Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self

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Expression of peripheral antigens in the thymus has been implicated in T cell tolerance and autoimmunity. Here we identified medullary thymic epithelial cells as being a unique cell type that expresses a diverse range of tissue-specific antigens. We found that this promiscuous gene expression was a cell-autonomous property of medullary epithelial cells and was maintained during the entire period of thymic T cell output. It may facilitate tolerance induction to self-antigens that would otherwise be temporally or spatially secluded from the immune system. However, the array of promiscuously expressed self-antigens appeared random rather than selected and was not confined to secluded self-antigens.

Early concepts of immunological self-tolerance postulated that lymphocytes are susceptible to induction of tolerance during the fetal and neonatal phases of life[1]. With the advent of T and B cell receptor–transgenic mice it became clear that individual lymphocytes are particularly susceptible to tolerance induction during the immature phase of their development[2,3]. However, a strict limitation of tolerance susceptibility imposed during the immature stage of lymphocytes—that is, during the intrathymic development of T cells—restricts the scope of T cell tolerance to those self-antigens that are presented by thymic antigen-presenting cells. It is hardly conceivable that this set of self-antigens should encompass all the self-antigens expressed by parenchymal organs. Accordingly, the thymus is regarded as the site of induction of tolerance to ubiquitously expressed proteins and to abundant blood-borne self-antigens. In contrast, the prevailing idea of tolerance to self-antigens that

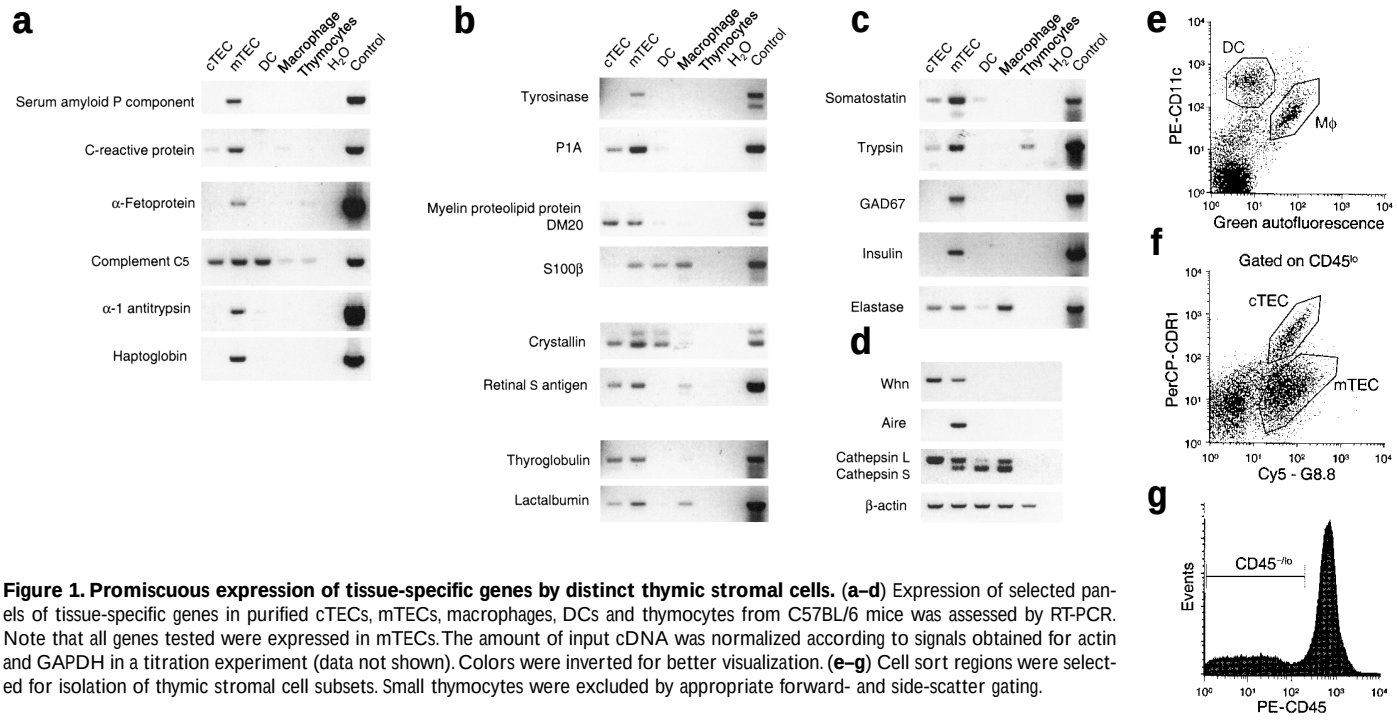

Figure 1. Promiscuous expression of tissue-specific genes by distinct thymic stromal cells. (**a–d**) Expression of selected panels of tissue-specific genes in purified cTECs, mTECs, macrophages, DCs and thymocytes from C57BL/6 mice was assessed by RT-PCR. Note that all genes tested were expressed in mTECs. The amount of input cDNA was normalized according to signals obtained for actin and GAPDH in a titration experiment (data not shown). Colors were inverted for better visualization. (**e–g**) Cell sort regions were selected for isolation of thymic stromal cell subsets. Small thymocytes were excluded by appropriate forward- and side-scatter gating.

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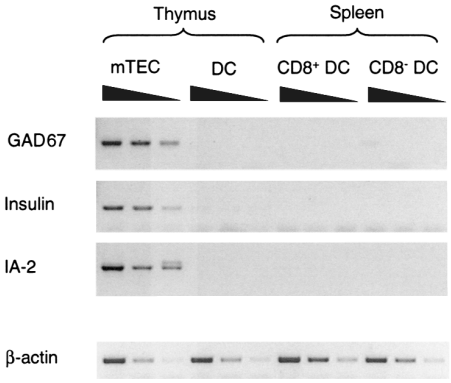

Figure 2. Expression of pancreatic autoantigens in mTECs *versus* thymic or splenic DCs. Expression of three pancreatic autoantigens in purified mTECs, thymic DCs and splenic myeloid and lymphoid DCs was assessed by RT-PCR. A fivefold cDNA dilution-series from the indicated populations were used for amplification.

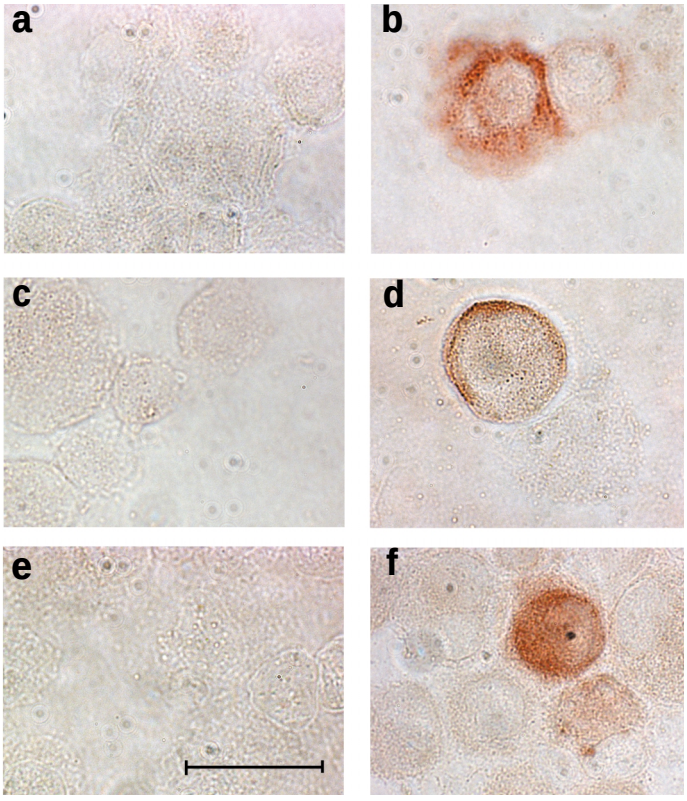

Figure 3. Protein expression of tissue-antigens by mTECs. Protein expression of (**a,b**) PLP (**c,d**) insulin and (**e,f**) P1A by purified mTECs. Control staining included (**a**) secondary antibody alone for PLP (**c**) specific blockade by free insulin for insulin and (**e**) preimmune serum for P1A. mTEC protein expression was heterogenous. Bar, 20 μm.

are confined to specific tissues is that it is facilitated by post-thymic peripheral mechanisms[4] or that such antigens may be ignored by the immune system[5]. This simplistic delineation of central *versus* peripheral tolerance has been challenged by the finding that expression of tissue-specific antigens in the thymus may be a more common occurrence than previously thought[6,7]. This phenomenon, which is termed ectopic or promiscuous gene expression, appears to be a specific feature of the thymus, rather than a reflection of general leakiness of tissue-specific genetic control. We advocate use of the term "promiscuous gene expression" to distinguish this physiological property from the term "ectopic", which is frequently used in the context of deliberate experimental expression of transgenes in an unphysiological context.

Accruing evidence suggests a functional role for promiscuous gene expression in the induction of T cell tolerance; allelic or strain-specific variations in the intrathymic expression of certain tissue antigens correlate with susceptibility to organ-specific autoimmunity[8–11]. Intrathymic expression of myelin proteolipid protein (PLP) confers central T cell tolerance in mice[12]. In addition, the absence of a particular protein segment (exon 3b) of PLP in the thymus due to alternative splicing leads to a lack of tolerance to this segment and offers an explanation for the role of this protein segment in major histocompatibility complex (MHC)-dependent susceptibility to experimental autoimmune encephalomyelitis (EAE)[12,13] and possibly multiple sclerosis in humans[14].

The cellular and molecular regulation of such promiscuous intrathymic expression of specific tissue antigens is as yet poorly characterized. Based on the analysis of pancreatic self-antigens, the existence of rare "peripheral antigen–expressing" (PAE) cells in the thymus is proposed[6,15]. The identity of such cells, however, remains controversial. Thymic insulin-expressing cells segregate into a low-density fraction that is enriched with dendritic cells (DCs)[15], and colocalization of insulin and other pancreatic hormones with markers of the DC- and macrophage-lineage has been reported in the murine[16] and human thymus[17]. In contrast, based on functional criteria or analysis of cell preparations enriched for cytokeratin$^+$ cells, other reports ascribe expression of tissue-specific genes to thymic epithelial cells (TECs)[15,18–20]. Thus, in several model systems where expression of transgenes controlled by tissue-specific promoters occurs in the thymus, the tolerogenic competence of the thymus is transplantable and persists after reconstitution with bone marrow of nontransgenic origin[15,18–20]. Intrathymic expression of acute-phase proteins, a class of inducible liver antigens, is restricted to rare medullary epithelial cells (mTECs)[20,21]. In addition, the expression of a transgenic human acute-phase protein in mTECs from mouse thymus mediates efficient negative selection of specific CD4$^+$ T cells[20,21].

This study aimed to assess the cellular expression pattern and regulation of promiscuous gene expression in mice based on the isolation of highly pure thymic stromal cell subsets. Our analysis identified mTECs as a specialized cell type in which promiscuous expression of a broad range of tissue-specific genes was an autonomous property[20,21].

Results

Peripheral tissue gene expression in the thymus

To address gene expression in thymic stromal cells, we enriched distinct subsets of these cells to high purity by a combination of density fractionation and cell sorting. Identification of the four major subsets of thymic stromal cells—cortical epithelial cells (cTECs, with the phenotype CD45$^{-/lo}$CDR1$^+$G8.8$^+$), mTECs (with the phenotype CD45$^{-/lo}$ CDR1$^-$G8.8$^+$), DCs (with the phenotype CD11c$^+$F4/80$^-$) and macrophages (with the phenotype CD11c$^-$F4/80$^+$)—was based on surface markers expressed by the respective subpopulations (**Fig. 1**). Complementary DNA was generated by reverse transcription of purified RNA and used to analyze expression of various genes by polymerase chain reaction (PCR). In addition to the surface markers used for cell sorting, expression of several indicator genes of known expression pattern was monitored to test the purity of stromal cell preparations. Thus, expression of *whn*, which encodes a transcription factor involved in thymus development[22], was restricted to epithelial cells. Among epithelial cells, the lack of expression of cathepsin-S in

Table 1. Summary of the cell type–specific pattern of promiscuous gene expression

	SAP	CRP	Complement C5	α-fetoprotein	Albumin	Haptoglobin	α1-antitrypsin	PLP	MOG	S100β	GAD67[a]	GAD65[a]	Somatostatin	Trypsin2	Elastase	Insulin[a]	Tyrosinase	Gp100	P1A	Thyroglobulin	Lactalbumin	α1-crystallin	Retinal S antigen	NAChRα1	IFABP	Amylase1
cTECs	–	–/+	+	–	–	–	–	+	–	–	–	–	–/+	–/+	+	–	–	+	–/+	+	–/+	+	+	–	–/+	+
mTECs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DCs	–	–	+	–	–	–	–	–/+	–	+	–	–	–/+	–	–/+	–	–	+	–/+	–	–	+	–	–	–	–
Macrophages	–	–	–/+	–	–	–	–	–/+	–	+	–	–	–	–	+	–	–	+	–	–	–/+	–/+	+	–	–	–/+

Genes are grouped according to their derivation from the organs of C57BL/6 mice. Note that all genes were expressed in mTECs. For designation of signal strength see Fig. 1. –, no signal ; –/+, no or weak signal in duplicate analysis; +, reproducible signal; nAChRα1, nicotinic acetylcholine receptor α1; gp100, melanosomal protein silver–Pmel17–gp87; iFABP, intestinal fatty acid binding protein; MOG, myelin oligodendrocyte glycoprotein. [a]Genes were also expressed by mTECs from NOD mice.

cTECs[23] and the restricted expression of *Aire*, which encodes the putative transcription factor autoimmune regulator (AIRE)[24,25] in mTECs, indicated efficient purification. Amplification of indicator genes was done with 30 cycles, whereas 40 cycles were used for the detection of tissue antigens.

The expression of a diverse set of genes that encompassed cell surface proteins, enzymes, hormones, structural proteins and genes of unknown function but restricted tissue distribution was analyzed in different thymic cell lineages. The analysis included genes that were originally assumed to be organ-specific and whose promotors are active in the thymus[26–28], as well as tissue-specific antigens whose expression in the thymus had not been addressed yet.

We found that all the genes assessed were expressed in mTECs. However, the expression pattern of tissue antigens was complex: antigens such as PLP and the complement component C5 were also detectable in cTECs and hematopoietic cells, respectively (**Fig. 1** and **Table 1**). Because the amount of cDNA used in individual reactions was normalized to β-actin amounts, in addition to qualitative assessment, the PCR analysis allowed a degree of quantitative assessment of gene expression. Thus we found that expression of the group of antigens that was detected in both mTECs and cTECs (PLP, elastase and thyroglobulin) appeared less strictly controlled and was frequently expressed by other cell types. In contrast, ~50% of the genes analyzed were far more abundantly, if not specifically, expressed in mTECs. This latter group included genes encoding serum amyloid P component (SAP), C-reactive protein (CRP), α-fetoprotein, glutamic acid decarboxylase (GAD67), insulin and tyrosinase.

Contrary to reports based on the histological examination of murine[16] and human[17,29] thymi, expression of the pancreatic autoantigens insulin, GAD67 and IA-2 (insulinoma-associated tyrosine phosphatase-like protein) could clearly be ascribed to mTECs. Under these PCR conditions no signal was obtained with cDNA prepared from thymic DCs or myeloid or lymphoid DCs isolated from the spleen (**Fig. 2**). Highly reproducible results were obtained from more than ten independent cell preparations from C57BL/6 mice. In addition, a selected panel of genes was analyzed in SJL/J and nonobese diabetic (NOD) mice; similar expression was observed (data not shown). Intrathymic expression of tissue antigens was consistently observed in mTECs and was therefore analyzed in more detail.

Specific protein expression was also examined in purified mTECs by immunostaining sorted thymic cells. In each case only a minor fraction of the mTECs stained strongly with the respective antibody (**Fig. 3**). While insulin-, somatostatin- and P1A-expressing cells were present at a frequency of 1 in 50–200, PLP$^+$ and S100β$^+$ cells were present more frequently (up to 1 in 20). The immunohistochemical data documented expression of tissue antigens by mTECs at the protein level, thus corroborating the RNA data. Protein expression appeared heterogenous with strong expression being restricted to a minor fraction of mTECs.

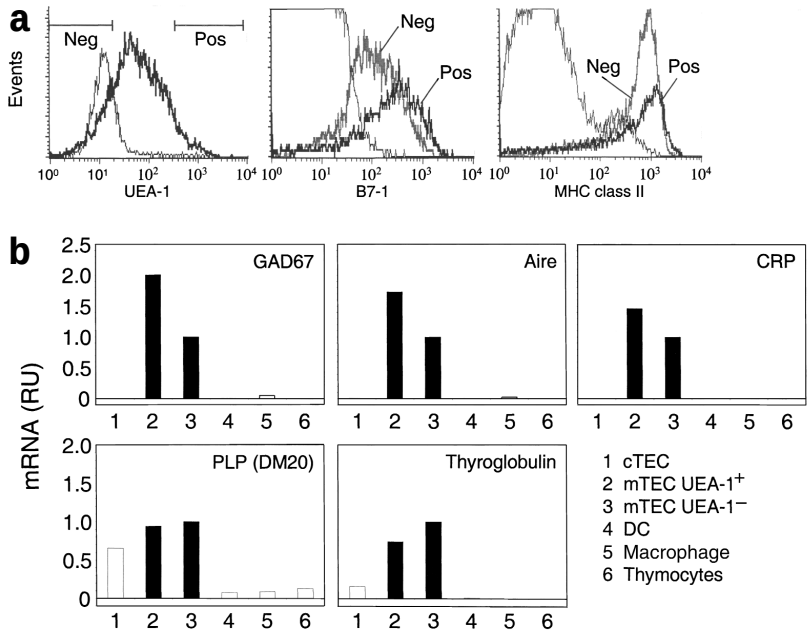

Figure 4. Promiscuous gene expression by subsets of mTECs. (**a**) Binding levels of the lectin UEA-1 were correlated with MHC class II IA^d and B7-1 expression in BALB/c mice. Medullary TECs, defined as negative or positive for UEA-1 binding in the left panel, were gated and analyzed for coexpression of B7-1 and MHC class II (middle and right panel). Control staining (thin lines) included UEA-1 binding on cTECs and B7-1 and MHC class II expression on thymocytes. (**b**) mTECs from C57BL/6 mice were subdivided into UEA-1$^+$– and UEA-1$^-$–binding subsets and promiscuous expression of a selected panel of genes was assessed by RT-PCR. Both subsets showed promiscuous gene expression. Signals were normalized to actin; expression of UEA-1$^-$ cells was arbitrarily defined as 1.0.

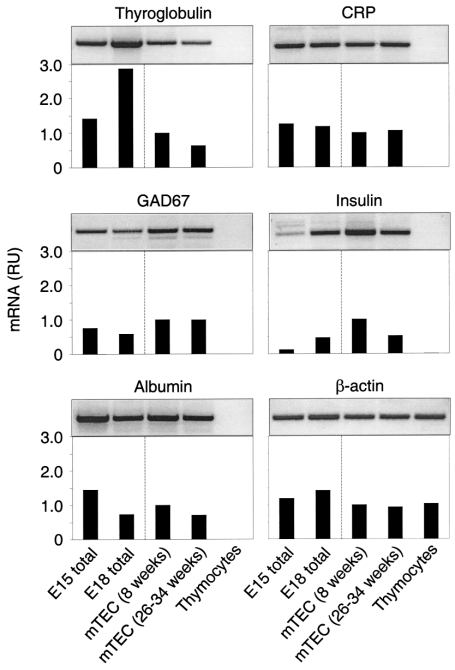

Figure 5. Promiscuous gene expression during ontogeny. Thymic expression of five tissue-specific genes was monitored during ontogeny. Expression of all genes tested was detectable at embryonic day 15 (E15) and was fully maintained into late adulthood. Due to different mTEC contents in the different preparations (note that prenatally unseparated thymus and postnatally purified mTEC were analyzed) approximately 30-fold more embryonic thymus cDNA was used in the RT-PCRs. mTEC expression in 8-week-old mice was arbitrarily defined as 1.0.

Promiscuous gene expression in mTEC subsets

Because only a small fraction of the G8.8$^+$CDR1$^-$ cells expressed a particular peripheral protein, we surmised that promiscuous gene expression might be segregated to a particular subset of mTECs. Medullary epithelial cells in the thymus are heterogenous with respect to phenotype and, possibly, function. There exists a distinct subset of mTECs that express a carbohydrate-epitope that binds to the lectin Ulex europaeus 1 (UEA-1)[30]. These UEA-1$^+$ mTECs have been implicated in the negative selection of endogenous superantigen-reactive thymocytes and may represent a more mature stage of mTEC development[30,31]. We addressed whether promiscuous gene expression may be a property of the UEA-1$^+$ mTEC subset. G8.8$^+$CDR1$^-$ mTECs were sorted into UEA-1$^-$ and UEA-1$^+$ cells, which showed that UEA-1 binding correlated positively with expression of B7-1 and, to a lesser degree, with the density of MHC class II (**Fig. 4a**). Analysis by RT-PCR revealed that expression of a number of representative tissue antigens, including myelin oligodendrocyte glycoprotein (MOG), glutamic acid decarboxylase 65 (GAD65) and acetylcholine receptor (AchR), was detectable in both subsets (**Fig. 4b** and data not shown), with a tendency for higher expression in UEA-1$^+$ mTECs. Promiscuous gene expression thus appeared to be a common property of phenotypically distinct mTEC subsets.

Promiscuous gene expression during ontogeny

The bulk of the T cell repertoire in mice is generated during the early postnatal phase. Thymic output declines after puberty but is still maintained into adulthood[32]. Thus expression of tissue-specific genes in the thymus, if it were to shape the self-reactive T cell repertoire, should be maintained as long as productive T cell differentiation proceeds. We

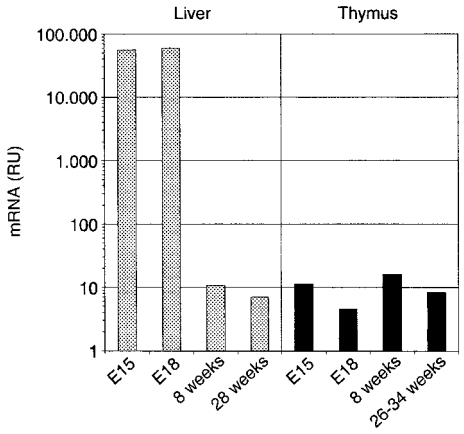

Figure 6. Promiscuous expression of a fetal tissue antigen. Pre- and postnatal expression of α-fetoprotein was compared in hepatocytes and thymi. Each sample was appropriately diluted to determine the relative signal strength within the linear amplification range.

therefore monitored promiscuous gene expression during ontogeny. Analysis of the fetal thymus was carried out on unseparated thymus cells, whereas purified mTECs were sampled from the postnatal thymus. A selected panel of genes was analyzed at gestation day 15 or 18; we found that they were all expressed prenatally (**Fig. 5**). When the expression of GAD67, CRP, thyroglobulin, insulin and albumin by mTECs from C57BL/6 mice aged 8 weeks and 26–34 weeks were compared, no age-related decline in gene expression was found. The same applied for expression of Whn and AIRE.

Fetal antigens are unusual with regard to their temporal gene regulation because their expression precipitously declines during the perinatal period. Tolerance to such fetal antigens may be compromised in adults as a result of limited exposure to the developing immune system. Thus, we compared thymic and tissue-specific regulation of α-fetoprotein, a prototypic fetal antigen. Although expression of α-fetoprotein was down-regulated by several orders of magnitude in adult liver compared to fetal liver, its expression in mTECs remained unaltered into adulthood (**Fig. 6**). This unabated postnatal expression in the thymus indicated a difference between the genetic control of a developmentally regulated gene in mTECs and hepatocytes.

The organization of stromal cell compartments in the thymus depends on unperturbed development of the T cell lineage[33,34]. Arrest of T cell development at distinct stages in various mouse mutants results in a failure to develop intact cortical and/or medullary compartments. We therefore assessed the dependence of promiscuous gene expression on unperturbed T cell development using two mutant lines.

In recombination-activating gene 2 (RAG-2)–deficient mice, T cell development is blocked at the CD4$^-$CD8$^-$ double-negative (DN) stage III (CD44$^-$CD25$^+$): immature CD4$^+$CD8$^+$ double-positive (DP) and mature CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ single-positive (SP) thymocytes are absent[35]. As a consequence, the thymic cortex and medulla are highly hypotrophic, yet the cTECs and mTECs are properly organized[34]. As expected, we found that the mTEC yield was reduced in thymi from RAG-2–deficient mice. When compared to expression in wild-type C57BL/6 mice, however, expression of various tissue-specific genes was unaffected. Epithelial cells from RAG-2–deficient mice defined by their cortical phenotype (CDR1$^+$G8.8$^+$) even expressed a wider range of genes than their wild-type counterparts, including *Aire* (**Fig. 7**). This

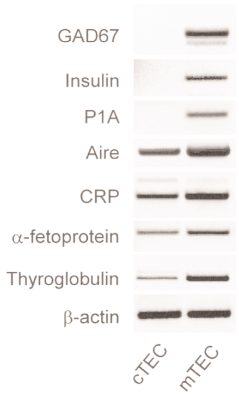

Figure 7. Promiscuous gene expression in RAG-2$^{-/-}$ mice. The expression pattern of a selected panel of promiscuously expressed genes in TECs from RAG-2$^{-/-}$ mice was analyzed.

pattern persisted after the reconstitution of RAG-2–deficient mice with wild-type bone marrow (data not shown).

In MHC class I and MHC class II double-deficient mice[36] T cell development is arrested at the transition from DP to SP thymocytes, which results in a condensed and hypotrophic medulla. Nevertheless, expression of five representative peripheral genes was clearly detectable in total thymus cDNA from these mice (data not shown).

Taken together, these findings indicated an autonomous regulation of promiscuous gene expression in thymic epithelial cells that was independent of age-related thymic involution, intact T cell development and developmental gene regulation in the respective peripheral tissue.

Promiscuous gene expression in thymus *versus* testis

At certain stages of male gametogenesis a broad range of genes is expressed[37]. The underlying mechanism and the biological significance of this phenomenon remain elusive, however. We asked whether the spectrum of ectopically expressed genes is distinct or shared between mTECs and testis. We analyzed the expression of several genes in cDNA prepared either from whole testis or highly enriched immature gametocytes (spermatogonia). Of 19 tissue-specific genes expressed in the thymus, 14 were also expressed in testis (**Table 2**). The transcription factors Whn and AIRE, both of which have been ascribed specific roles in thymus biology, were also found in male gametocytes.

Table 2. Expression of tissue-specific genes in thymus *versus* testis

Genes expressed in thymus and testis	Genes expressed in thymus but not in testis
Complement C5	α-fetoprotein
CRP	Elastase
Somatostatin	Insulin
Trypsin2	SAP
GAD67	Tyrosinase
P1A	
S100β	
PLP/DM20	
α1-crystallin	
Retinal S antigen	
Thyroglobulin	
Lactalbumin	
Aire	
Whn	

Gene expression was analyzed in young adult C57BL/6 mice. Testis fraction II was highly enriched in immature gametocytes (spermatogonia). Genes absent in fraction II were also absent from mature sperm and unfractionated testis cell preparations.

Promiscuous gene expression and tolerance

Although a link between intrathymic expression of tissue-specific genes and tolerance induction has been presumed, it remains to be demonstrated for each antigen individually whether thymic expression is sufficient and/or necessary for tolerance induction. Here, we have addressed tolerance to the major acute phase protein in mice, SAP[38,39]. SAP belongs to the group of peripheral genes whose expression was restricted to mTECs (**Fig. 1**). Immunization of SAP-deficient[40] and wild-type mice with purified SAP showed a strongly diminished *in vitro* recall response in wild-type compared to SAP-deficient mice, which indicated CD4$^+$ T cell tolerance to SAP (**Fig. 8a**). To dissect the contribution of mTEC- or liver-derived antigen to SAP-specific tolerance, we generated two sets of chimeric mice. Expression of SAP was either exclusively restricted to mTECs (a SAP$^{+/+}$ thymus was transplanted into thymectomized SAP$^{-/-}$ recipients) or SAP expression by mTECs was excluded (a SAP$^{-/-}$ thymus was transplanted into thymectomized SAP$^{+/+}$ recipients). In the latter

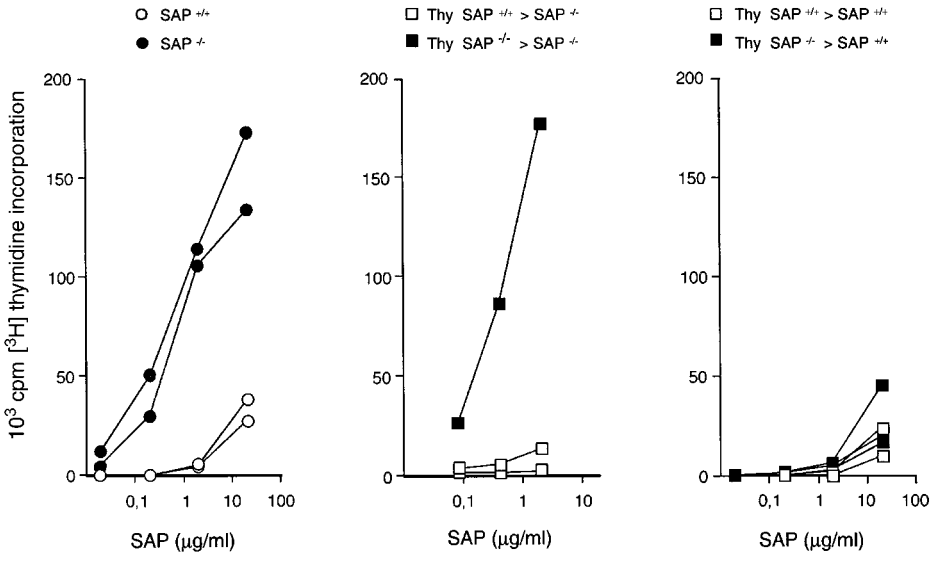

Figure 8. Expression of SAP in mTECs is sufficient, but not necessary for T cell tolerance. The *in vitro* recall proliferative response of local lymph node cells 9 days after immunization with SAP was measured and the responses of individual mice with a comparable PPD response are shown. (**a**) Comparison of the SAP-specific immune response of C57BL/6 wild-type mice and SAP$^{-/-}$ mice. (**b**) Thymectomized SAP$^{-/-}$ (–/–) animals that had been grafted with a wild-type (+/+) thymus and, after lethal irradiation, reconstituted with SAP$^{-/-}$ bone marrow were immunized with SAP. (**c**) Thymectomized SAP wild-type (+/+) animals that had been grafted with a SAP$^{-/-}$ thymus (–/–) and, after lethal irradiation, reconstituted with wild-type bone marrow were immunized with SAP

case the liver most likely represented the exclusive source of SAP. Two months after irradiation and reconstitution with bone marrow, the chimeric mice were immunized with purified mouse SAP and tested for the proliferative response of draining lymph node T cells *in vitro*. T cell reactivity was highly diminished in mice that were exclusively expressing SAP in the thymus, that is, the tolerant wild-type phenotype was recapitulated (**Fig. 8b**). Corresponding inverse chimeras in which mTEC SAP expression was specifically lacking were analyzed in the same manner. Again, the response upon immunization with SAP was markedly diminished compared to SAP-deficient controls (**Fig. 8c**). This indicated that tolerance to SAP could also be maintained by liver-derived serum-borne SAP. Thus, in the case of SAP, promiscuous expression in mTECs was sufficient, but not necessary, to induce $CD4^+$ T cell tolerance.

Discussion

We have shown that intrathymic expression of tissue-specific genes is a distinctive characteristic of mTECs. Such a broad range of tissue-antigen expression was not found in other epithelial cells, that is, cells from the duodenum and salivary gland (data not shown). Because, without exception, transcription of all the tissue antigens tested was detectable in mTECs, we surmise that a much wider range of self-antigens is expressed in mTECs. Promiscuous expression of tissue antigens in mTECs is distinct from illegitimate transcription, which refers to the existence of one or a few transcripts of any gene in any cell[41,42]. The expression pattern of peripheral genes in stromal cells other than mTECs appeared complex and did not seem to correlate with the structural or functional commonalities of their gene products or with the cell lineage or germ layer–derivation of the respective peripheral tissue.

Such indiscriminate gene expression may seem incompatible with the intact physiology of a highly differentiated cell type. Promiscuous gene expression, however, is not unique to mTECs; it also occurs in developing spermatocytes[37]. We found there was a considerable overlap of the genes that were promiscuously expressed in both cell types. Although the biological significance of widespread gene expression in male gametocytes has not yet been determined, it obviously does not interfere with their complex physiology.

Several features of promiscuous gene expression described here appeared particularly conducive to the induction of T cell tolerance. The scope and level of promiscuously expressed genes was maintained during ontogeny along with the productive generation of T cells, even in the case of genes that showed stringent developmental regulation in the respective peripheral tissue. In addition, promiscuous gene expression remained unaffected when T cell development was perturbed and thus appeared to be an autonomous property of mTECs.

Analysis of gene expression profiles in yeast revealed a degree of discordance between the relative expression levels of mRNA species and the corresponding proteins[43]. Because of this, it was important to confirm the expression of proteins. The expression pattern of tissue antigens at the protein level was characterized by strong positive staining of an mTEC subset. The frequency of expressing cells varied within a range of 1 in 20–200, depending on the antigen. Given an average yield of 22,000 mTECs per thymus, the absolute numbers of $antigen^+$ cells ranged between 100–1000 per thymus for a particular antigen. These frequencies, which represented minimal estimates due to unspecific losses during the isolation procedure, agreed well with previous estimates based on histological analysis[15].

The finding that, when analyzed in single cells, promiscuous gene expression appeared confined to a minor fraction of cells in the medulla[15,21,44] has been interpreted to indicate the existence of a specialized subtype of thymic stromal cells[6]. We found that both UEA^+ and UEA^- epithelial cells showed promiscuous gene expression, albeit in slightly different amounts. An alternative explanation for the paucity of tissue antigen–expressing cells may be that gene expression in the mTECs was subject to temporal fluctuations within individual cells. Such variations in time may mitigate the eventual deleterious effects of an unphysiological gene product, yet they warrant the expression of a diverse array of peripheral antigens within the cell populations.

The RNA expression analysis presented here needs to be extended not only to the corresponding proteins but also to peptide presentation and ultimately tolerance imposition. The latter analysis must be conducted for each individual antigen; only broad data collection will allow an assessment of the overall contribution of promiscuous gene expression to the maintenance of self-tolerance. Spontaneous or induced autoimmunity does not preclude a degree of incomplete tolerance to the relevant autoantigens. Potentially autoreactive T cells may represent a censored residual repertoire that has escaped tolerance-induction due to low affinity or specificity for cryptic determinants, as is the case for autoantigens of the central nervous system[45,46].

We found no evidence that intrathymic expression of a particular gene evolved from the necessity to maintain tolerance to its gene product by this mechanism alone, as might be assumed for self-antigens expressed in a temporarily or spatially restricted manner. Thus, expression of SAP in mTECs was sufficient, but not necessary, for T cell tolerance. Previously we reported an analogous observation for PLP tolerance, in this case expression by hematopoietic cells substituted for TECs in tolerance induction[12]. In addition, circulating C5 was found to be sufficient to mediate central tolerance by intrathymic presentation of serum-derived protein[47]; yet C5, as well as albumin (another abundant serum protein), were both expressed by mTECs. It appears that the range of promiscuously expressed genes is random rather than the reflection of an obvious immunological requirement. Thus, mTECs may indeed represent an immunological homunculus, in that they mirror and anticipate the peripheral self[48,49].

Expression of certain unmutated tumor antigens, such as tyrosinase and P1A, is confined to specific cell types, that is, melanocytes and male gametocytes, respectively. This restricted tissue expression is thought to limit their exposure to the immune system. Consequently, tolerance to these antigens may be compromised or absent, rendering them potential targets for the immunotherapy of cancer[50]. A certain degree of tolerance to these antigens that results from intrathymic expression, however, may obviate this approach or limit it to low-avidity T cells[51,52]. A conclusive assessment of T cell tolerance to unmutated tumor antigens and the eventual contribution of intrathymic expression must await comparison of the specific T cell repertoire in the respective knockout *versus* wild-type mice.

Self-tolerance through expression of tissue antigens in mTECs may not only operate *via* T cell deletion, it may also operate *via* the selection of regulatory T cells. There is accumulating evidence for the intrathymic generation and selection of regulatory T cells[53,54]. A T cell receptor–transgenic model was used to show that the generation of antigen-specific $CD4^+CD25^+$ T regulatory cells is apparently dependent on the expression of a neoself-antigen in radioresistant cells of the thymus[55]. Medullary TECs may induce the selection of a repertoire of regulatory T cells that encompass a wide spectrum of self-reactivity, as determined by promiscuous gene expression. This would explain the involvement of thymus-derived regulatory T cells in the control of various tissue-specific autoimmune diseases[53,54] and classic observations concerning dominant tolerance to tissue grafts after transplantation of

thymic epithelium[56]. In fact, considering the low probability with which thymocytes could interact with such infrequent stromal cells, selection of regulatory T cells is a more plausible explanation than negative selection for tolerance to tissue antigens expressed by rare mTECs[57].

The division of labor among thymic stromal cells may broaden the scope of central T cell tolerance[21,57]: mTECs can focus their self-antigen display on the peripheral self, whereas DCs are specialized to induce tolerance to the hematopoietic self[21,57]. If mTECs have such a prominent role in the induction of tolerance to parenchymal organs, one would expect the disturbance of the function of this cell type to have severe immunological consequences. It is intriguing that mutations in *Aire*, which encodes a putative transcription factor predominantly expressed in mTECs, result in autoimmunity against multiple peripheral tissues[58]. Understanding the immunological implications of promiscuous gene expression in mTECs as well as deciphering its molecular regulation and control, which may be akin to gene regulation in male gametocytes during spermatogenesis, should prove highly insightful as well as challenging.

Methods

Animals. C57BL/6, BALB/c and SJL/J mice were from Charles River WIGA (Sulzbach, Germany) and NOD mice from M&B A/S (Ry, Denmark). MHC class I^- and MHC class II^- ($H\text{-}2^b$)[36], $SAP^{-/-}$[40] and $RAG\text{-}2^{-/-}$ ($H\text{-}2^b$) mice were bred at the animal facilities of the Deutsches Krebsforschungszentrum (Heidelberg, Germany). All mice were kept under specific pathogen–free conditions. $RAG\text{-}2^{-/-}$ mice were from B. Arnold (Deutsches Krebsforschungszentrum).

Generation and immunization of chimeric animals. Thymectomy, fetal thymus grafts and construction of bone marrow radiation chimeras were done as described[20]. Chimeras were immunized with 50 μg of mouse SAP in complete Freund's adjuvant (CFA), which was injected into one footpad 8 weeks after reconstitution. Mouse SAP was purified as described[59]. Eight days later, popliteal and inguinal lymph nodes were removed. Cells (4×10^5 cells/well) were cultured in triplicate for 72 h in round-bottomed 96-well plates in serum-free medium (HL-1, BioWhittaker, Verviers, Belgium) in the presence or absence of protein. Proliferation was measured by incorporation of [^{3}H]thymidine, which was added for the last 24 h of culture (1 μCi/well). Only animals with comparable control responses to the CFA component–purified protein derivative (PPD) were included in the analyses.

RNA preparation and cDNA synthesis. RNA was isolated from tissues with the RNeasy Mini Kit (Qiagen, Hilden, Germany) or from single-cell suspensions with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Total RNA (4 μg of tissue-extracted RNA or an equivalent of 40,000–200,000 single cells) was reverse transcribed into cDNA with $Oligo(dT)_{20}$ Primer and Superscript II Reverse Transcriptase (Gibco-BRL, Eggenstein, Germany), this was followed by RNase H digestion (Promega, Madison, WI). RNA integrity was assessed by inspection of 28*S* and 18*S* band intensities after agarose gel electrophoresis.

RT-PCR analysis. PCRs were done in a final volume of 50 μl, with 1.25 U of REDTaq DNA Polymerase (Sigma, Munich, Germany). Final concentrations of the PCR mix were: 250 nM for each primer, 200 μM dNTP (MBI Fermentas, St. Leon-Rot, Germany), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.1 mM $MgCl_2$ and 0.01% gelatin. The reaction mix was overlaid with mineral oil (Sigma) and amplification carried out in a RoboCycler 40 (Stratagene, Amsterdam, Netherlands) under standard thermal cycler conditions: a single denaturing step at 94 °C for 3 min followed by either 40 (for detection of promiscuously expressed genes) or 25 cycles (for actin normalization) of 94 °C for 1 min, 52–66 °C for 1 min and 72 °C for 2 min, followed by a final extension step of 72 °C for 3 min. The following oligonucleotide pairs were used (sense and antisense respectively): nAChRα1: 5′–TGGGCTCCGAACATGAGA–3′ and 5′–CGATGACGGTGATGATGATG–3′; β-actin: 5′–TGGAATCCTGTGGCATCCATGAAAC–3′ and 5′–TAAAACGCAGCTCAGTAACAGTCCG–3′; Aire: 5′–ACCATGGCAGCTTCTGTCCAG–3′ and 5′–GCAGCAGGAGCATCTCCAGAG–3′; albumin: 5′–GTAGAAGAGCCTAAGAACTTGG–3′ and 5′–TAATCTGCTTCTCCTTCTCTGG–3′; amylase1 5′–ATCTCATTGACATTGGCGTAGC–3′ and 5′–AGAATGTCAAGATGGATGCTCC–3′; α1-antitrypsin: 5′–GGCGCAGAAGGTTAGTCCAG–3′ and 5′–CTGTAGCATCAGGCACGAGC–3′; cathepsin-L: 5′–AAAGGGTTGTGTGACTCC–3′ and 5′–TCTTGCTGCTACAGTTGG–3′; cathepsin-S: 5′–ACATGACCGAAGCTTTCC–3′ and 5′–CATCAAGAGTCCCATAGC–3′; complement C5: 5′–GTGTGGAAGCTGACTGTG–3′ and 5′–GCTTGACAGGATGGACAC–3′; CRP: 5′–CCATGGAGAAGCTACTCTG–3′ and 5′–GTGTAGCCCTTGTGCAG–3′ or 5′–CCCAAGATGATGCTTGC–3′; α1-crystallin: 5′–ACTGTTCGACCAGTTCTTCG–3′ and 5′–TCAGGAGAGAAGTGCTTCAC–3′; elastase: 5′–ATGTCCAGCTGGGTGTTCCG–3′ and 5′–TCCAGAGCGGACTCCATCTC–3′; iFABP: 5′–AGACGGAACGGAGCTCAC–3′ and 5′–GCTCTTCAGCGTTGCTCC–3′; α-fetoprotein: 5′–CTGGATGTCAGGACAATCAG–3′ and 5′–AAACAGACTTCCTGGTCCTG–3′; GAD65: 5′–TGGAATCCTCACAAGATGATGG–3′ and 5′–TCTTCATTGTCTTCCAGAGTGC–3′; GAD67: 5′–TGCAACCTCCTCGAACGCGG–3′ and 5′–CCAGGATCTGCTCCAGAGAC–3′; gp100: 5′–GTCAAGACGGTGGCCTTGG–3′ and 5′–CTGCTGTCCACTGAGGAGC–3′; haptoglobin: 5′–CAAGTATGTCATGCTGCCTG–3′ and 5′–AGCAGTGCAGGGCTAGAACC–3′; IA-2: 5′–AGACAGGGCTCCAGATTTTGC–3′ and 5′–GGCATGGTCATAGGGTAGGAAG–3′; insulin I and II: 5′–CAGCCCTTAGTGACCAGC–3′/5′–CAGCCCTAAGTGATCCGC–3′ and 5′–TCTCCAGCTGGTAGAGGG–3′; lactalbumin: 5′–CTCTTCCAGATCAGTGACAG–3′ and 5′–AGGCAGATCATCTTCACAGG–3′; MOG: 5′–GTGTGCTGACTCTCATCG–3′ and 5′–CTCCAGGAAGACACAACC–3′; P1A: 5′–ATGGGTGCTGGCGCTAACTGT–3′ and 5′–TCTTCTGGGCTTTGCAACTGC–3′; PLP: 5′–ACTACAAGACCACCATCTGC–3′ and 5′–CCATACATTCTGGCATCAGC–3′; retinal S-antigen: 5′–TGACTACCTACCCTGTTCAG–3′ and 5′–TTCACTGGATGTGAGCTCTC–3′; S100β: 5′–TCAAGGAGCAGGAAGTGG–3′ and 5′–ATGGTGGTCAGCAACACG–3′; SAP: 5′–CAAGCATGGACAAGCTG–3′ and 5′–CCCAAGTGGTACATAGG–3′ or 5′–CAACAATGCCAGAGGAG–3′; somatostatin: 5′–CTGCGACTAGACTGACCC–3′ and 5′–CTTGCAGCCAGCTTTGCG–3′; thyroglobulin: 5′–TGGCCAGCATACACCTTCTC–3′ and 5′–GCATAGTCGTCTGTGGAGTG–3′; trypsin 2: 5′–CACCAACAACATGATCTGTG–3′ and 5′–CTGATTGAAGAGAGACTAGG–3′; tyrosinase: 5′–TGCACAGATGAGTACTTG–3′ and 5′–ACCATGTAAGAGTCTCTG–3′; whn: 5′–ACACCAGCAGCCATTGTTCC–3′ and 5′–CCAAGCTGTCATCCTTCAGC–3′. All primer pairs were synthesized at the oligonucleotide synthesis facility of the German Cancer Research Center and were located in different exons to exclude PCR products that originated from contaminating genomic DNA. Reaction products were separated on 1×TAE (40 mM Tris-acetate, 1 mM EDTA) 2% agarose gels that contained 30 ng/ml of ethidium bromide (Roth, Karlsruhe, Germany). PCR products were revealed with the Lumi-Imager (Roche), bands were quantified with LumiAnalyst 3.0 software (Roche). For semiquantitative PCR, the different cDNA preparations were normalized to β-actin expression before testing expression of the gene of interest.

Immunohistochemistry. Cytospin preparations of isolated mTECs were fixed in ice-cold acetone for 5 min and then air-dried. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 in PBS for 15 min. Unspecific protein binding was blocked by a mixture of 3% nonfat dry milk, 0.5% Tween and 1% goat serum (in the case of PLP and insulin) and 1% donkey serum (in the case of P1A). Insulin expression was detected by a guinea pig anti–pig insulin serum (Dako Diagnostica, Hamburg, Germany) and developed with the ABC kit (Vectorstain, Burlingame, CA), according to the manufacturer's instructions. To control for the specificity of staining, free insulin (30 nM, Sigma) was added to the pretitered anti-insulin serum. PLP was detected with the monoclonal antibody (mAb) O10[60] and developed with a peroxidase-conjugated goat anti–mouse μ chain–specific serum (Southern Biotechnology, Biozol, Eching, Germany). The specificity of this staining was verified on thymus section of $PLP^{-/-}$ mice. P1A expression was detected by a rabbit–anti-P1A peptide–specific antiserum[61] and developed by a peroxidase-conjugated donkey–anti-rabbit serum (Jackson Immunoresearch, Dianova, Hamburg, Germany). The corresponding preimmune serum was used as a specificity control. Staining was also verified on positive tissue, that is, pancreas, brain and testis. All staining was developed with AEC substrate (Sigma) and analysed with an Axiophot-2 microscope (Zeiss, Oberkochen, Germany).

Isolation of thymic cells. Thymic stromal cells were purified as described[12]. Briefly, rosettes were enriched from collagenase-digested thymi on a fetal calf serum (FCS) gradient, dissociated with EDTA and stained with biotin-conjugated mAb N418–Red^{670}–conjugated streptavidin (Gibco-BRL) and fluorescein isothiocyanate (FITC)–F4/80 (Serotec Oxford, UK). $N418^+$ (DCs) and $F4/80^+$ cells (macrophages) were sorted with a FACSVantage Plus cell sorter (Becton Dickinson, Heidelberg, Germany). Alternatively, cells were only stained with phycoerythrin (PE)-N418 and macrophages were defined by their strong autofluorescence. TECs were enriched on a discontinuous Percoll density gradient from thymi sequentially digested by collagenase-dispase and trypsin. The TEC-enriched fraction was stained with biotin-CDR1–Red^{670}-streptavidin, FITC-G8.8 and PE–anti-CD45. Cortical TECs ($CD45^{-/lo}CDR1^+G8.8^+$) and mTECs ($CD45^{-/lo}CDR1^-G8.8^+$) were sorted with purity >98 % upon reanalysis. For four-color detection the following combinations were used: FITC–UEA-1 (Sigma), biotin-CDR1–peridinine chlorophyll protein (PerCP)-streptavidin (Becton Dickinson), PE–anti-CD45 and Cy5-G8.8. Alternatively, PE–anti-CD45 was replaced by PE–anti-B7.1 (clone 16-10A1) or PE–anti-MHC class II IA^d (clone AMS-32.1, both from PharMingen, Hamburg, Germany). Staining was always preceded by blocking with the anti-FcR mAb 2.4G2. The average cell yields per thymus were 5400 cTECs and 22,000 mTECs in C57BL/6 mice and 4400 mTECs in $RAG\text{-}2^{-/-}$ mice. These numbers represented minimal estimates due to unspecific losses at the various steps of the isolation procedure.

Isolation of spleen cells. Myeloid and lymphoid DCs from spleen were isolated as described[62]. In brief, a collagenase-dispase digested suspension of eight spleens was incubated in the presence of 10 mM EDTA for 5 min, subjected to erythrocyte lysis and filtered through a 70-μm cell strainer. The suspension was loaded on a 14.5% Accudenz gradient (Accurate Chemical & Scientific Corporation, New York, NY) and centrifuged at 530*g* for 20 min at room temperature. The low-density fraction was collected, washed and stained with PE-CD11c, FITC-CD11b and cychrome-CD8. Sorted myeloid DCs ($CD11c^+CD11b^{hi}CD8^-$) or lymphoid DCs ($CD11c^+CD11b^{lo}CD8^+$) (1×10^6) were used for generation of cDNA.

Isolation of testis cells. Testis was either gently mechanically dissociated or subjected to differential enzymatic dissociation (one round of 15 min with collagenase at 25 °C to

remove Leydig cells, followed by two rounds of 20 min with collagenase-dispase at 37 °C to dissociate the seminiferous tubuli). The collagenase-dispase fractions were pooled, filtered and subjected to density centrifugation on a discontinuous Percoll gradient as described[63]. Low-density cells collected in fraction II (interface ρ 1.03/1.045) were highly enriched in immature male sperm cells (spermatogonia), whereas the sediment was highly enriched in mature sperms as judged by morphological criteria.

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