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Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets

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SUMMARY

T-cell development is under the tight control of thymic microenvironments. Conversely, the integrity of thymic microenvironments depends on the physical presence of developing thymocytes, a phenomenon designated as 'thymic crosstalk'. We now show, using three types of immunodeficient mice, i.e. CD3ε transgenic mice, RAG^{null} mice and RAG^{null}-bone-marrow-transplanted CD3ε transgenic mice, that the control point in lymphoid development where triple negative (CD3$^-$,CD4$^-$,CD8$^-$) thymocytes progress from CD44$^+$CD25$^-$ towards CD44$^-$CD25$^+$, influences the development of epithelial cells, critically inducing the extra, third dimension in the organization of the epithelial cells in the cortex. This tertiary configuration of the thymic epithelium is a typical feature for the thymus, enabling lymphostromal interaction during T-cell development. Crosstalk signals at this control point also induce the formation of thymic nurse cells. Moreover, our data indicate that establishment of a thymic cortex is a prerequisite for the development of the thymic medulla. Thus, differentiating thymocytes regulate the morphogenesis of thymic microenvironments in a stepwise fashion.

Abbreviations: RAG^{null}, mice deficient for the recombinase activation gene; tgε26, transgenic mice expressing the human CD3ε gene; TN, triple negative (CD3$^-$,CD4$^-$,CD8$^-$) thymocyte; DP, double positive (CD4$^+$,CD8$^+$) thymocyte; SP, single positive (CD4$^+$, resp. CD8$^+$) thymocyte; TCR, T-cell receptor; MHC, major histocompatiblity complex

Key words: Thymus, Microenvironment, Crosstalk, Epithelium, Lymphostromal interaction, Mouse, RAG^{null}

INTRODUCTION

The thymus is a primary lymphoid organ crucially involved in the differentiation of T lymphocytes. Its stroma creates several distinct microenvironments, which control defined steps in T-cell development. Thymic microenvironments are composed of a network of epithelial reticular cells, present in both the cortex and medulla (van Ewijk, 1988, 1991). Non-epithelial stromal cells, such as fibroblasts, macrophages and interdigitating reticular cells are integrated in this network (Boyd et al., 1993). In adult mice, progenitor T cells derived from the bone marrow enter the thymic stroma at the border between cortex and medulla and migrate, while differentiating, through distinct microenvironments in the subcapsular cortex, the deep cortex, the corticomedullary junction and the medulla (Kyewski et al., 1987; van Ewijk, 1991; Anderson et al., 1996). During T-cell development, thymocytes interact with thymic stromal cells within these microenvironments. Lymphostromal interaction induces proliferation and ensures that a T-cell repertoire is selected, reactive to foreign epitopes but tolerant to self epitopes.

It has been shown, on the basis of avidity of the interaction between T-cell receptors (TCR) on the surface of thymocytes and peptide-MHC complexes expressed on stromal cells, that the fate of the developing thymocytes is determined by the thymic stroma (Ashton-Rickardt and Tonegawa, 1994). Thus, epithelial cells in the cortex mediate positive selection (Anderson et al., 1993; Bevan, 1997; de Koning et al., 1997; Jameson and Bevan 1998; reviewed by Laufer et al., 1999), whereas interdigitating cells and epithelial cells in the medulla mediate negative selection of developing T cells (Sprent et al., 1988; Lo et al., 1997; Laufer et al., 1999). The net balance between these two processes apparently determines the thymic output of selected T cells (Anderson et al., 1998).

Because of its embryological origin, the thymus differs from secondary lymphoid organs. While secondary lymphoid organs are mesodermal in origin, the thymus is ectodermal and endodermal in origin, explaining the epithelial nature of thymic stromal cells (Owen and Jenkinson, 1984). Importantly, the organization of the epithelial cells in the thymus differs completely from that in other (non-lymphoid) organs. Rather than forming a sheet of cells positioned on a basement membrane, thymic epithelial cells form a three-dimensional (3-D) sponge-like meshwork of elongated cytoplasmic extensions,

interconnected through desmosomes. This 3-D configuration enables migration of developing T cells through the stroma and facilitates intimate cell surface contact between stromal cells and developing T cells. Moreover, the composition of the thymic stroma creates microenvironments, as defined by distinct structural and functional features (van Ewijk, 1991).

The unique structural organization of the thymic stroma is already present early in ontogeny. Several control points determine the development of thymic microenvironments, either intrinsically regulated by the genetic program expressed within stromal cells themselves (Boehm et al., 1995; Manley and Capecchi, 1995; Nehls et al., 1996; Blackburn et al., 1996), or extrinsically regulated, for example by developing lymphoid cells (van Ewijk et al., 1994; Holländer et al., 1995a; van Ewijk et al., 1999). (1) The first control point regulates the fusion of ectoderm and endoderm in the region of the 3rd pharyngeal cleft and pouch, respectively (Owen and Jenkinson, 1984), and is induced by neural crest cells derived from the hindbrain in the embryo migrating into this region (Auerbach, 1960; Bockman and Kirby, 1984; Itoi and Amagai, 1998). Thus, around day 10 in gestation, two laterally positioned thymic primordia are created, which subsequently migrate ventrally to fuse in the midline of the body, on top of the heart. (2) At a second control point, both the Whn transcription factor, a product of the *nude* locus, and Pax1, a transcriptional regulatory protein, are expressed around day 11 in gestation (Nehls et al., 1996; Wallin et al., 1996). Next, Ikaros$^+$ hematopoietic precursor cells are attracted to these differentiated primordia (M. Itoi and T. Amagai, personal communication). Sustained interaction between neural-crest-derived mesenchymal cells and epithelial cells induces lobulation, which is followed by angiogenesis of the thymic 'anlage' (Bockman and Kirby 1984; M. Itoi and T. Amagai, personal communication). (3) During the third control point at day 12 of gestation, epithelial cells differentiate into cortical and medullary types (M. Itoi and T. Amagai, personal communication). (4) The fourth control point, at day 14 in gestation, regulates the positioning of the cortex and medulla (van Vliet et al., 1985). (5) Finally, the fifth control point is reached by day 16 in ontogeny, when the thymic stroma gains functional competence to select developing thymocytes (van Vliet et al., 1985; Fairchild and Austin, 1995).

In the present paper, we focus on two control points that regulate the organization and positioning of cortical and medullary microenvironments, respectively. We show that both control points are influenced by different subsets of developing thymocytes. Our study utilizes two strains of immunodeficient mice, each harboring a block at a different stage of early T-cell development. Thus, mice expressing a high copy number of the human CD3ε gene (tgε26 mice) reveal a block at the stage of early triple negative (TN) CD44$^+$CD25$^-$ cells (Wang et al., 1994, 1995). Such mice not only have a small thymus with low cellularity but they also show heavily disturbed cortical and medullary microenvironments. In particular, a distinct border between cortex and medulla is lacking and many epithelial cells are organized in a typical two-dimensional (2-D) fashion (Holländer et al., 1995a). Compared to tgε26 mice, T-cell development in RAG^{null} mice progresses to a slightly later stage in T-cell development, i.e. at the TN CD44$^-$CD25$^+$ stage. Such mice show a thymus with increased cellularity and a well-developed thymic cortex, but medullae are still absent.

Here, we identify subsets of developing T cells involved in the establishment of thymic microenvironments in the cortex and the medulla. By transplanting tgε26 mice with RAG^{null} bone marrow, we show that progression in T-cell development from TN CD44$^+$CD25$^-$ towards TN CD44$^-$CD25$^+$ stimulates the conversion of 2-D organized epithelial sheets into a 3-D organized epithelial network. Subsequent transplantation of these (RAG^{null}→tgε26) chimeric mice with wild-type bone marrow cells enables normal thymopoiesis and is paralleled by correct organization of medullary microenvironments. Our data indicate that maturing thymocytes control the development of thymic stroma in a stepwise fashion, resulting first in the induction of cortical microenvironments, followed by the establishment of medullary microenvironments.

MATERIALS AND METHODS

Mice

tgε26 mice (Wang et al., 1995) were maintained through sib breeding in the animal facility of the Beth Israel Hospital. RAG-2^{null} mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Antibodies

All antibodies used in the present study are indicated in Table I.

Bone marrow transplantation

Neonatal tgε26 mice (2 to 4 days post partum) were injected intraperitoneally with 5×10^6 bone marrow cells derived from adult RAG^{null} mice in a volume of 50 μl. No precondition treatment was performed to the neonatal recipients. 5-10 weeks after the neonatal bone marrow transplantation, these mice were killed for analysis, or used for subsequent transplantation with wild-type bone marrow cells.

Bone marrow transplantation to adult tgε26 mice was performed as described (Holländer et al., 1995a). Basically, the adult tgε26 mice (8-10 weeks old), which were injected with RAG^{null} bone marrow cells at birth, were pretreated with superlethal irradiation (650 rad + 450 rad). 10^7 wild-type bone marrow cells treated with two rounds of anti-Thy-1.2 + complements were injected into the tail vein of these recipient mice. Such mice are defined as WT→(R→tgε26). These mice were analyzed 6-10 weeks after the second bone marrow transplantation.

Flow cytometric analysis

Single-cell suspensions of thymocytes were obtained by mechanical disruption of the thymus. Cells were first preblocked with purified anti-mouse Fc receptor antibody and then stained for three-color flow cytometric analysis as described previously (Holländer et al., 1995a).

Microscopy

For histology, and transmission and scanning electron microscopy,

Table 1. Antibodies used

Antibody	Target	Reference
Ra3-6B2	B220	Coffman and Weisman (1981)
7D4	CD25	Malek et al. (1983)
IM7	CD44	Lesley and Trowbridge (1982)
DS-1	IgM	Sieckmann et al. (1984)
ER-TR 4	cortical epithelial cells	Van Vliet et al. (1994)
ER-TR 5	medullary epithelial cells	Van Vliet et al. (1984)
145-2C11	CD3ε	Leo et al. (1987)
H75-597	TCRαβ	Kubo et al. (1989)
53-2. 1	Thy-1. 2	Ledbetter and Herzenberg (1979)
RM4-5	CD4	Frederickson and Bash (1987)
53-6. 7	CD8	Ledbetter and Herzenberg (1979)
2-4G2	FcR	Unkeless (1979)

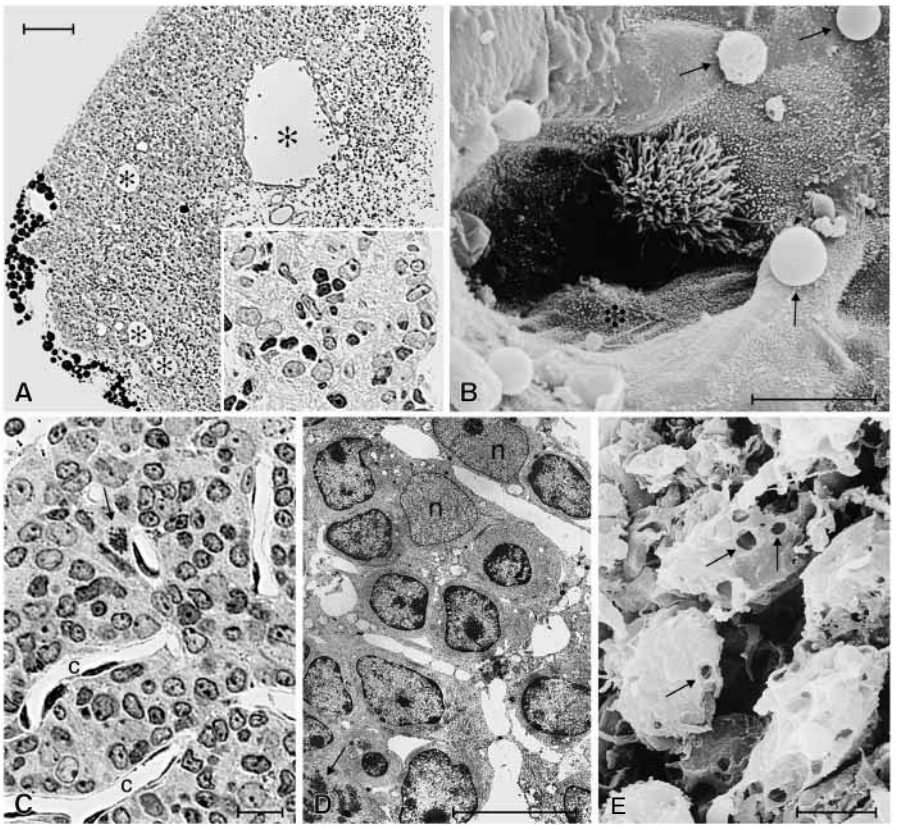

Fig. 1. (A) The thymus of a tgε26 mouse is characterized by a low cellularity and by the presence several thymic cysts (asterisks). At higher magnification (A, inset), pale staining epithelial cells can be noted packed together, with a few scattered dark staining lymphocytes. (B) Scanning electron microscopic of a thymic cyst in a tgε26 mouse, revealing two-dimensionally organized epithelial cells normally not present in the thymus, such as ciliated cells, absorptive cells with short microvilli (asterisk), and mucus secreting goblet cells (arrows). (C) In contrast to the thymus of tgε26 mice, the thymus of RAG^{null} mice shows increased cellularity with proliferating lymphocytes (arrow). Angiogenesis in the RAG^{null} thymus is manifested by the presence of many capillaries (c). (D) Transmission electronmicrograph of the thymic stroma of RAG^{null} mice, showing several thymic nurse cells (TNC), comprising medium-sized proliferating (arrow) lymphocytes. TNC are interconnected through desmosomes and are part of the integral 3-D epithelial network. n, nucleus of epithelial cell. (E) Scanning electron micrograph of the RAG^{null} thymus, showing four thymic nurse cells in close proximity to each other in for part of a 3-D epithelial network. Note presence of TNC characteristic caveolae (arrows). Bars, (A) 130 μm; (B) 6.5 μm; (C) 12.5 μm; (D) 20 μm; (E) 13 μm.

mice were fixed by total body perfusion, as described previously (van Ewijk, 1988). Fixed tissues were further processed according to standard technical procedures.

Immunohistology

For immunohistology, tissues were immersed in OCT compound and snapfrozen in liquid nitrogen. 5 μm frozen sections were cut using a Leitz cryostat. Sections were collected on gelatin-precoated microscope slides, and stored at room temperature in a desiccator, for not longer than 3 days. Sections were stained with rat monoclonal antibodies (Table 1) and incubated with a rabbit anti-rat horse radish peroxidase conjugate (DAKO). To prevent background staining, all antibodies were optimally titrated. The conjugate was visualized by incubation with a solution of 1% diaminobenzidine.

RESULTS

Thymic microenvironments in tgε26 tg and RAG^{null} mice differ in architecture

The thymus of young adult tgε26 mice is very small in size, containing around 10^6 lymphoid cells, with no clear distinction between cortex and medulla (Fig. 1A). Interestingly, several large cysts are present within the thymic stroma (Fig. 1A,B). Epithelial cells are palely stained and do not display the characteristic network configuration observed in the thymus of wild-type mice (Fig. 1A, inset).

At the ultrastructural level, epithelial cells lack the typical long cytoplasmic extensions (data not shown) and form many thymic cysts. Such cysts contain at least three types of 'classical' epithelial cells also found in other tissues (Fig. 1B), i.e. (1) cells with microvilli (as in the gastrointestinal tract), (2) mucin-secreting goblet cells (as in the gastrointestinal tract and in the respiratory tract), and (3) cells expressing cilia (as in the respiratory tract). Lymphoid cells in the tgε26 thymus are generally small in size; they show an electron-dense nucleus while vacuoles are frequently found in the cytoplasm (data not shown). These features indicate a state of apoptosis, most probably caused by overexpression of the CD3ε gene, early in T-cell development (Wang et al., 1995).

Compared to tgε26 thymi, cellularity in the RAG^{null} thymus has increased to 3-5×10^6 thymocytes. Lymphoid cells are generally medium-sized and actively proliferating. Furthermore, the RAG^{null} thymus is well vascularized and contains many capillaries (Fig. 1C). Transmission electron microscopy reveals a network of epithelial cells with extended cytoplasmic processes, interconnected through desmosomes (Fig. 1D). Most strikingly, we found a high frequency of thymic nurse cells (TNC) in the thymus of RAG^{null} mice, frequently associated with capillaries (Fig. 1D,E). TNC comprise medium-sized thymocytes, and a fraction of these cells appear to be cycling, as shown by the presence of metaphase chromosomes (Fig. 1C,D). Thymic cysts are absent in the thymus of RAG^{null} mice.

Injection of tgε26 mice with RAG^{null} bone marrow converts the thymic phenotype to that of RAG^{null} mice

To define the phenotype of thymocytes responsible for the development of the thymic stroma in cortex and medulla, we have generated two types of chimeric mice. Type 1 chimeric mice were created by transplantation of bone marrow cells derived from RAG^{null} mice into newborn tgε26 mice

(RAG^{null}→tgε26). Type 2 chimeric mice were created by transplantation of normal bone marrow cells into type 1 chimeric mice in adult life, and are designated as WT→(R→tg26ε). If crosstalk operates at the level of defined thymocyte subsets, we reasoned that the type 1 chimeric mice should develop thymic microenvironments identical to those found in RAG^{null} mice, whereas the double-transplanted tgε26 mice should reveal a thymic phenotype comparable to normal mice.

Flow cytometric analysis of the development of thymocytes in type 1 chimeric mice is presented in Fig. 2A. Most thymocytes in control tgε26 mice are Thy-1$^-$ CD44$^+$CD25$^-$ and express B220 and IgM. In contrast, lymphocytes in the RAG^{null} thymus are all Thy-1$^+$ and have reached to the CD44$^-$ CD25$^+$ stage, where further maturation is blocked (Holländer et al., 1995a). Injection of tgε26 mice with bone marrow cells from RAG^{null} mice leads to a moderate increase in cellularity (to 2-5×10^6 cells/thymus), and a dramatic change in thymocyte phenotype. More than 90% of the cells are now Thy-1$^+$, and a large majority of these cells has differentiated to the TN CD44$^-$ CD25$^+$ phenotype, identical to that of thymocytes in RAG^{null} mice. Moreover, the frequency of B220$^+$ cells in the tgε26 thymus has decreased close to the levels found in wild-type thymi.

Immunohistological analysis of the thymus of RAG^{null}→tgε26 mice reveals an organization of thymic stromal cells comparable to that found in the thymus of RAG^{null} mice (see Holländer et al., 1995a). We present here the microscopic data from one type 1 mouse, representative of the series of 21 successfully transplanted mice, showing that the two thymic lobes were differentially reconstituted.

The small right lobe (Fig. 3) was not reconstituted, and showed a stromal organization comparable to the thymus of untransplanted tgε26 mice (see Holländer et al., 1995). In contrast, the medium-sized left lobe was reconstituted with donor-derived thymocytes, and displayed a stromal architecture similar to that in RAG^{null} mice (see van Ewijk et al., 1999).

The unreconstituted right lobe (Fig. 3A-C) shows large cysts, and the cortical epithelial cells, defined by the antibody ER-TR4, have changed their orientation. While in the normal thymus cortical epithelial cells are oriented perpendicularly towards the capsule (van Vliet et al., 1984), thus guiding migration of developing thymocytes towards the medulla, thymic epithelial cells in the unreconstituted lobe are aligned parallel to the capsule. Moreover, a well-defined medulla is lacking and ER-TR5$^+$ medullary epithelial cells are found scattered throughout the thymus (Fig. 3B). The unreconstituted lobe was further characterized by a high frequency of ER-TR7$^+$ fibroblasts (Fig. 3C). Within this lobe, B lymphocytes occurred at high frequency, localized especially in close proximity to thymic fibroblasts (Fig. 3D).

The left thymic lobe of the bone marrow transplanted tgε26 mouse demonstrates in contrast a well-developed cortical reticulum, displaying many round to oval ER-TR4$^+$ structures (Fig. 4A,B), comparable in size to TNCs found in RAG^{null} mice (see also Fig. 1D,E). Although small patches of ER-TR5$^+$ cells are present, well-defined medullas are still lacking (Fig. 3D). Large cysts are absent and the frequency of B220$^+$ cells has decreased to that found in the RAG^{null} thymus (Fig. 4D). The many capillaries running through the thymic stroma show that there is increased angiogenesis after reconstitution (Fig. 4A,C).

The observed discrepancies between the stromal architecture of the left and right thymic lobes indicate that, under the present experimental conditions, only a limited number of T-cell precursors repopulated the thymus, giving rise to a progeny of cells arrested at the CD44$^-$CD25$^+$ stage. Importantly, progression to this stage in T-cell development rescues the 3-D organization of the thymic reticulum, reduces the frequency of fibroblasts and induces a high frequency of thymic nurse cells.

Injection of *RAG^{null}→tgε26* chimeras with normal bone marrow leads to a complete restoration of the architecture of the thymic stroma

Injection of adult Rag^{null}→tgε26 chimeras with bone marrow cells derived from normal mice restores, as expected, T-cell development completely, with thymic cellularity dramatically increasing to 1.1×10^8 cells. Phenotypical analysis with antibodies directed to CD4, CD8 and TCRαβ shows similar patterns between the WT→(R→tg26ε) mice and untransplanted wild-type controls (Fig. 2B). Normal numbers of mature T cells are also present in peripheral lymphoid organs of double-transplanted mice (data not shown).

Immunohistological analysis of the thymus of double-transplanted mice shows a normal distribution of CD3$^+$ thymocytes (Fig. 5A) and a complete restoration of cortical thymic microenvironments (Fig. 5B-D). In contrast to the

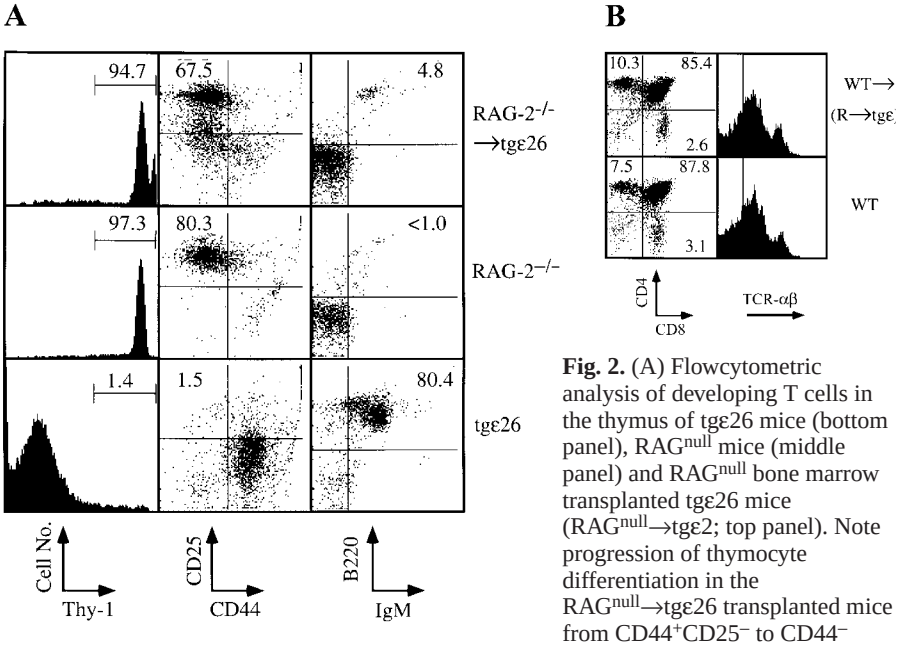

Fig. 2. (A) Flowcytometric analysis of developing T cells in the thymus of tgε26 mice (bottom panel), RAG^{null} mice (middle panel) and RAG^{null} bone marrow transplanted tgε26 mice (RAG^{null}→tgε2; top panel). Note progression of thymocyte differentiation in the RAG^{null}→tgε26 transplanted mice from CD44$^+$CD25$^-$ to CD44$^-$ CD25$^+$. (B) Flowcytometric analysis of developing thymocyte subsets in the thymus of wild-type mice (bottom panel) and 'double' transplanted, i. e. WT→(R→tg26ε) mice (top panel). Note endstage maturation of thymocytes in the double-transplanted mice.

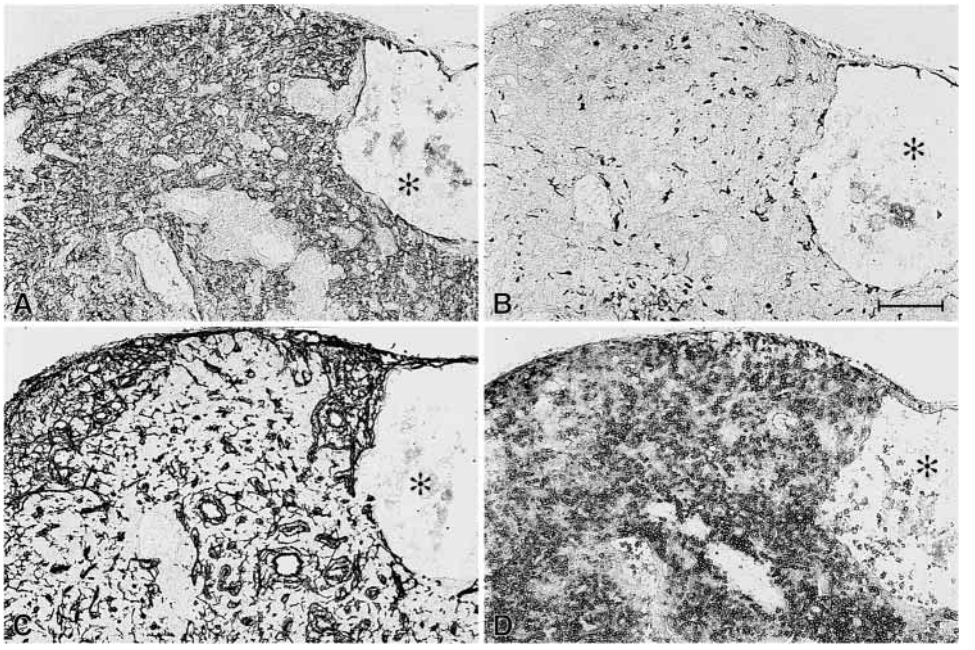

Fig. 3. Four adjacent frozen sections of the right unreconstituted thymic lobe of a tgε26 mouse, transplanted with RAG^{null} bone marrow cells. The phenotype of cells in the right lobe is similar that in thymic lobes of untreated tgε26 mice (Holländer et al; 1995). Sections were incubated with monoclonal antibodies directed to cortical epithelial cells (A; ER-TR4); medullary epithelial cells (B; ER-TR5); fibroblasts (C; ER-TR7) and B lymphocytes (D; B220). The asterisk indicates a thymic cyst, lined by ER-$TR4^+$ and ER-$TR5^+$ epithelial cells. Bar, 75 μm.

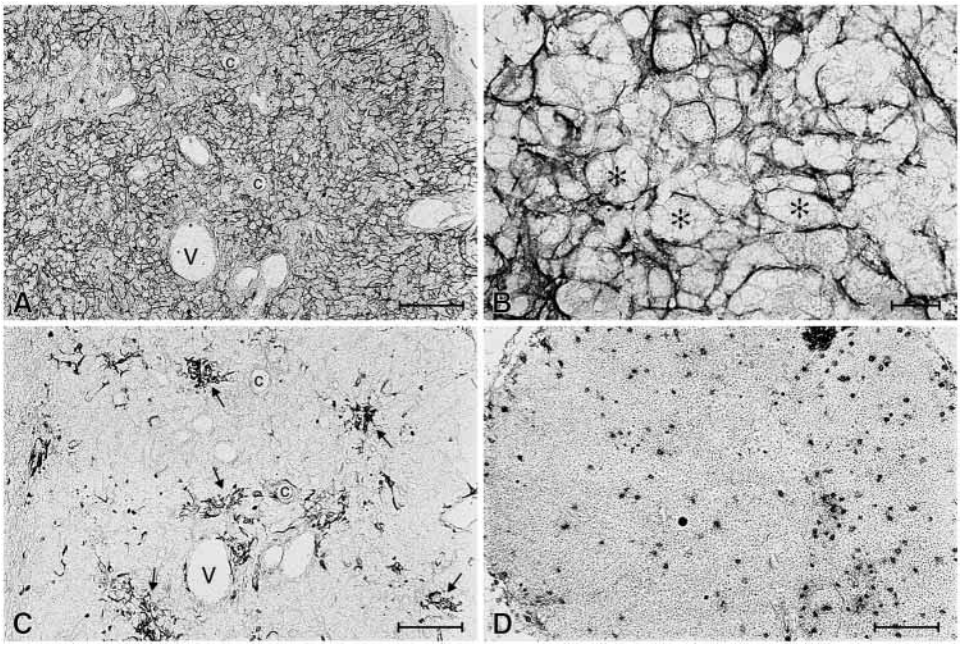

Fig. 4. Frozen sections of the left thymic lobe of the same tgε26 mouse, transplanted with RAG 2^{null} bone marrow. Comparison to Fig. 3 shows that the left thymic lobe is reconstituted showing a stromal architecture similar to thymic lobes in RAG^{null} mice (van Ewijk et al., 1999). Sections were incubated with monoclonal antibodies specific for cortical epithelial cells (A,B; ER-TR4), medullary epithelial cells (C; ER-TR5), and B lymphocytes (D; B220). Note loss of thymic cysts, establishment of a thee-dimensional cortical epithelial network (A), induction of thymic nurse cells (B, asterisks), loss of B cells (D). This type of reconstitution does not restore the medulla, only small clusters of medullary epithelial cells (C, arrows) are found. Bars, 75 μm (A,C,D); 13 μm (B).

thymus in RAG→tgε26 chimeras, the thymus in the double-transplanted tgε26 mice now also reveals expansion of the ER-$TR5^+$ medullary epithelial cells, which have regrouped into discrete medullary areas (Fig. 5C).

Importantly, a difference in reconstitution was observed between the present double-transplanted mice and the single bone marrow transplanted adult tgε26 mice, reported earlier (Holländer et al., 1995b). In the latter mice, T-cell development remained abnormal, and these mice developed a severe colitis, leading to death of the mice 5-8 weeks after bone marrow transplantation. In contrast, in the present experiments, none of seven double-transplanted mice analyzed showed any sign of colitis or other defects (data not shown).

DISCUSSION

Early stages in T-cell development are defined by absence of CD3, CD4 and CD8 (triple negative [TN] thymocytes). TN thymocytes can be further subdivided into four differentiation stages defined by antibodies directed to the markers CD44 (PgP-1) and CD25 (the α chain of the IL2 receptor). Thus, TN thymocytes progress subsequently from TN $CD44^+CD25^-$, TN $CD44^+CD25^+$, TN $CD44^-CD25^+$ to TN $CD44^-CD25^-$. Productive rearrangement of the TCRβ locus occurs in the TN $CD44^-CD25^+$ stage (Fehling and von Boehmer, 1997), immediately leading to the TN $CD44^-CD25^-$ stage. Thymocytes in this stage of development are precursors for

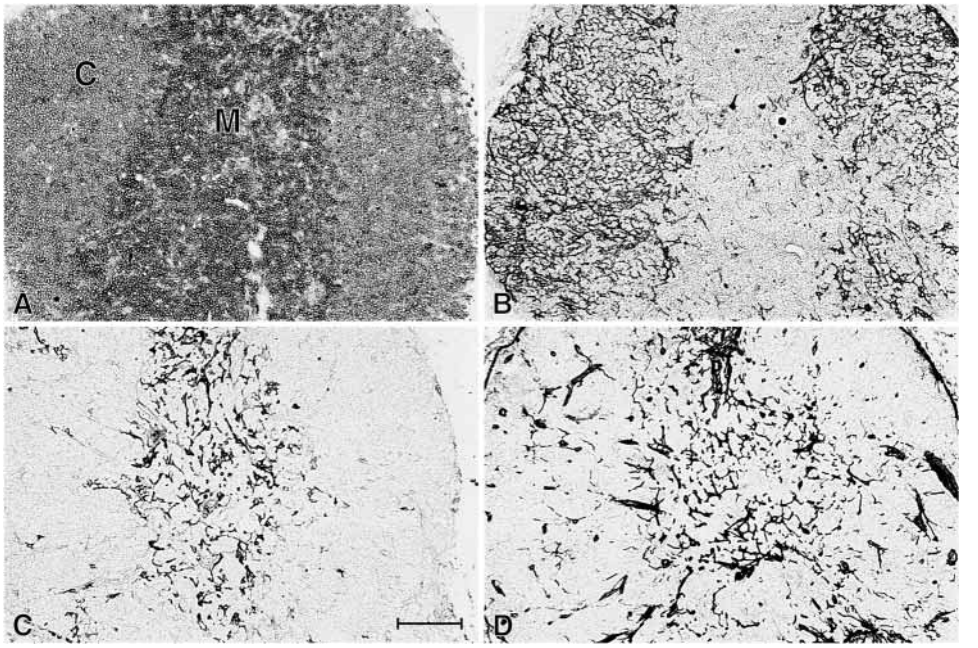

Fig. 5. Four adjacent frozen sections of a 'double' transplanted tgε26 mouse, incubated with monoclonal antibodies directed to the TCR-CD3 complex (KT3) on developing thymocytes (A); cortical epithelial cells (B; ER-TR4); medullary epithelial cells (C; ER-TR5). and fibroblasts (D; ER-TR7). Double-transplanted tgε26 mice (= normal bone marrow transplanted RAGnull→tgε26) show completely restored thymic microenvironments in cortex (C) and medulla (M). Bar, 75 μm.

$CD4^+$, $CD8^+$ (double positive, DP) thymocytes, which in turn develop in mature $CD4^+$ or $CD8^+$ (single positive, SP) thymocytes.

The two immunodeficient mouse strains used in the present study have a block in T-cell development within the window of TN thymocytes. While in tgε26 mice, due to the overexpression of the human CD3ε gene, T-cell development is blocked at the TN $CD44^+CD25^-$ stage, the null mutation in the Rag locus results in a slightly later arrest in T-cell development, phenotypically defined as: TN $CD44^-CD25^+$. Apparently, this small phenotypical shift in differentiation stage has a major influence on the composition and organization of the thymic stroma. Thus, a blockade in lymphoid development at the level of TN $CD44^+CD25^-$ leads to loss of the typical organization of the epithelial cells. In the normal thymus, epithelial reticular cells are oriented perpendicular to the thymic capsule, promoting cell traffic from cortex to medulla. By contrast, in tgε26 mice, epithelial cells are positioned parallel to the thymic capsule, impairing proper cell traffic through the thymus. Furthermore, the 3-D orientation of the epithelial network is replaced by a 2-D orientation in these mice, resulting in the formation of thymic cysts, which contain types of differentiated epithelial cells normally not present in the thymus but typical for the respiratory and gastrointestinal tracts. The loss of a 3-D epithelial network and the subsequent presence of thymic cysts is not a specific feature of tgε26 phenotype. Similar changes in thymic architecture have also been observed in other immunodeficient mouse strains, such as in Ikaros dominant –/– mice (W. v. E. and K. Georgopoulos, unpublished data), indicating that the 3-D→2-D conversion reflects a typical feature of thymic epithelium lacking inductive signals derived from developing thymocytes.

Our transplantation studies reflect the impressive plasticity of the thymic stroma. Moreover, the data provide direct evidence that phenotypically defined subsets of T-cell precursors regulate its morphogenesis. Apparently, the phenotypical transition of TN $CD44^+CD25^-$ cells into TN $CD44^-CD25^+$ cells is of crucial importance for the differentiation of cortical microenvironments. This developmental progression leads to three important changes in the architectural organization of the thymic stroma in RAG^{null}→tgε26 transplanted mice: (1) thymic nurse cells (TNC) are formed at a high frequency, (2) thymic fibroblasts decrease in frequency, and (3) the 2-D oriented epithelium is replaced by 3-D structured epithelial network,

The induction of a high frequency of TNC in the thymus of RAG^{null} mice and RAG^{null}→tgε26 mice is unexpected. Although precursors of TNC might be present in the thymus of untransplanted tgε26 mice, we were unable to detect their presence, because of the low frequency of thymocytes and due to lack of a specific marker for TNC. TNC were initially described in vitro, as lymphoepithelial complexes (Wekerle et al., 1980), and their existence was later confirmed in situ (van Ewijk, 1988). The function of TNC remains to be determined; they have been implicated in the induction of thymocyte proliferation (Gao et al., 1993), in positive selection (Pezzano et al., 1996) and in the induction of apoptosis (Aguilar et al., 1997). A recent ultrastructural study shows that the thymus contains at least three different types of TNC, each with different functions (Brelinska and Warchol, 1997). The TNC in RAG^{null} mice resemble the described type 1 TNC, which are indeed thought to be involved in the early expansion of T-cell progenitors, since many TNC complexes in RAG^{null} contain dividing lymphoid cells. Proliferating cells in TNC probably represent TN thymocytes, shifting from $CD44^+CD25^+$to $CD44^-CD25^+$. This latter subpopulation is subject to β selection (Fehling and von Boehmer, 1997), which raises the possibility that type 1 TNC control this crucial checkpoint in thymopoiesis.

The tgε26 thymus is characterized by a high frequency of B lymphocytes (Takoro et al., 1998). These cells predominantly develop within the thymus and co-localize with thymic fibroblasts rather than with thymic epithelial cells. The

capacity of the thymic microenvironment to support B lymphopoieses has also been demonstrated in recent in vitro experiments, using deoxyguanosin-treated fetal thymic lobes seeded with fetal-liver-derived B-cell progenitors. These thymic lobes show considerable growth and maturation of B lymphocytes. However, as in the tgε26 thymus, B lymphocytes prefer localization in areas where fibroblasts are predominantly present. Moreover, B lymphocytes crosstalk to fibroblasts and stimulate their expansion. In contrast, seeding of de-Guo lobes with T-cell progenitors restores thymopoieses, induces the 3-D organization of the epithelial network and reduces the frequency of thymic fibroblasts (W. v. E., W. T. V. Germeraad, H. Kawamoto and Y. Katsura, unpublished data). Likewise, injection of tgε26 mice with RAG^{null} bone marrow decreases both the frequency of B lymphocytes and the frequency of fibroblasts in the thymus. It seems likely therefore that, under experimental conditions where T-cell development is promoted in the thymic microenvironment, B lymphopoiesis decreases, resulting in diminished crosstalk to thymic fibroblasts.

Our experiments further indicate that generation of a regular cortex is a prerequisite for the development of the medulla. In earlier experiments (Holländer et al., 1995a), we observed that bone marrow transplantation of adult tgε26 mice with wild-type bone marrow did not lead to a complete restoration of T-cell development. The stromal architecture of the thymus in these transplanted mice remained disturbed. As a consequence, T-cell selection remained abnormal and the mice died after several weeks from severe colitis (Holländer et al., 1995b). In contrast, our present data show that sequential transplantation of tgε26 mice with RAG^{null} bone marrow at neonatal age, followed by bone marrow transplantation with wild-type cells at adult age, results in normal T-cell development. Thus, thymic epithelial cells in the tgε26 mutant mice are susceptible to crosstalk signals and can create a regular thymic architecture. Our experiments clearly demonstrate that these signals are provided by thymocytes beyond the TN $CD44^{+}CD25^{-}$ developmental stage and, more precisely, that TN $CD44^{-}CD25^{+}$ T-cell precursors present in the RAG^{null} (but not the tgε26) mice can mediate this inductive event for cortical epithelial cells. Moreover, these experiments also demonstrate that this induction of a regular cortex occurs during a developmentally restricted 'window' ranging from day 14.5 in gestation to the first days after birth (this paper; S. Zuklys, B. W., G. H., unpublished results). 'Preconditioning' of the cortical stroma by transplantation of RAG^{null} bone marrow cells is a prerequisite for a successful second transplantation with wild-type bone marrow at adult age. Apparently, T-cell progenitors in the normal bone marrow inoculate are able to migrate in this 'cortex-only' thymus, and are allowed to differentiate into TCRαβ-CD3-expressing DP thymocytes. In turn, these more mature thymocytes crosstalk to medullary epithelial cells, leading to reconstitution of medullary microenvironments (van Ewijk et al., 1994). At this stage, after complete repair of thymic microenvironments in both cortex and medulla, thymopoiesis results in the appearance of SP thymocytes. This corticomedullary interdependence was also noted in normal mice during ontogeny, where the thymic cortex (as defined by the presence of an ER-$TR4^{+}$ epithelial network) develops prior to the medulla (van Vliet et al., 1985).

So far, we have shown that plasticity of the thymic epithelial stroma is crucially important in the induction of thymic microenvironments. A logical question is whether the capacity to remodel is maintained throughout life. Analysis of the thymus in older mice indicates that the organization of medullary epithelial cells is affected from 6 months of age, while the cortical stroma is disrupted from 12 months of age (Hirokawa et al., 1994; Takeoka et al., 1996). These defects are, however, not a permanent feature of the old thymus. Unexpectedly, orchidectomy in mice even at an age of 24 months results in restored thymopoiesis, paralleled with complete restoration of cortical and medullary microenvironments (R. Boyd, personal communication), indicating that the plasticity of the thymic stroma is indeed maintained throughout life.

The cellular events regulating the induction of a normal cortical architecture have not yet been elucidated. At present, it is still unclear whether crosstalk between developing T cells and thymic epithelial cells induces proliferation and differentiation of epithelial cells, or more repositioning of thymic epithelial cells already differentiated into cells with a cortical and medullary phenotype, respectively. In this regard, two findings are of interest. First, a subpopulation of embryonic and neonatal thymic epithelial cells concomitantly express both cortical and medullary markers (Röpke et al., 1995). Such cells also exist at low frequency in the adult thymus and it has been postulated that these cells form a pool of thymic epithelial precursors. Second, Penit et al. (1996) have shown that, in RAG^{null} mice transplanted with normal bone marrow, cortical rather than medullary epithelial cells start to proliferate, while medullary cells are found 10-15 days after bone marrow transfer. This observation is consistent with the notion that medullary epithelial cells could differentiate from epithelial precursor cells in the cortex. In line with this observation is a recent report by Klug et al. (1998) indicating the presence of epithelial cell precursors at the corticomedullary junction.

The molecular mechanism underlying the plasticity of the thymic stroma is presently a focus of attention. *Wnt*-mediated signals are likely involved because Wnt proteins regulate proliferation, differentiation and cell fate of epithelial cells (Cadigan and Nusse, 1997), Moreover, Wnt proteins are expressed in a differential pattern by thymocytes at various maturational stages during intrathymic ontogeny. Recently performed co-cultures with thymic epithelial cell lines indicate that, upon lymphostromal interaction, the *Wnt* signaling pathway is activated, in turn leading to upregulation of *Fz* (Frizzled) members on epithelial cells (G. H., G. Balciunaite and S. Zuklys, unpublished data). Furthermore, angiogenesis is induced in tgε26 tg mice transplanted with RAG^{null} bone marrow cells, and angiopoetin was recently found to be differentially expressed in RAG thymi compared to unmanipulated tgε26 thymic tissue (G. H. and S. Zuklys, unpublished data). In line with this notion, we have found that, using in vitro cultured fetal thymic lobes, the oxygen concentration in the culture system critically regulates the induction of a 3-D organized thymic stroma in the thymic lobes (W. T. V. Germeraad, H. Kawamoto, Y. F. Jiang, Y. Katsura and W. v. E., unpublished data). Thus, increased blood supply to the reconstituted tgε26 thymus may be reflective of a role for oxygen and nutrients in the recovery of thymic microenvironments

Taken together, our data indicate that developing thymocytes

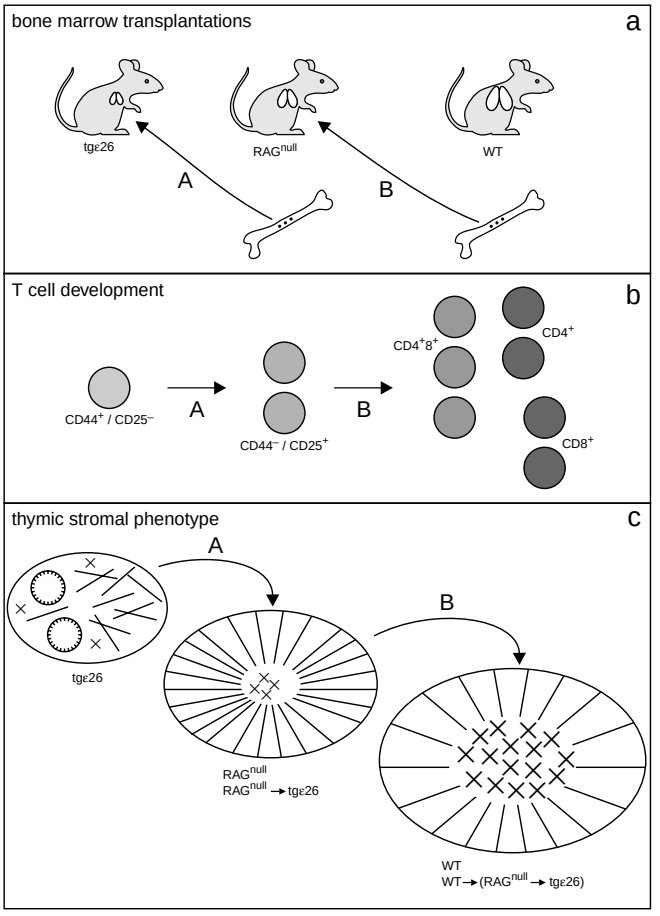

Fig. 6. Stepwise development of the thymic stroma. tgε26 mice display an arrest in T-cell development at the stage of TN $CD44^+$,$CD25^-$ and demonstrate in consequence severely disturbed thymic microenvironments, with loss of cortical and medullary areas and formation of thymic cysts. Transplantation of neonatal tgε26 mice with T-cell precursors able to progress to the stage of TN $CD44^-$,$CD25^+$ (transfer with RAG^{null} bone marrow) restores epithelial microenvironments in the cortex, but not in the medulla, creating a 'cortex only' thymus. In contrast, progression of T-cell development to the stage of late double positive, or mature single positive thymocytes (induced by a second transplantation with wild-type bone marrow) induces full restoration of epithelial microenvironments in the medulla.

play an important role in the functional organization of thymic microenvironments. As illustrated in Fig. 6, crosstalk between thymocytes and epithelial cells regulates the creation of thymic microenvironments in a stepwise fashion. Thus, early developing TN thymocytes provide signals to thymic cortical epithelial cells, which in turn start creation of functional 3-D organized cortical microenvironments. These differentiated microenvironments allow further progression in thymopoiesis, leading to the development of DP, TCRαβ-expressing thymocytes. Subsequently, TCR-expressing thymocytes crosstalk to medullary epithelial cells, leading to the generation of correct microenvironments in the medulla, enabling final steps in the maturation of single positive T cells. Hence, differentiating thymocytes control the morphogenesis of the thymic epithelium in a stepwise fashion.

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REFERENCES

Aguilar, L. K., Aguilar-Cordova, E., Cartwright, J. and Belmont, J. W. (1994). Thymic nurse cells are sites of thymocyte apoptosis. *J. Immunol.* **152**, 2645-2651.

Anderson, G., Moore, N. C., Owen, J. J. T. and Jenkinson, E. J. (1996). Cellular interactions in the thymocyte development. *Annu. Rev. Immunol.* **14**, 73-99.

Anderson, G., Jenkinson, E. J., Moore, N. C. and Owen, J. J. T. (1993). MHC class II positive epithelium and mesenchyme are both required for T cell development in the thymus. *Nature* 362, 70-73.

Anderson, G., Partington, K. M. and Jenkinson, E. J. (1998). Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J. Immunol.* **15**, 6599-6603.

Ashton-Rickardt, P. G. and Tonegawa, S. (1994). A differential-avidity model for T cell selection. *Immunol. Today* **15**, 362-366.

Auerbach, R. (1960). Morphogenetic interactions in the development of the mouse thymus gland. *Dev. Biol.* **2**, 271-284.

Bevan, M. J. (1997). In thymic selection, peptide diversity gives and takes away. *Immunity* **7**, 175-178.

Blackburn, C. C., Augustine, C. L., Li, R., Harvey, R. P., Malin, M. A., Boyd, R. L., Miller, J. F. A. P. and Morahan, G. (1996). The nude gene acts cell autonomously and is required for differentiation of thymic epithelial progenitors. *Proc. Natl. Acad. Sci. USA* **93**, 5742-5746.

Bockman, D. E. and Kirby, M. L. (1984). Dependance of thymus development on derivatives of the neural crest. *Science* **223**, 498-500.

Boehm, T., Nehls, M. and Kyewski, B. (1995). Two genetically separable steps in the differentiation of thymic epithelium. *Immunol. Today* **16**, 555-556.

Boyd, R. L., Tucek, C. L., Godfrey, I. D., Izon, D. J., Wilson, T. J., Davidson, N. J., Bean, A. G. D., Ladyman, H. M., Ritter, M. A. and Hugo, P. (1993). The thymic microenvironment. *Immunol. Today* **14**, 445-459.

Brelinska, R. and Warchol, J. B. (1997). Thymic nurse cells: their functional ultrastructure. *Microsc. Res. Tech.* **38**, 250-266.

Cadigan, K. M. and Nusse, R. (1997). Wnt signalling: a common theme in animal development. *Genes Dev.* **15**, 3286-3305.

Coffman, R. C. and Weissman, I. L. (1981). A monoclonal antibody that recognizes B cells and B cell precursors in mice. *J. Exp. Med.* **153**, 269-279.

de Koning, J., Molfetto, L. di, Reilly, C., Wei, Q., Havran, W. L. and Lo, D. L. (1997). Thymic cortical epithelium is sufficient for the development of mature T cells in RelB-deficient mice. *J. Immunol.* **158**, 2558-2566.

Fairchild, P. J. and Austin J. M. (1995). Developmental changes predispose the fetal thymus to positive selection of $CD4^+CD8^-$ T cells. *Immunology* **85**, 292-298.

Fehling H. J. and von Boehmer, H. (1997). Early αβ T cell development in the thymus of normal and genetically altered mice. *Curr. Opin. Immunol.* **9**, 263-275.

Frederickson, G. G. and Basch, R. S. (1989). L3T4 antigen expression by hemopoietic precursor cells. *J. Exp. Med.* **169**, 1473-1478.

Gao, X., Nishimura, T., Takeuchi, Y., Sudo, T. and Habu, S. (1993). Thymic nurse cell clone supports the differentiation of $CD4^-8^-$ thymocytes into CD4+8+ thymocytes in vitro. *Immunol. Lett.* **35**, 169-175.

Hirokawa, K., Utsuyama, M., Kasai, M., Kurashima, C., Ishijima, S. and Zeng, Y., X. (1994). Understanding the mechanism of the age-change of thymic function to promote T cell differentiation. *Immunol. Lett.* **40**, 269-277.

Holländer, G. A., Wang, B., Nichogiannopoulou, A., Platenburg, P. P., van Ewijk, W., Burakoff, S. J., Gutierrez-Ramos, J.-C. and Terhorst, C. (1995a). Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* **373**, 350-353.

Holländer, G. A., Simpson,S., Mizoguch, E., Nichogiannopoulou, A., She,

J., Bhan, A., Burakoff, S. J., Wang, B. and Terhorst, C. (1995b). Severe colitis resulting from aberrant thymic selection. *Immunity* **3**, 27-39,

Itoi, M. and Amagai, T. (1998). Role of fibroblastic cell lines in development of the mouse thymus anlage in organ culture. *Cell. Immunol.* **183**, 32-41.

Jameson, S. C. and Bevan, M. J. (1998). T cell selection. *Curr. Opin. Immunol.* **10**, 214-219.

Klug, D. B., Carter, C., Crouch, E., Roop, D., Conti, C. J. and Richie, E. R. (1998). Interdependence of cortical thymic epithelial cell differentiation and T lineage commitment. *Proc. Natl. Acad. Sci. USA* **95**, 11822-11827.

Kubo, R. T., Born, W., Kappler, J. W., Marrack, P. and Pigeon, M. (1989).). Characterization of a monoclonal antibody which detects all murine alpha-beta T cell receptors. *J. Immunol.* **142**, 2736-2742.

Kyewski, B. A., Momberg, F. and Schirrmacher, V. (1987). Phenotype of stromal cell-associated thymocytes in situ is compatible with selection of the T cell repertoire at an 'immature' stage of thymic T cell differentiation. *Eur. J. Immunol.* **17**, 961-967.

Laufer, T., Glimcher, L. H. and Lo, D. L. (1999). Using thymus anatomy to dissect T cell repertoire selection. *Sem. Immunol.* **11**, 65-70.

Ledbetter, J. A. and Herzenberg, L. A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47**, 63-90.

Leo, O., Foo, M., Sachs, D. H., Samilson, L. E. and Bluestone, J. A. (1987). Identification of a monoclonal antibody specific for murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* **84**, 1374-1378.

Lesley, J. and Trowbridge, I. S. (1982). Characterization of a polymorphic murine cell-surface glycoprotein. *Immunogenetics* **15**, 313-320.

Lo, D. L., Reilly, C. R., Burkly, L. C., de Koning, J., Laufer, T. M. and Glimcher, L. H. (1997). Thymic stromal cell specialization and the T cell receptor. *Immunol. Res.* **16**, 3-14.

Malek, T. R., Robb, R. J. and Shevach, E. M. (1983). Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA* **80**, 5694-5698.

Manley, N. R. and Capecchi, M. R. (1995). The role of Hoxa-3 in mouse thymus and thyroid development. *Development* **121**, 1989-2003.

Nehls, M., Kyewski, B., Messerle, M., Waldschütz, R., Schüddekopf, K., Smith, A. J. H. and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science* **272**, 886-889.

Owen, J. J. T. and Jenkinson, E. J. (1984). Early events in T lymphocyte genesis in the fetal thymus. *Am. J. Anat.* **170**, 301-310.

Penit, C., Lucas, B., Vasseur, F., Rieker, T. and Boyd, R. L. (1996). Thymic medulla epithelial cells acquire specific markers by post mitotic maturation. *Dev. Immunol.* **5**, 25-36.

Pezzano, M., Philp, D., Stephenson, S., Li, Y., Reid, V., Maitta, R. and Guyden, J. C. (1996). Positive selection by thymic nurse cells requires IL1-beta and is associated with an increased Bcl-2 expression. *Cell. Immunol.* **169**, 174-184.

Röpke, C., Soest, P., van Platenburg, P. P. and van Ewijk, W. (1995). A common stem cell for murine cortical and medullary thymic epithelial cells. *Dev. Immunol.* **4**, 149-156.

Sieckmann, D. (1984). A monoclonal mouse antibody that recognizes a mouse IgM allotypic determinant (Igh-6. 1). *Federation Proceedings* **43**, 488.

Sprent, J., Lo, D. L., Gao, E. K. and Ron, Y. (1988). T cell selection in the thymus. *Immunol. Rev.* **101**, 173-190.

Takeoka, Y., Chen S. Y., Yago, H., Boyd, R. L., Suehiro, S., Schultz, L. D, Ansari A. A. and Gershwin, M. E. (1996). The murine thymic microenvironment: changes with age. *Int. Arch. All. Immunol.* **111**, 5-12.

Takoro, Y., Sugawara, T., Yaginuma, H., Nakauchi, H., Terhorst, C., Wang, B. and Takahama, Y. (1998). A mouse carrying a genetic defect in the choice between T and B lymphocytes. *J. Immunol*. **161**, 4591-4598.

Unkeless, J. C. (1979). Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte receptors. *J. Exp. Med.* **150**, 580-596.

van Ewijk, W. (1988). Cell surface topography of thymic microenvironments. *Lab. Invest.* **59**, 579-590.

van Ewijk, W. (1991). T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol.* **9**, 591-615.

van Ewijk, W., Shores, E. W. and Singer, A. (1994). Crosstalk in the mouse thymus. *Immunol. Today* **15**, 214-217.

van Ewijk, W., Wang, B. P., Holländer, G., Kawamoto, H., Spanopoulou, E., Itoi, M., Amagai, T., Jiang, Y., Germeraad, W. T. V., Chen, W. F. and Katsura, Y. (1999). Thymic microenvironments, 2-D versus 3-D? *Sem. Immunol.* **11**, 57-64

van Vliet, E., Jenkinson, E. J., Kingston, R., Owen, J. J. T. and van Ewijk, W. (1985). Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur. J. Immunol.* **15**, 675-681.

van Vliet, E., Melis M. and van Ewijk, W. (1984). Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur. J. Immunol.* **14**, 524-529.

Wallin, J., Eibel, H., Neubuser, A., Willing, J., Koseki, H. and Balling, R. (1996). Pax-1 is expressed during development of the thymus epithelium and is required for normal T cell maturation. *Development* **122**, 22-30.

Wang, B., Biron, C., She, J., Higgins, K., Sunshine, MJ., Lacy, E., Lonberg, N. and Terhorst, C. (1994). A block in both early T lymphocyte and natural killer cell development in transgenic mice with high copy numbers of the human CD3ε gene. *Proc. Natl. Acad. Sci. USA* **91**, 9402-9406.

Wang, B., Levelt, C., Salio, M., Zheng, D., Sancho, J., Liu, C. P., She, J., Huang, M., Higgins, K., Sunshine, M. J., Eichmann, K., Lacy, E., Lonberg, N. and Terhorst, C. (1995). Over-expression of CD3ε transgenes blocks T lymphocyte development. *Int. Immunol.* **7**, 435-448.

Wekerle, H., Ketelsen, U.-P. and Ernst, M. (1980). Thymic nurse cells: Lymphoepithelial cell complexes in murine thymuses. Morphological and serological characterization. *J. Exp. Med.* **151**, 925-944.