-TR10 stained a subpopulation of medullary stromal cells, differing in numbers and morphology from the small medullary subpopulation stained by ER-TR4 (compare with figure 3a). The stromal cells in the medulla showed long cytoplasmic extensions and differed in morphology from the ER-TR5$^+$ medullary epithelial cells (compare with Fig. 3b). ER-TR10 did not stain the subcapsular epithelial cells. The general staining pattern identifies ER-TR10 as a CTES III.B mAb (3,24). As ER-TR4 was also determined as a CTES III.B mAb, it is clear that even a CTES subgroup is still heterogenous. ER-TR10 did not stain sections of spleen and mesenteric lymph node (data not shown).

ER-TR11 and **ER-TR12** (Fig. 4b and 4c) displayed comparable staining patterns. They both stained the majority of medullary TEC, and subpopulations of cortical and subcapsular TEC. However, ER-TR12 detected more cortical TEC, with a higher intensity compared to ER-TR11, indicating that subpopulations of cortical TEC can be identified by differential staining with ER-TR11 and/or ER-TR12. The staining patterns of these mAb do not fit into the five main staining patterns defined by the CTES nomenclature and, therefore, they are classified as cluster XX mAb. No staining could be observed on sections of spleen and mesenteric lymph node (data not shown).

ER-TR13 (Fig. 5) stained the majority of cortical TEC but not medullary or subcapsular TEC. The cortical staining pattern was not uniform, as in some areas of the cortex only a minority of TEC stained with ER-TR13 (data not shown). Interestingly, ER-TR13 displayed more intense staining of cortical epithelial cells at the cortico-medullary junction compared to the outer cortex (Fig. 5b). Simultaneous staining with ER-TR5 indicated that these cells marked the border between the cortex and the

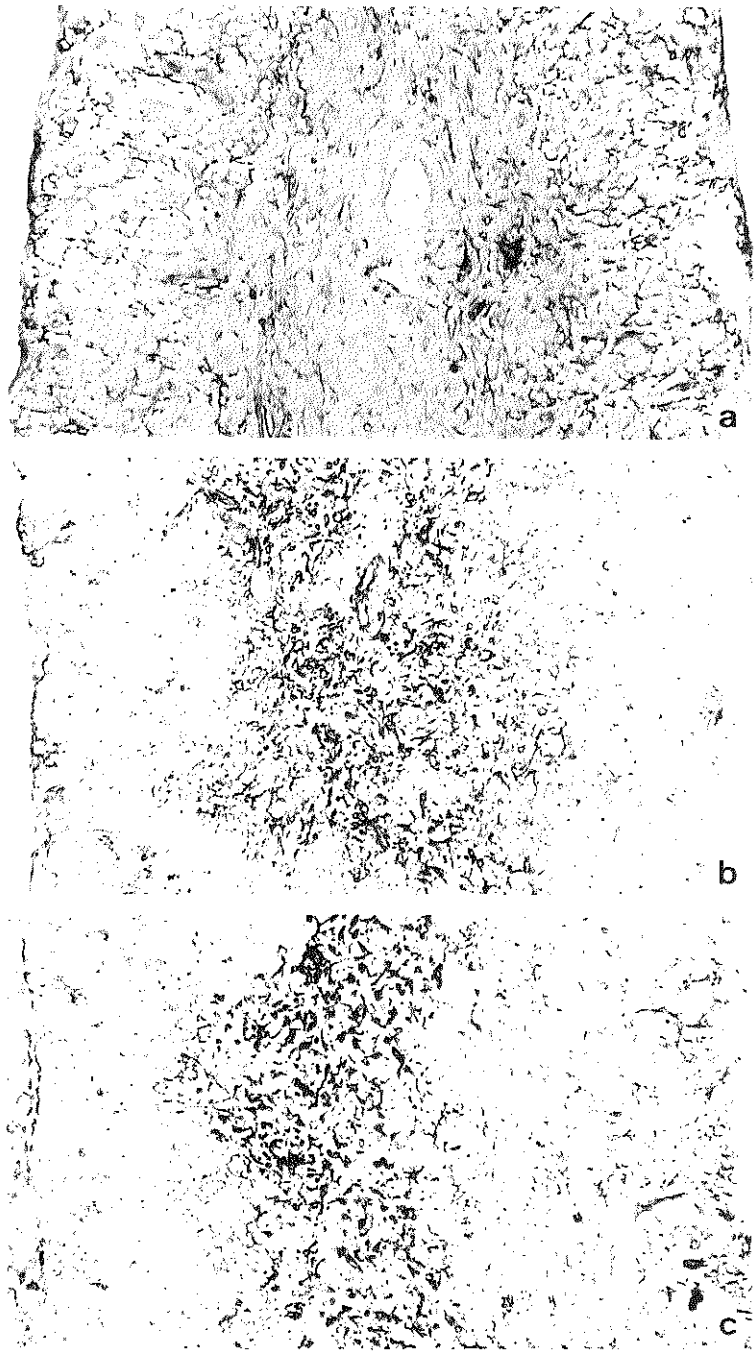

Figure 4. Thymic microenvironments characterized by new mAb against epithelial cells. Frozen sections of adult BALB/c thymus were stained with the mAb ER-TR10 (4a), ER-TR11 (4b), and ER-TR12 (4c). In each section, the medulla (in the center) is on both sites flanked by cortex. Magnification: x 100.

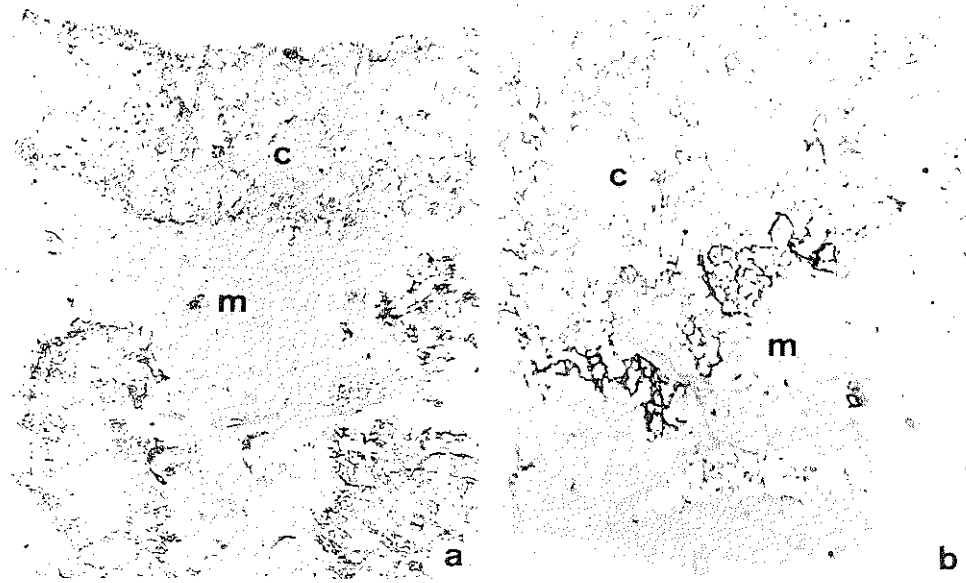

Figure 5. Thymic microenvironment characterized by the mAb ER-TR13. Frozen sections of adult BALB/c thymus were stained with the mAb ER-TR13. Abbreviations: c, cortex; m, medulla. Magnification: x 60 (a), x 140 (b).

medulla, i.e. the cortico-medullary junction (data not shown). This antibody was classified as a CTES III.A mAb. No staining could be observed on sections of spleen and mesenteric lymph node (data not shown).

Our results demonstrate the heterogeneity of cortical TEC, as the four anti-stromal mAb, presented here, all have different staining patterns with epithelial cells in the cortex. In addition, a new subpopulation of medullary stromal cells is defined by ER-TR10.

Non-epithelial thymic marker

ER-TR14 (Fig. 6a) did not react with thymic epithelial cells, but instead stained medullary thymocytes as well as endothelial cells lining the blood vessels and capillaries (in cortex and medulla; Fig. 6a). The staining of the thymocytes was focal (*inset* of Fig. 6a), resembling "caps". However, flow cytometric analysis of thymocytes with ER-TR14 did not reveal any surface staining above background (data not shown). Instead, staining of fixed thymocytes showed that the mAb reacts with a cytoplasmic molecule (Fig. 7). Around 30% of adult thymocytes were stained with the mAb (Fig. 7C), exceeding the normal number of medullary thymocytes (12-15%; ref. 47).

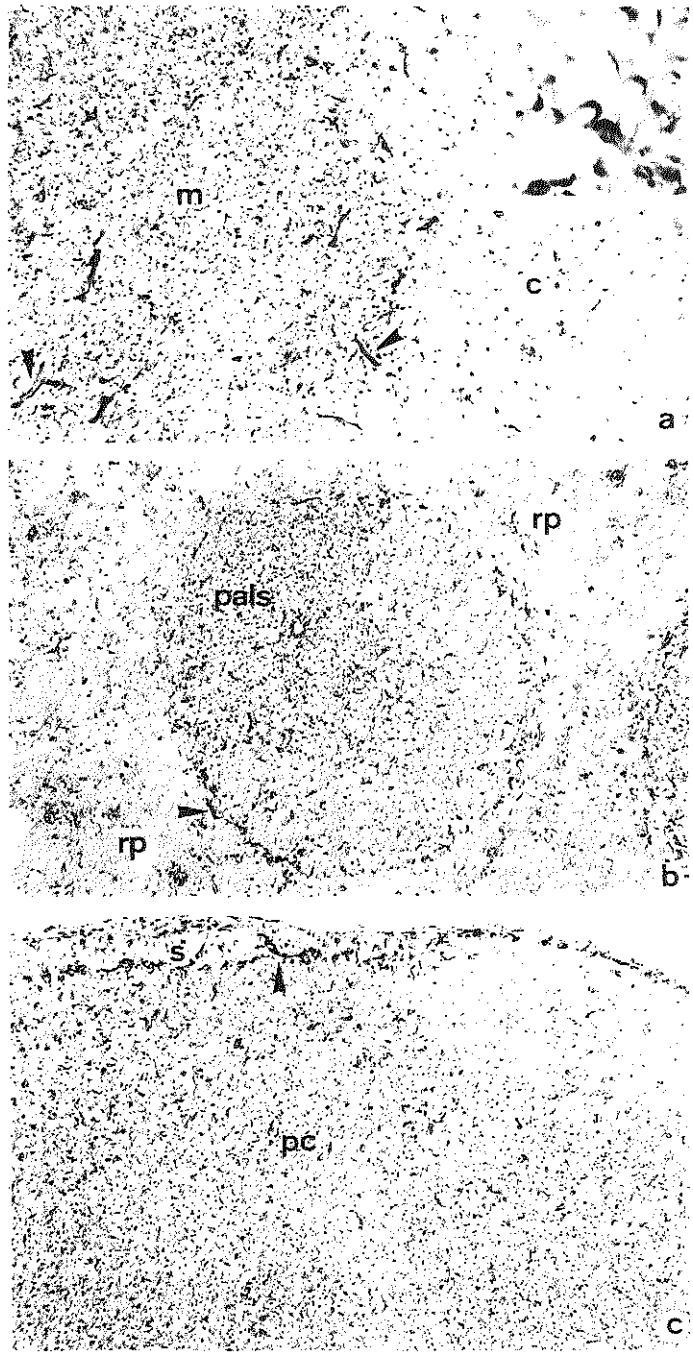

Figure 6. Lymphoid organs stained with the mAb ER-TR14. Frozen sections of adult BALB/c thymus (6a), spleen (6b), and mesenteric lymph node (6c) were stained with the mAb ER-TR14. The inset in figure 6a shows the focal ("caps") staining pattern observed on medullary thymocytes. Abbreviations: c, cortex; m, medulla; pals, peri-arteriolar sheath; rp, red pulp; pc, paracortex; s, subcapsular marginal sinus. ER-TR14$^+$ endothelial cells in the lymphoid organs are marked by arrowheads. Magnification: x 90 (inset: x 600).

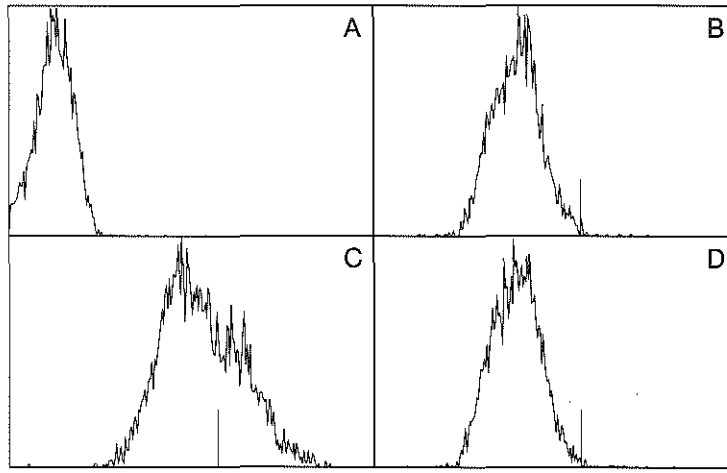

Figure 7. Flowcytometric detection of the ER-TR14 antigen in the cytoplasm of the thymocytes. Paraformaldehyde-fixed thymocytes were stained with PBS (7b) or the mAb ER-TR14 (7c) and ER-TR13 (7d), followed by FITC-conjugated anti-rat mAb. For comparison, the fluorescence signal of unfixed, unstained thymocytes is shown (7a).

However, this percentage can be an overestimation caused by background staining, due to fixation (compare Fig. 7A and 7B) and staining with an IgM mAb (observe the peak of negative cells for two IgM mAb in Fig. 7C and 7D).

In peripheral lymphoid organs, T lymhocytes present in the PALS of the spleen (Fig. 6b) and in the paracortex of the lymph node (Fig. 6c), stained with a similar focal staining pattern as observed for medullary thymocytes. Weak focal staining could also be observed on a subpopulation of B-lymphocytes in spleen and, to a lesser extent, in lymph node. Endothelial cells in spleen and lymph node, like in thymus, also clearly reacted with ER-TR14 mAb. In the spleen, endothelial cells of and/or close to the marginal sinus (surrounding the white pulp) were positive (Fig. 6b). In the lymph node, reactivity with endothelial cells of the subcapsular marginal sinus (Fig. 6c), the high-endothelial venules, and the medullary sinus was detected (data not shown). These results indicate the existence of cross-reactive antigenic epitopes in mature T cells and endothelial cells.

Discussion

The anti-idiotypic approach towards defining ligand-receptor pairs has been successfully pursued in numerous applications (reviewed in ref. 27,48), ever since

Sege and Peterson (49) illustrated the insulin receptor binding properties of anti-anti-insulin antibodies. When one member of a ligand-receptor pair remains unidentified, as is the case here for the putative CD44 receptor on thymic stromal cells, it is no trivial matter to prove the true anti-idiotypic nature of the antibodies, derived from Ab1 immunized animals, that react with the proposed target cells. Although hyaluronic acid has clearly been shown to be a receptor (ligand) for CD44 in various organs (32,50,51) it appears unlikely that mAb IM7.8.1 recognizes the hyaluronic acid binding site on CD44: the murine M1 myeloid cell line, used as immunogen to elicit the IM7.8.1 antibody specificity in rats, expressed CD44, being at the same time uncapable of binding hyaluronic acid (30). Monoclonal antibody IM7.8.1 binds CD44 on both hyaluronic acid binding (BW5147, see ref. 30) and non-binding (M1 myeloid) cells. Moreover, Miyake et al. (31) showed that IM7.8.1 was likely to bind near to, but not into the hyaluronic acid binding site. As mAb IM7.8.1 stains lymphoid cells in both the cortex and medulla of the thymus, it appeared likely that anti-idiotypic antibodies should recognize mainly thymic stromal cells (e.g. ER-TR10-13 type staining patterns). It cannot be excluded, however, that non-hyaluronic acid-CD44 receptor may also occur on lymphoid cells, giving rise to antibodies with the ER-TR14 type staining pattern. Although no direct evidence could be presented to prove the anti-idiotypic nature of mAb ER-TR10-14, neither can it be excluded that these antibodies do not bear relation to the CD44 ligand-receptor pair. As the anti-idiotypic activity of antiserum could only be demonstrated at titers of 100 or less in both CIFA (Fig. 1) and cell aggregation assay (Fig. 2), the possibility exists of low affinity anti-idiotypic antibodies (52). As hybridoma culture supernatants contain antibodies at concentrations approximately 3 orders of magnitude lower than antiserum, both CIFA and cell aggregation assays may not have sensitivity enough to illustrate anti-idiotypic inhibition.

Irrespective of whether a relationship can still be shown between CD44 and the new antibodies ER-TR10 to ER-TR14, we believe that these mAb may contribute to elucidating the complex interrelationships between thymic stromal cells and the developing thymocyte, due to their unique staining pattern.

Of the five mAb, presented in this paper, two displayed thymic epithelial staining patterns that could not be classified according to the CTES nomenclature (24,25). These mAb, ER-TR11 and ER-TR12 that stain all medullary TEC and subpopulations of cortical and perivascular TEC, are grouped in cluster XX. Moreover, their staining patterns are new to this cluster: they do not fit in with the existing CTES XX subsets and they do not stain thymocytes as most CTES XX mAb do (17,21,22,24,25,53). Nevertheless, they resemble other CTES XX mAb in the apparent absence of staining distinct thymic compartments. They stain TEC of the subcapsular, cortical as well as medullary compartment, but in different proportions. Such a variable expression of a stromal cell-associated surface molecule does not contradict a function for this

molecule at a distinct stage of T cell development. For example, it has been shown that ICAM-1, a ligand for LFA-1 expressed by most thymocytes, is expressed by subcapsular and cortical TEC as well as isolated stromal elements in the medulla and at the cortico-medullary junction (54-56). Nevertheless, mAb against LFA-1 as well as ICAM-1 result in the impaired generation of $CD4^+8^+$ thymocytes (57). Thus, identification of the molecules recognized by CTES XX molecules is essential for understanding their function in T cell development.

The mAb ER-TR13, defined as a CTES III.A mAb, displayed a new staining pattern, not observed before. The intense staining of the innermost cortical epithelial cell layer, lining the cortico-medullary junction, is suggestive of a specialized microenvironment for T cell development. In this cortico-medullary region thymocytes are probably at a late $CD4^+8^+$ stage, close to developing into mature single positive thymocytes. It could well be that this transition is supported by these deep cortical TEC. On the nature of the ER-TR13 antigen can only be speculated: a variety of molecules is expressed by thymic stromal cells, including (1) adhesion molecules, e.g. ICAM-1 (discussed above); (2) receptors for soluble factors: cytokines and neuroendocrine hormones influence TEC function (58,59); (3) ectoenzymes (60). It can be imagined that TEC of the deep cortex express an adhesion molecule, necessary for specific interaction with $CD4^+8^+$ thymocytes, or a receptor for a soluble factor that will induce specialized TEC function, essential to distinct stages of T cell development in this microenvironment. The specialized function of the TEC could also be mediated by ectoenzymes, a new category of surface molecules (60). These enzymes not only serve as activation signal-transducing molecules or adhesion molecules, but can also use their digestive activity in regulating biologically active peptides (60). In this manner, TEC can activate a soluble factor essential for maturing cells in this local microenvironment. Several peptidases have now been identified on the surface of thymic cells, for example, aminopeptidase-A (identified by mAb 6C3) on cortical epithelial cells (15,61) and dipeptidyl peptidase IV (THAM, CD26) on $CD4^-8^-$ thymocytes (62,63).

The mAb ER-TR10 is identified as a CTES III.B mAb by staining a majority of cortical TEC and a subpopulation of medullary TEC. The population of medullary TEC stained by ER-TR10 differs in morphology and number from the few medullary TEC stained by the cortical TEC marker ER-TR4, the medullary marker ER-TR5 and other medullary TEC-specific mAb (64). This indicates the presence of a new medullary TEC subpopulation. Other medullary TEC subpopulations have been defined by expression of MHC molecules: cells express either conventional MHC class I and II molecules or a new class of MHC molecules, termed H-2O (64-66). Thus, phenotypically different microenvironments of medullary TEC can be identified. However, a function for these microenvironments as well as a function for the medullary compartment as a whole is not well established. A recent study shows that only MHC class II^+ cortical TEC and

mesenchymal cells are essential for proper T cell development (67), whereas other studies indicate that the development of medullary TEC is dependent on the presence of mature TcR^+ cells (68,69). Identification of medullary TEC-specific molecules would greatly facilitate our understanding of this compartment and of microenvironments within.

The mAb ER-TR14 is not a TEC specific marker, instead it recognizes medullary thymocytes and peripheral T cells. This indicates that this mAb is a marker for mature cells of the T cell lineage. Its focal staining pattern, with caps, suggests a surface molecule, but instead we observed only cytoplasmic staining. Such a focal cytoplasmic staining pattern has been described for the enzyme acid α-naphthyl acetate esterase (ANAE), that defines the mature stages of the human T cell lineage (70). It suggests that the ER-TR14 antigen can be a cytoplasmic enzyme. A dot-like cytoplasmic staining pattern of medullary thymocytes and peripheral T cells was also observed with a CD21 mAb, recognizing the complement receptor CR2 (71). In the latter study it was shown that the dotlike staining localized to the Golgi region. Thus, the ER-TR14 antigen probably localizes to the Golgi region of mature T cells; an enzyme or a CR2 related peptide are possible candidates.

The ER-TR14 antigen is also weakly expressed by peripheral B cells, and by vascular endothelium in thymus, lymph node and spleen. The expression of the antigen by mature T cells as well as endothelial cells is suggestive of a molecule involved with cell adhesion (72), but remains questionable as we were not able to detect surface expression of the ER-TR14 antigen. Rather, the staining of both cell populations indicates the existence of cross-reactive antigenic epitopes in mature T cells and endothelial cells.

This paper shows that thymic stroma is more complex in nature than sofar thought: still new thymic microenvironments can be defined. Further characterization of the different stromal cell types, constituting these microenvironments, and their molecules will indicate whether they are functional subpopulations capable of supporting a distinct stage of T cell development.

Acknowledgements

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CHAPTER 8

General discussion

The thymus is the primary lymphoid organ that supports the maturation and differentiation of T lymphocytes. Precursor T lymphocytes enter the thymus from the bloodstream and will, through a complex multi-step process, develop into mature effector T cells. This process can be monitored by analyzing surface expression of the TcR/CD3 complex and its coreceptors CD4 and CD8 on the developing thymocyte. Thus, phenotypically distinct thymocyte subpopulations have been identified and a lineage relationship between these subsets is well established (see fig. 2 in chapter 1; ref. 1,2). In this multi-step process, several control points have been identified: (1) entry into the thymus, (2) induction of proliferation, (3) induction of differentiation into different lineages, (4) positive and negative selection, and (5) the exit out of the thymus. As the developing T cell migrates through the thymus (3,4), different thymic stromal cells, i.e. epithelial cells (TEC), interdigitating cells (IDC) and macrophages (Mø), govern these control points. During their development, thymocytes express various surface molecules that bind either ligands present on these stromal cells (cellular interaction) or cytokines produced by the stromal cells (humoral interaction). Thus, microenvironments are created, where thymocytes receive short range, maturation-inducing signals from the stromal cells. In this respect, it has been shown that perivascular TEC attract precursor T cells from the bloodstream, cortical macrophages may induce proliferation, TEC in the subcapsular region of the thymus probably induce differentiation of the different lineages, and positive and negative selection are induced by cortical TEC and IDC, respectively. In figure 3 of chapter 1, the available evidence on the specific role of the various stromal cells in promoting sequential stages of T cell development has been combined in a model of T cell differentiation.

The studies presented in this thesis are concerned with the different control points that occur along the pathway of T cell development. The results of these studies will be discussed accordingly.

Entry of precursor T cells into the thymus involves attachment to the endothelium of the thymic blood vessels and subsequent migration through the endothelial layer as well as the perivascular epithelial layer into the surrounding thymic tissue. It has been shown that entry of precursor T cells into the thymus can be inhibited by Mab against phagocytic glycoprotein-1 (Pgp-1 or CD44; ref. 5,6). Pgp-1 is a cell adhesion molecule that may have multiple ligands. Possible ligands include vascular addressins as well as extracellular matrix proteins (e.g. collagens, fibronectin). Recently, it was shown that Pgp-1 has a function in binding hyaluronic acid (7). Pgp-1 is present on the surface of thymus-homing cells and the earliest prothymocytes, suggesting that the putative ligand for Pgp-1 might be present on endothelial cells, stromal cells, or extracellular matrix proteins within the thymus. To identify the putative ligand for phagocytic glycoprotein-1 (Pgp-1 or CD44) within the thymus, we employed the *anti-*

idiotype strategy (chapter 7). Anti-idiotype mAb, recognizing the Pgp-1 ligand, are raised by using an anti-Pgp-1 mAb as an immunogen. However, despite the presence of anti-idiotype activity in the serum of the rats, we were not able to produce mAb with a true anti-idiotype nature. The anti-idiotype reactivities, if existent, of the mAb that we produced were probably of low affinity and could not be detected in our assays. Nevertheless, mAb were developed that identified new subpopulations of TEC. ER-TR10 defines a subset of medullary TEC, whereas the ER-TR13 antigen is strongly expressed by cortical TEC at the cortico-medullary junction (chapter 7). Likewise, heterogeneity was reported for thymic IDC, based on the expression of CD8 (8), as well as medullary TEC, based on MHC class II expression (9). This heterogeneity, as established with mAb, suggests the presence of different stromal cell populations with different functions.

The molecules recognized by the large variety of anti-stromal mAb, identifying different microenvironments, need to be characterized functionally. This can be achieved by treating fetal thymus in organ culture (FTOC) with the respective anti-stromal mAb. This *in vitro* model system supports the development of immature $CD4^-8^-$ cells into mature $CD4^+8^-$ and $CD4^-8^+$ $\alpha\beta$ T cells and also the development of $\gamma\delta$ T cells (10). Lymphostromal interactions can be studied in the FTOC by treating this culture system, either with mAb, that recognize cell adhesion molecules or cytokine receptors, or with cytokines. As a result, T cell development may be modulated and analysis of the phenotype of the thymocytes in such cultures will indicate the stage of T cell development that is controlled by specific cellular interactions or cytokines (10-12). This method has been employed in our studies, for instance, to demonstrate the role of the transferrin receptor in early T cell development.

After entry into the thymus, thymocytes will enter a phase of T cell development which is marked by intense proliferation of the cells (2,3,13). The majority of the immature $CD4^-8^-3^-$ ('triple negative', TN), $CD4^-8^+3^-$, and $CD4^+8^+3^-$ thymocytes is rapidly cycling (2). In general, dividing cells require iron, and this essential element mainly internalized through the transferrin receptor (CD71), expressed on the cell surface during cell cycle. According to this reasoning, CD71 might be a useful marker for large immature thymocytes. Therefore, we analyzed CD71 expression by thymocytes. Indeed, we found that CD71 is predominantly expressed by immature thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$ and $CD4^+8^+3^-$ phenotype (chapter 3). These cells were predominantly present in the subcapsular region, but also scattered throughout the cortex of the adult thymus. Downregulation of CD71 occurs when proliferation ceases, i.e. within the $CD4^+8^+3^-$ thymocyte subpopulation. As a consequence, CD71 is not expressed on mature $\alpha\beta TcR^+$ thymocytes. Indeed, analysis of organ cultures of day 14 fetal treated with a CD71 mAb showed that (1)

proliferation of thymocytes was inhibited, and (2) no $\alpha\beta$TcR expressing cells developed (chapter 4). However, $CD44^-25^+$ TN thymocytes and its precursors developed in normal numbers, indicating that the progeny of the $CD44^-25^+$ TN cells is affected by CD71 treatment. Interestingly, organ cultures of day 16 fetal thymus (containing mainly $CD44^-25^+$ and $CD44^-25^-$ TN cells) were not inhibited by CD71 mAb treatment. Together, these data indicate that the transition of $CD44^-25^+$ to $CD44^-25^-$ TN cells is most dependent on external iron suppletion. In day 16 fetal thymus apparently enough $CD44^-25^-$ TN cells have formed and decreasing iron uptake by cells beyond this point is unable to block further T cell proliferation and differentiation. The $CD44^-25^+$ TN cells are at a major control point where cells of the $\gamma\delta$ T cell lineage and the $CD4^-8^-$ $\alpha\beta$ T cell lineage diverge from the main $\alpha\beta$ T cell lineage (14). This latter lineage is blocked by iron depletion. Surprisingly, the development of $\gamma\delta$ T cells, that also express CD71, was not affected by CD71 treatment. This indicates that $\gamma\delta$ T cells are either less iron-dependent or possess alternative iron-uptake mechanisms.

The results mentioned above show that iron deficiency, caused by CD71 treatment, leads to an inhibition of T cell development. Iron deficiency, caused by malnutrition, is one of the most common health problems in third-world countries (15), and may lead to impaired cell-mediated immunity (16,17). The quantative and qualitative inhibition of T cell development that we observed provides an explanation for this phenomenon (18).

The cytokine interleukin-2 (IL-2) is also an important growth factor during the exponential growth phase at the early stage of T cell development (12). Both the IL-2-producing cells as well as the cells expressing the IL-2 receptor (IL-2R) are found within the $CD4^-8^-3^-$ subpopulation. Thus, treatment of fetal and adult thymi (*in vivo* and *in vitro*) with an anti-IL-2R mAb results in an arrest in proliferation and differentiation: thymocytes do not develop beyond the TN stage (19-21). Surprisingly, treatment of FTOC with IL-2 also results in a growth arrest at the TN stage (22,23). In such cultures, NK-like cells preferentially expand (23). We established whether IL-2 treatment also affected the development of thymic stromal cells (chapter 6). We found that IL-2 treatment caused a dramatic thymic disorganization: cortical TEC were virtually absent, whereas medullary TEC had increased in numbers. As cortical TEC do not express an IL-2R, our data suggest that the cortex has collapsed, because this compartment has no function in the absence of $\alpha\beta$ T cell development. However, others have reported that cortical TEC are still present in e.g. day 14/15 fetal thymus and SCID thymus, that also lack terminal differentiation of $\alpha\beta$ T cells. In our study, we feel that the $Fc\gamma RII^+$ $CD4^-8^-3^-$ cells with large granular lymphocyte morphology are responsible for the observed thymic disorganization. This cell type preferentially expanded in the IL-2 treated cultures and by producing a variety of cytokines probably disturbs the delicate cytokine balance in the thymus. Thus, our study indicates that

cytokines play a key role in proper development of thymic microenvironments.

After the phase of expansion, thymocytes arrive at a new control point in their development, i.e. positive selection. For positive selection of thymocytes, which occurs at the $CD4^+8^+$ stage of T cell development, interactions of the TcR and the coreceptor (CD4 or CD8) with the MHC molecules on thymic stromal cells proves essential (24-31). Treatment of FTOC with a mAb against MHC class II molecules inhibits the development of mature $CD4^+8^-$ thymocytes (chapter 5). The mAb treatment affects the $CD4^+8^+3^{low}$ cells, because CD4 was found to be upregulated in this population. However, mAb treatment probably also affects $CD3^-$ cells, since CD4 upregulation was also observed on the surface of $CD4^+8^+3^-$ cells. This finding suggests that CD4 may be used as an early "anchor" to establish contact of MHC class II expressing stromal cells. CD4 upregulation is clearly an attempt of the potential MHC class II restricted thymocyte to be positively selected. However, in the presence of anti-MHC class II antibodies it fails to do so because mAb treatment abrogates the physical interaction between TcR and MHC class II on thymic stromal cells. Indeed, immunohistological analysis showed that MHC class II molecules of cortical TEC were saturated with the anti-MHC class II antibodies. Consequently, cortical TEC can not support positive selection of the developing thymocytes under these conditions.

After passing the control points of positive and negative selection, the mature $\alpha\beta TcR^+$ thymocytes are found in the medulla. A function for this compartment in adult thymus still has to be established. Moreover, it was shown that the medulla is absent under conditions where mature T cells do not develop, indicating that developing T cells influence the integrity of the medullary compartment (32-34). In FTOC, we observed that medullary epithelial cells were only present in small numbers, not organized in a medullary compartment and with the morphology of Hassall's corpuscles (chapter 6). As untreated FTOC supports the development of mature $\alpha\beta$ and $\gamma\delta$ T cells, our results suggest that (1) the medulla in FTOC is not essential to support T cell development, and (2) the development of the medulla in FTOC is not induced by mature T cells. If results with FTOC can be extended to normal fetal thymus, it suggests that development of the fetal thymic medulla is not induced by maturing T cells, in contrast to adult thymic medulla. In conclusion, thymocytes may be important for development of the medulla, but what is the role of the medulla in T cell development (Ref. 35)?

The results presented in this thesis provide insight into some of the control points of T cell development. We have shown that (1) development of mature $\alpha\beta$ T cells is arrested at the $CD44^-25^+$ $CD4^-8^-3^-$ stage when iron-uptake of thymocytes through the transferrin receptor is inhibited, (2) cytokines are essential for proper development of thymic microenvironments, (3) CD4 may be used by both $CD3^+$ and $CD3^-$ as an *anchor* to establish contact with MHC class II expressing thymic stromal cells, (4) the

development of the fetal thymic medulla is not induced by mature T cells. Furthermore we produced new mAb that recognize new thymic microenvironments as well as new thymocyte antigens. To gain more insight into T cell development, it will be essential that the molecules recognized by the large variety of anti-stromal mAb, and the cells expressing them become characterized functionally. In this respect, the use of stromal cell lines, hesitantly, starts to produce some scientific output. Some cell lines have now been reported that support distinct stages of T cell development (36-39), whereas other cell lines were capable of inducing positive selection (40,41). However, progress in this field remains slow, since thymic stromal cells are difficult to maintain in culture, and, moreover, tend to loose their phenotype *in vitro* and may therefore loose functional capacity as well (chapter 2). More promising is the approach to manipulate stromal cells by the production of gene "knock-out" mice. In this way, mice have been produced, deficient in MHC class II molecules; as a result mature $CD4^+8^-$ thymocytes do not develop (24). The production of mice lacking a specific stromal cell antigen or even a complete stromal cell subpopulation, using this approach, will certainly opeen new areas in T cell development research.

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Summary

The thymus provides an optimal environment for the development of T lymphocytes. Precursor T cells (produced in the bone marrow) enter the thymus from the bloodstream and will, through a complex multi-step process, develop into mature effector T cells. Phenotypically distinct thymocyte subpopulations have been identified, based on the expression of the TcR/CD3 complex and the coreceptor molecules CD4 and CD8, and a lineage relationship between most of these subsets is now well established (**chapter 1**; see also figure 2). The maturation of the thymocytes is promoted by the (non-lymphoid) stromal cells of the thymus, i.e. the epithelial cells (TEC), interdigitating cells (IDC), and macrophages (Mø). Developing thymocytes receive maturation-inducing signals from these stromal cells, either directly through cell-cell contacts (cellular interaction) or indirectly via cytokines produced by the stromal cells (humoral interaction). Thus, thymocytes pass through a number of control points at which crucial developmental changes occur: (1) entry into the thymus, (2) induction of proliferation, (3) induction of differentiation into different lineages, (4) positive and negative selection, and (5) the exit out of the thymus. At most, if not all, control points, lymphostromal interactions play a pivotal role. The studies presented in this thesis focus on the different control points in T cell development and the function of the stromal microenvironment.

The different stromal cell types can now be identified by monoclonal antibodies (**chapter 2**). In this respect, monoclonal antibodies have been developed that specifically recognize distinct subpopulations of thymic epithelial cells. Based on the thymic staining patterns of the TEC-specific mAb, at least four subpopulations of TEC can be distinguished: (a) subcapsular and perivascular TEC; (b) cortical TEC; (c) medullary TEC; and (d) Hassall's corpuscles. Together with mAb against macrophages and interdigitating cells, at least six different categories of stromal cells have been identified phenotypically. A major application of these mAb lies in the characterization of possibly aberrant thymic microenvironments under various experimental conditions. For example, immunohistological analysis of thymi of cortisone-treated mice has shown that a collapse of the cortical compartment accompanies the cortisone-induced absence of $CD4^+8^+$ cells normally present in this compartment. Thus, the inhibition of T cell development may cause changes in stromal cell composition. It should be noted, at this point, that the molecules recognized by the large variety of mAb against stromal cells still need to be characterized functionally.

The earliest phase of T cell development is marked by intense proliferation of the thymocytes. In general, dividing cells require iron, and this essential element is mainly internalized through the transferrin receptor (CD71), expressed on the cell surface. Non-dividing cells, however, express CD71 only in exceptional cases. To determine

whether CD71 could be a useful marker for immature cycling thymocytes, we analyzed CD71 expression by thymocytes. We found that CD71 is expressed by immature, cycling thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$ and $CD4^+8^+3^-$ phenotype (**chapter 3**). These cells were predominantly localized in the subcapsular region, but also scattered throughout the cortex of the adult thymus. Downregulation of CD71 occurs when proliferation ceases, i.e. within the $CD4^+8^+3^-$ thymocyte subpopulation. As a consequence, CD71 is not expressed on mature $\alpha\beta TcR^+$ thymocytes.

To examine which stages of T cell development depend mostly on iron for proliferation and/or differentiation, we cultured fetal thymi in the presence of a CD71 mAb (**chapter 4**). Such treatment will result in a reduced iron-uptake by the CD71 positive cells. In the presence of a CD71 mAb, we observed that (1) the proliferation of the thymocytes is strongly inhibited, and (2) no $\alpha\beta$TcR expressing cells developed. Our results also showed that an arrest in the development of $\alpha\beta$ T cells occurred between the $CD44^-25^+$ and $CD44^-25^-$ $CD4^-8^-3^-$ thymocyte stages, indicating that this transition is most dependent on external iron suppletion. The $CD44^-25^+$ $CD4^-8^-3^-$ cell is at a major control point where cells of the $\gamma\delta$ T cell lineage and the $CD4^-8^-$ $\alpha\beta$ T cell lineage diverge from the main $\alpha\beta$ T cell lineage. Terminal differentiation of this latter lineage is blocked completely by iron depletion. In contrast, the development of fetal $\gamma\delta$ T cells was not affected by CD71 mAb treatment, although these cells expressed CD71. This indicates that $\gamma\delta$ T cells are either less iron-dependent or possess alternative iron-uptake mechanisms.

After the phase of expansion, thymocytes arrive at a new control point in their development, i.e. positive selection. For selection of thymocytes, which occurs at the $CD4^+8^+$ stage of T cell development, interactions of the TcR and the coreceptor (CD4 or CD8) with the MHC molecules on thymic stromal cells are essential. Treatment of fetal thymus organ culture (FTOC) with a mAb against MHC class II molecules inhibits the development of mature class II-restricted $CD4^+8^-$ thymocytes, but not of class I-restricted $CD4^-8^+$ cells (**chapter 5**). The mAb treatment already affects the $CD4^+8^+3^{low}$ cells, because CD4 was found to be upregulated in this population. Surprisingly, mAb treatment probably also affects TcR^- cells, since CD4 upregulation was also observed on the surface of $CD4^+8^+3^-$ cells. This finding suggests that CD4 may be used as an early "anchor" to establish contact with MHC class II expressing stromal cells. CD4 upregulation may be an attempt of the potential MHC class II restricted thymocyte to be positively selected, but it fails to do so, as anti-MHC class II mAb treatment abrogates the required TcR-MHC interaction between the developing thymocyte and the stromal cells. Indeed, immunohistological analysis has shown that MHC class II molecules on cortical TEC, the stromal cell type that induces positive selection, are saturated with the anti-MHC class II mAb in these experiments.

After passing the control points of selection, the mature $\alpha\beta TcR^+$ thymocytes arrive

in the medulla. A function for this compartment in the development of T lymphocytes has not yet been established. Moreover, it was even observed that the adult thymic medulla is absent under conditions where mature $\alpha\beta$ T cells fail to develop. Surprisingly, we found that medullary epithelial cells in FTOC were only present in small numbers, not organized in a medullary compartment and with the morphology of Hassall's corpuscles (**chapter 6**). As FTOC yet supports the development of mature $\alpha\beta$ and $\gamma\delta$ T cells, our results suggest that (1) the medulla in FTOC is not essential to support T cell development, and (2) the development of the medulla in FTOC is not induced by mature T cells. Provided that FTOC reflects normal fetal thymus development, these results suggest that development of fetal thymic medulla is not induced by maturing T cells, in contrast to adult thymic medulla. When FTOC is treated with IL-2, normal $\alpha\beta$ T cell development is blocked and, therefore, we used the IL-2-treated FTOC to study the possible concomitant changes in thymic microenvironments. After treatment with IL-2, we observed a dramatic thymic disorganization. Cortical TEC were virtually absent, whereas medullary TEC strongly increased in numbers. However, medullary TEC were not organized as a clearcut medulla. The observed thymic disorganization is likely caused by the FcγRII$^+$ CD4$^-$8$^-$3$^-$ cells with large granular lymphocyte morphology that preferentially expanded in the IL-2 treated cultures. By producing a variety of cytokines these cells probably disturb the delicate cytokine balance in the thymus. Thus, our study indicates that cytokines play a key role in proper development of thymic microenvironments.

In **chapter 7** the development of new anti-stromal mAb is described using the anti-idiotype strategy. We used this strategy to identify the putative ligand for Pgp-1 (CD44) within the thymus. The CD44 cell adhesion molecule is present on the surface of thymus-homing cells and on the earliest prothymocytes. Others have shown that interaction between Pgp-1 and its ligand is important for entering the thymus and/or the early phase of T cell development. The putative ligand for Pgp-1 might be present on endothelial cells, stromal cells, or extracellular matrix proteins within the thymus. An anti-Pgp-1 mAb was used as an immunogen to induce anti-idiotype mAb reactive with the Pgp-1 ligand. Although anti-idiotype activity was present in the serum of the immunized rats, we were not able to produce mAb with a true anti-idiotype nature. Nevertheless, mAb were developed that exhibited new stromal staining patterns. For example, the mAb ER-TR13 strongly stains cortical TEC at the cortico-medullary junction. The staining patterns of four other mAb are also presented in this chapter. It's still possible that the ligand for Pgp-1 is recognized by one of the newly developed mAb.

Taken together, the studies in this thesis show that (1) the transferrin receptor is a marker for immature cycling thymocytes, (2) iron is essential for proliferation and differentiation of immature thymocytes, (3) CD4 is used by both CD3$^+$ and CD3$^-$

thymocytes as an *anchor* to establish contact with MHC class II expressing stromal cells, and (4) cytokines affect thymic microenvironments. Furthermore, we produced mAb that recognize new thymic microenvironments as well as new thymocyte antigens. To gain more insight into T cell development, it will be essential that the molecules recognized by the large variety of anti-stromal antibodies and the cells expressing them become functionally characterized. In this respect, the production of gene "knock-out" mice that lack expression of a specific stromal cell antigen or even a complete stromal cell subpopulation is at present a promising approach.

Samenvatting

De thymus biedt de optimale omgeving voor de ontwikkeling van T-lymfocyten. Voorloper T-cellen (afkomstig uit het beenmerg) komen de thymus binnen vanuit de bloedbaan en zullen zich, via een complex proces, ontwikkelen tot rijpe effector T-cellen. Verschillende thymocytsubpopulaties kunnen worden onderscheiden, gebaseerd op de expressie van het TcR/CD3 complex en de coreceptor moleculen CD4 and CD8 (**hoofdstuk 1**; zie ook figuur 2). De rijping van de thymocyten wordt geïnduceerd door de niet-lymfoïde stromale cellen van de thymus, namelijk de epitheliale cellen (TEC), de interdigiterende cellen (IDC), en de macrofagen (Mø). Thymocyten ontvangen tijdens hun ontwikkeling rijping-inducerende signalen van deze stromale cellen, zowel direct via cel-cel contacten (cellulaire interactie) als indirect via cytokinen geproduceeerd door de stromale cellen (humorale interactie). Op deze manier passeren de thymocyten een aantal controlepunten waar cruciale veranderingen in de T-celontwikkeling plaatsvinden: (1) binnenkomst in de thymus, (2) inductie van proliferatie, (3) inductie van de verschillende differentiatielijnen, (4) positieve en negatieve selectie, en (5) het verlaten van de thymus. Op de meeste, zo niet alle, controlepunten spelen lymfo-stromale interacties een centrale rol (zie figuur 3 in hoofdstuk 1). De studies in dit proefschrift richten zich op de verschillende controlepunten in de T-celontwikkeling en op de functie van de stromale micro-omgeving.

De verschillende stromale celtypen kunnen nu geïdentificeerd worden met behulp van monoclonale antistoffen (**hoofdstuk 2**). Gebaseerd op het reactiepatroon van de antistoffen gericht tegen de epitheliale cellen, kunnen op zijn minst vier TEC subpopulaties onderscheiden worden: (a) subcapsulaire en perivasculaire TEC; (b) corticale TEC; (c) medullaire TEC; en (d) lichaampjes van Hassall. Samen met antistoffen tegen macrofagen en interdigiterende cellen kunnen er tenminste zes verschillende categoriën van stromale cellen herkend worden. Een belangrijke toepassing van deze monoclonale antilichamen ligt in de karakterisatie van mogelijk afwijkende micro-omgevingen onder verschillende experimentele omstandigheden. Uit de immunohistologische analyse van thymi van cortison-behandelde muizen is bijvoorbeeld gebleken dat de cortison-geïnduceerde afwezigheid van $CD4^+8^+$ cellen gepaard gaat met een sterke reductie van de corticale micro-omgeving. Overigens dient te worden opgemerkt dat de moleculen die herkend worden door een grote verscheidenheid aan anti-stromale antistoffen nog steeds op functionele karakterisatie wachten.

De vroegste fase in de T-celontwikkeling is herkenbaar aan de sterke proliferatie van de thymocyten. Alle delende cellen hebben ijzer nodig en dit essentiële element wordt voornamelijk door de cel opgenomen via de transferrinereceptor (CD71). Niet-delende

cellen, echter, brengen CD71 alleen in uitzonderlijke gevallen tot expressie. We hebben de CD71 expressie van thymocyten geanalyseerd om na te gaan of CD71 als marker voor onrijpe delende thymocyten gebruikt zou kunnen worden. We vonden dat CD71 tot expressie komt op onrijpe delende thymocyten met een $CD4^-8^-3^-$, $CD4^-8^+3^-$ en $CD4^+8^+3^-$ fenotype (**hoofdstuk 3**). Deze cellen zijn voornamelijk aanwezig in het subcapsulaire gebied van de volwassen thymus, maar komen ook verspreid door de cortex voor. Expressie van CD71 neemt af als de proliferatie ophoudt, en dit vindt plaats binnen de $CD4^+8^+3^-$ thymocytsubpopulatie. Dientengevolge komt CD71 niet tot expressie op rijpe $\alpha\beta TcR^+$ thymocyten.

Om te bepalen welke stadia van de T-celontwikkeling het meest afhankelijk zijn van ijzer voor hun proliferatie en/of differentiatie, werden foetale thymi gekweekt in de aanwezigheid van een CD71 monoclonale antistof (**hoofdstuk 4**). Zo'n behandeling leidt tot een gereduceerde opname van ijzer door de CD71-positieve cellen. In de aanwezigheid van een CD71 antistof werd waargenomen dat (1) de proliferatie van de thymocyten sterk geremd werd en (2) er geen $\alpha\beta$TcR-positieve cellen tot ontwikkeling kwamen. Onze resultaten lieten ook zien dat een blokkade in de ontwikkeling van de $\alpha\beta$ T-cellen plaatsvindt tussen de $CD44^-25^+$ en $CD44^-25^-$ $CD4^-8^-3^-$ stadia, wat aangeeft dat deze overgang het meest gevoelig is voor een tekort aan ijzer. De $CD44^-25^+$ $CD4^-8^-3^-$ cel bevindt zich op een belangrijk controlepunt waar cellen van de $\gamma\delta$ T-cellijn en van de $CD4^-8^-$ $\alpha\beta$ T cellijn zich afscheiden van de hoofdlijn van $\alpha\beta$ T celontwikkeling. De terminale differentiatie van deze laatste ontwikkelingslijn wordt door het ijzertekort volledig geblokkeerd. Daarentegen wordt de ontwikkeling van foetale $\gamma\delta$ T cellen niet beïnvloed door de CD71 behandeling, ook al brengen deze cellen CD71 wel tot expressie. Dit geeft aan dat $\gamma\delta$ T-cellen hetzij minder ijzer-afhankelijk zijn voor hun ontwikkeling, danwel beschikken over alternatieve mechanismen om ijzer op te nemen.

Na de expansie-fase komen de thymocyten aan bij een nieuw controlepunt in hun ontwikkeling, namelijk positieve selectie. Voor de selectie van thymocyten, die plaatsvindt op het $CD4^+8^+$ stadium van de T-celontwikkeling, zijn interacties van de TcR en de coreceptor (CD4 of CD8) met de MHC moleculen op de stromale cellen van de thymus essentieel. Behandeling van de foetale thymus orgaan cultuur (FTOC) met een monoclonale antistof tegen MHC klasse II moleculen leidt tot een remming van de ontwikkeling van rijpe klasse II-gerestricteerde $CD4^+8^-$ thymocyten, maar niet van klasse I-gerestricteerde $CD4^-8^+$ cellen (**hoofdstuk 5**). De antistofbehandeling bleek al effect te hebben op de $CD4^+8^+3^{low}$ cellen, omdat de expressie van CD4 door deze populatie verhoogd was. Opmerkelijk was de bevinding dat antistofbehandeling vermoedelijk ook een effect heeft op TcR^- cellen, want CD4 werd ook door de $CD4^+8^+3^-$ cellen verhoogd op het oppervlak tot expressie gebracht. Deze bevinding suggereert dat CD4 als een 'anker' gebruikt wordt om contact te maken met MHC

klasse II positieve stromale cellen. Door de verhoogde expressie van CD4 zou een potentieel MHC klasse II gerestricteerde thymocyt de interactie met stromale cellen kunnen verhogen om daarmee toch nog positief geselecteerd te worden. Dit vindt echter niet plaats, omdat de noodzakelijke TcR-MHC interactie geblokkeerd is als gevolg van de behandeling met een anti-MHC klasse II antistof. Immunohistologische analyse in deze experimenten laat inderdaad zien dat de MHC klasse II moleculen op de corticale TEC, het stromale celtype dat positieve selectie induceert, verzadigd zijn met de anti-MHC klasse II antistoffen.

Nadat de controlepunten van selectie gepasseerd zijn, arriveren de rijpe $\alpha\beta$TcR$^+$ thymocyten in de medulla. Een functie voor de medulla in de ontwikkeling van de T-lymfocyten is nog steeds niet vastgesteld. Bovendien lijkt de ontwikkeling van de medulla juist van de aanwezigheid van rijpe T-cellen af te hangen: de medulla van de volwassen thymus is afwezig onder condities waar rijpe $\alpha\beta$ T-cellen niet tot ontwikkeling kunnen komen. Opmerkelijk is dat in de FTOC, medullaire epitheelcellen slechts in kleine aantallen aanwezig zijn, niet georganiseerd in een medulla zijn en de morfologie hebben van de lichaampjes van Hassall (**hoofdstuk 6**). Aangezien de FTOC toch de ontwikkeling van rijpe $\alpha\beta$ en $\gamma\delta$ T cellen ondersteunt, geven onze resultaten aan, dat (1) de medulla in de FTOC niet essentieel is voor het ondersteunen van de T-celontwikkeling, en (2) de ontwikkeling van de medulla in de FTOC niet wordt geïnduceerd door rijpe T-cellen. Op voorwaarde dat de FTOC de normale foetale thymusontwikkeling weerspiegelt, suggereren deze resultaten dat de ontwikkeling van de medulla in de foetale thymus, i.t.t. de medulla in de volwassen thymus, niet door rijpe T-cellen geïnduceerd wordt.

Als de FTOC met IL-2 behandeld wordt, wordt de normale $\alpha\beta$ T celontwikkeling geremd en om die reden is de IL-2-behandelde FTOC gebruikt om de mogelijke veranderingen in de micro-omgevingen van de thymus te bestuderen. Na de behandeling met IL-2 werd een sterk verlies van structurele organisatie van de thymus waargenomen. Corticale TEC waren vrijwel afwezig, terwijl medullaire TEC juist sterk in aantal waren toegenomen. Echter, de medullaire TEC waren niet georganiseerd in een duidelijk te onderscheiden medulla. De waargenomen disorganisatie van de thymus wordt waarschijnlijk veroorzaakt door de FcγRII$^+$ CD4$^-$8$^-$3$^-$ cellen met 'large granular lymphocyte' morfologie, die zich bij voorkeur ontwikkelden in de IL-2-behandelde kweken. Deze cellen verstoren vermoedelijk de delicate cytokine balans in de thymus door een verscheidenheid aan cytokinen te produceren. Daarom geeft deze studie aan dat cytokinen belangrijk zijn voor een goede ontwikkeling van de micro-omgevingen van de thymus.

In **hoofdstuk 7** wordt de ontwikkeling beschreven van nieuwe monoclonale anti-stromale antistoffen die gegenereerd zijn m.b.v. de anti-idiotype strategie. Deze strategie werd gebruikt om de vermeende ligand van Pgp-1 (CD44) in de thymus te

identificeren. Het CD44 celadhesie molecuul is aanwezig op het oppervlak van voorloper T-cellen die naar de thymus migreren en op de jongste prothymocyten in de thymus. Anderen hebben laten zien dat de interactie van Pgp-1 met zijn ligand belangrijk is voor het binnentreden in de thymus en/of de vroege fase van de T-celontwikkeling. De vermeende ligand voor Pgp-1 zou aanwezig kunnen zijn op endotheelcellen, stromale cellen, of extracellulaire matrix-eiwitten in de thymus. Een anti-Pgp-1 antistof werd gebruikt als immunogeen om anti-idiotype antistoffen, reactief met de Pgp-1-ligand, op te wekken. Hoewel er anti-idiotype activiteit in het serum van de geïmmuniseerde ratten aanwezig was, bleek het niet mogelijk om monoclonale antistoffen te maken met aantoonbare anti-idiotype activiteit. Toch werden er antilichamen ontwikkeld die nieuwe reactiepatronen met thymusweefsel gaven. Zo reageert de monoclonale antistof ER-TR13 sterk met corticale TEC gelegen aan de cortico-medullaire junctie. De reactiepatronen van vier andere antistoffen worden ook in dit hoofdstuk gepresenteerd. Het is mogelijk dat een van deze nieuwe antistoffen de ligand voor Pgp-1 herkent.

Concluderend laten de studies in dit proefschrift zien dat (1) de transferrine recptor een marker is voor onrijpe delende thymocyten, (2) ijzer een belangrijke rol speelt in de proliferatie en differentiatie van onrijpe T-cellen, (3) CD4 door zowel $CD3^+$ als $CD3^-$ thymocyten als een *anker* gebruikt wordt om contact te maken met de MHC klasse II-positieve stromale cellen, en (4) cytokinen de micro-omgevingen van de thymus beïnvloeden. Bovendien, hebben we monoclonale antistoffen geproduceerd die zowel nieuwe micro-omgevingen in de thymus definiëren als nieuwe thymocytantigenen herkennen. Om een beter inzicht in de T-celontwikkeling te verwerven is het noodzakelijk dat de moleculen, die door een grote variatie aan anti-stromale antistoffen herkend worden, en de cellen die ze tot expressie brengen, functioneel gekarakteriseerd worden. In dit opzicht lijkt de productie van muizen, waarbij de betreffende genen door homologe recombinatie geïnactiveerd zijn, of die zelfs een complete stromale celsubpopulatie missen, een veelbelovende benadering.

Abbreviations

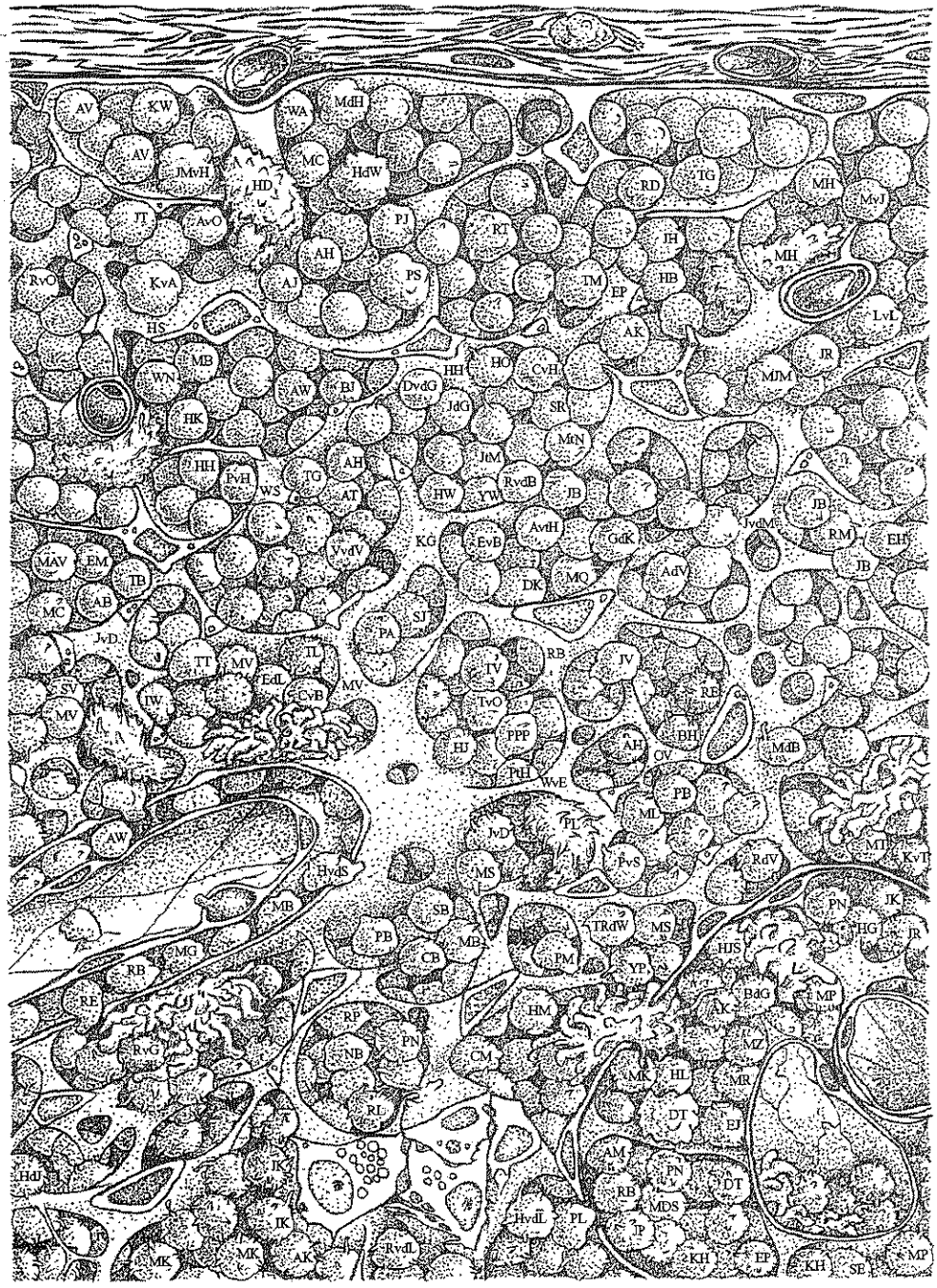
BSA : bovine serum albumin
C : constant
CD : cluster of differentiation
CK : cytokeratin
CMJ : cortico-medullary junction
cort : cortex
CsA : cyclosporin A
cTEC : cortical TEC
CTES : clusters of thymic epithelial staining
D : diversity
DC-ROS : dendritic cell rosettes
DN : double negative ($CD4^-8^-$)
DP : double positive ($CD4^+8^+$)
ECM : extracellular matrix
FCS : fetal calf serum
FITC : fluorescein isothiocyanate
Fn : fibronectin
FnR : fibronectin receptor
FTOC : fetal thymus organ culture
FTS : facteur thymique serique
GD : gestational day
HAb : hybrid antibody
HC : Hassall's corpuscles
HSA : heat-stable antigen
ICAM : intercellular adhesion molecule
IDC : interdigitating cell
IFN : interferon
IL : interleukin
IL-2R : interleukin-2 receptor
J : joining
kDa : kilo Dalton
KLH : keyhole limpet hemocyanin
LFA : lymphocyte function antigen
mAb : monoclonal antibody/antibodies
med : medulla
MHC : major histocompatibility complex
Mls : minor lymphocyte stimulating
Mø : macrophage
Mø-ROS : macrophage rosettes
mTEC : medullary TEC
MW : molecular weight
OT : oxytocin
P-TR : phagocytic cell of the thymic reticulum
PBS : phosphate buffered saline
PE : phycoerythrin
Pgp-1 : phagocytic glycoprotein-1
PI : propidiumiodide
pv : perivascular
pvTEC : perivascular TEC
RAG : recombination activating genes
sc : subcapsular
SCF : stem cell factor
SCID : severe combined immunodeficiency
scTEC : subcapsular TEC
SEM : standard error of the mean
SP : single positive ($CD4^+8^-$ or $CD4^-8^+$)
TcR : T cell receptor
TEC : thymic epithelial cell
TEM : transmission electron microscopy
TfR : transferrin receptor
TGF : transforming growth factor
TMF : thymic microenvironmental factor
TN : triple negative ($CD4^-8^-3^-$)
TNC : thymic nurse cell
TNF : tumor necrosis factor
TSTGF : thymic stroma-derived T cell growth factor
V : variable
VLA : very late antigen
VP : vasopressin

Dankwoord

Dit boekje beschrijft hoe een thymocyt tijdens zijn ontwikkelingsreis door de thymus continu ervaring op doet door contacten te leggen met andere thymus cellen. Uiteindelijk, met zijn wijsheid op zak, verlaat hij de thymus.

Iedereen hartelijk dank voor de prettige samenwerking

Pieter B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