On the role of *aire* and *smad4* in thymic development

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Summary

The thymus is critical for the evolutionary fitness of mammals. It allows the efficient development of T cells, a highly potent defense system, while restraining the developing T cells to prevent self-destruction. In contrast to the extensively studied thymocyte development, the thymic stroma supporting this development has by far been less studied. As a consequence, a precise understanding of the molecular mechanisms guiding thymic epithelial cell development is largely lacking. Thymic tolerance induction is a prerequisite for an organism to bear the tremendously powerful T cells. Failure of tolerance ultimately leads to potentially fatal self-destruction. Despite this perception, the molecular mechanisms underlying tolerance induction await to be unraveled.

It is still unresolved how endogenous self-antigens are being physiologically used for the control of self-tolerance. The phenomenon of ectopic thymic gene expression of peripheral self-antigens has been described in recent years. Humans lacking a functional Autoimmune Regulator (AIRE) gene develop a spontaneous, multiorgan autoimmune syndrome termed Autoimmune Polyendocrinopathy Ectodermal Dystrophy (APECED) Syndrome. Studies in mice proposed that the transcription factor *aire* might be critical in regulating ectopic gene expression, a possible prerequisite for negative selection. The first part of this PhD project seeked to generate a mouse model of the human APECED syndrome and to establish a genetic tool enabling direct in vivo manipulation of the rare cells of Aire expressing thymic medullary epithelial cells (mTEC). The work presented here describes the successful generation of such a mouse model and discusses the limitations of the created aire-cre mice to study *in vivo* tolerance induction by Aire expressing mTECs.

The second part of the thesis is devoted to the role of Smad4 in thymic epithelial cell development and function. Thymic epithelial cells constitute the most abundant component of the stroma, form a 3-D meshwork and express soluble and membrane bound molecules critical for T cell development. In turn, thymocytes deliver signals that control TEC differentiation. The molecular nature of this lympho-epithelial cross-talk is incompletely understood. Members of the TGF-β family of signalling molecules (TGF-β, Bmp and Activins) are critical in embryonic development of many tissues. Signalling of these molecules occurs via the cytoplasmic second messenger Smad4. To test whether Smad4 plays a role in thymic organogenesis, we generated mice specifically deficient for Smad4 expression in TECs. While lack of Smad4 expression allows for the formation of a thymus, mutant embryos and postnatal mice display a profoundly reduced thymic cellularity. Thymocyte development, ap-

pears, however, surprinsingly normal. Nevertheless, peripheral T cell biology is substantially affected by the thymic epithelial cell specific Smad4 deficiency. A persistent T cell lymphopenia and a substantial shift in the balance of naive to regulatory T cells are unexpected results. These striking findings illustrate that the importance of thymic epithelial cells beyond the thymus knows no bounds.

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Abbreviations

Units are indicated according to the SI (système international) if not indicated otherwise.

A = adenosine
AI = autoimmune
AICD = activation induced cell death
AIRE = human autoimmune regulator protein
aire = autoimmune regulator gene
Aire = murine autoimmune regulator protein
ALPS = Autoimmune Lymphoproliferative Syndrome
APC = Allophycocyanin
APC = antigen presenting cell
APECED = autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
BM = bone marrow
bp = base pair
BSA = Bovine serum albumine
C = cytosine
cγ = common γ chain of cytokines IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21
CD = cluster of differentiation
CDR = complementarity determining region
cds = coding sequence
CFSE = carboxyfluoroscein diacetat succinimidyl ester
c-kit = CD117, receptor for stem cell factor
CLP = common lymphoid precursor
cpm = counts per minute
CRE = enzyme derived from bacteriophage P1 that *c*auses *re*combination
CTLA4 = cytotoxic T-lymphocyte antigen 4
DC = dendritic cell
DM = diabetes mellitus
DN = double negative
DNA = Deoxyribonucleic acid
DP = double positive
ds = double stranded
E xy = embryonic day xy
ECM = Extracellular matrix
EF cell = embryonic feeder cell
ELISA = Enzyme linked immunosorbent assay
ES cell: embryonic stem cell
FACS = Fluorescence activated cell sorting
FCS = Fetal calf serum
FGF = Fibroblast growth factor
FITC = Fluorescein Isothiocyanate
Flp = Flipase
FoxN1 = Gene belonging to the winged helix family of transcription factors
G = guanin

GAD65/67 = Glutamine Decarboxylase 65/67
GFP = green fluorescent protein
GP-33 = glycoprotein 33 of LCMV
H-2 = Mouse MHC (see also HLA and MHC)
HA tag= hemagglutinin used as an epitope tag
HBSS = Hank's balanced salt solution
Hh = Hedgehog (a signalling cascade of the Hh family)
HLA = Human leukocyte antigen (see also MHC and H-2)
HSC = Hematopoietic stem cell
i.p. = intraperitoneally
i.v. = intravenously
IL = Interleukin
IMDM = Iscove modified Dulbecco medium
IRES = internal ribosomal entry site
ISP = immature single positive
ITAM = immunoreceptor tyrosine-based activation motif
kb = kilobase
KGF = FGF 7 = Keratinocyte growth factor = Fibroblast growth factor 7
LB = Luria Bertani broth
LCMV = lymphocytic choriomeningitis virus
LIP = lymphopenia induced proliferation
LN = Lymph nodes
loxP = locus of crossover (P1 bacteriophage)
LPC = lymphoid precursor cell
MALT = Mucosa associated lymphoid tissue
MC 57 cells = Fibroblast cell line on $H2^b$ MHC background
MHC = Major Histocompatibility complex (See also HLA and H-2)
milliQ = deionized H_2O purified to $\geq$ 18 MΩ electrical resistance
moAb(s) = monoclonal antibody (ies)
MS = multiple sclerosis
MΦ = Macrophage
NCC = neural crest cells
NIP-OVA = Nitroiodophenol conjugated Ovalbumin
NK cell = natural killer cell
NLS = nuclear localization signal
oligo = oligodeoxyribonucleic acid nucleotide
ORF = open reading frame
PBS = phosphate buffered saline
pc = post conceptionem; detection of the vaginal plug is defined as day 0
PCR = polymerase chain reaction
PE = R-Phycoerythrin
PerCP = Peridinin Chlorophyll Protein
PHD finger (Plant homeodomain) finger
PI = Propidium iodide
pIV = promoter IV
PLP = Proteolipid protein (a component of myelin)
pp = pharyngeal pouch
pTα = pre T α chain

RA = rheumatoid arthritis
Rag = Recombination activation gene
RIP = rat insulin promoter
ROSA26 = reverse orientation splice acceptor 26
R-smad = receptor smad
RT = Room temperature
RTE = recent thymic emigrant
RT-PCR = reverse transcription polymerase chain reaction
SA = streptavidin
Shh = sonic hedgehog
Smad = Human/Mouse homolog of **Sma** (C.elegans) and **M**others **A**gainst **D**ecapentaplegic (MAD) (Drosophila)
spf = specific pathogen free
ss = single stranded
T = tyrosine
TCE = T cell clonal expansion
TCR = T cell receptor
TE = Tris-Hcl pH 8.5
TEC = Thymic epithelial cell
TGF-β = transforming growth factor-β
T_{Reg} cell = T regulatory cell
TSA = tissue specific antigen
UEA-1 = Ulex europaeus antigen –1 (lectin)
Vα, Vβ = variable region of the α (or β) chain of the TCR
wnt = Family of evolutionarily conserved secreted glycoproteins (wingless)

Introduction

Do we need (a) thymus?

I am convinced that the importance of the mammalian thymus has long been underestimated and that it is crucial for survival in an environment challenging one's adaptive immune system daily. This is best illustrated by two diseases in which thymic development cannot fully take place: Di George syndrome and thymic aplasia as part of the nude phenotype, both in humans and mice. Patients lacking a specific region on chromosome 22 in the case of the Di George Syndrome or a functional *FoxN1* gene for the nude phenotype do not have a fully developed thymus and succumb without appropriate isolation from the environment and antibiotic treatment within the first weeks to months of life due to life-threatening infections. Furthermore, prolonged life-span in Western countries and the advent of new therapeutic options such as hematopoietic stem cell transplantation render life-long proper thymic function desireable.

Despite intensive research many questions related to thymic development and to the molecular mechanisms that control the mature thymus' unique function remain still unanswered. The work of the last four years of my life focused on a better understanding of the molecular and cellular mechanisms that account for normal thymus organogenesis and function.

Due to the complexity of genetic networks involved in developmental processes, I concentrated on two genes that are *smad4* and aire. *Smad4* is an important intracellular signal transduction molecule involved in the development of many organs. The function of aire was unknown at the start of my thesis but an association to tolerance induction could be assumed, as patients lacking functional aire develop multiple autoimmune diseases.

Thymic organogenesis from E10-E12

In vertebrates, cells from all germinal layers interact for thymic organogenesis in a precise sequence of inductive events that control both proliferation and differentiation of epithelial cells. In mouse development, the early thymic primordium is first detectable from approximately embryonic day 10 (E 10) on. After this positioning, inductive signals stimulate cells from the ventral endodermal lining of the third pharyngeal pouch to adapt the fate of thymic

epithelial cells (TEC) [1, 2]. In contrast, dorsal aspects of the 3rd pharyngeal pouch (pp) develop into the parathyroid glands [3]. Two transcription factors are known to be differentially expressed between the ventral and the dorsal aspect of the 3rd pp. Gcm2 is expressed in the dorsal part, FoxN1 is exclusively found in the ventral aspect. Around E11, lateral budding of the endoderm occurs, forming the thymic anlage. Mesenchyme of the third and fourth pharyngeal arches, surround the thymic anlage and provide signals (FGF7, FGF10) for further proliferation of thymic epithelial cells. However, mesenchymal support is not required for further differentiation of TECs at this developmental stage [4]. Initially derived both from mesoderm and neural crest, the mesenchymal cells migrate subsequently into the epithelial anlage where they eventually establish an intrathymic network of fibroblasts and blood vessels. The role of ectodermally derived cells in thymic development is controversial. It appears that signals from the endoderm attract ectodermally derived neural crest cells (NCC) that in turn provide further proliferation signals [1]. The importance of neural crest cells for thymus organogenesis is illustrated by genetic mouse models ($Pax3^{-/-}$) where NCC migration is impaired, leading to thymic hypoplasia. These results are confirmed by surgical removal of cephalic NCC, a procedure that also leads to thymic hypoplasia. Although the role of NCC-derived mesenchyme to support thymus development is clear, it appears that mesenchymal cells from other sources can substitute in experimental systems to provide the necessary signals. In a recent report, NCC lacking TGF-βRII migrated to the thymic primordium at E13.5 but the thymus remained hypoplastic [5].

At E11.5 hematopoietic precursor cells seed to the thymus anlage although its epithelial cells are yet incompetent to fully support T cell development. In mice and humans, the two thymic lobes then move medially, ventrally and caudally to reach its final position, the midline above the heart by E 12.5. Differentiation and migration seem to be independently regulated as nude mice have correctly positioned thymic rudiments lacking proper differentiation while on the other hand several mutants with development of normal but ectopic thymic lobes are known. Mice with $Hoxa3^{+/-}$, $Hoxb3^{-/-}$, $Hoxd3^{-/-}$ mutations reflect a migration defect rather than an impairment of differentiation [1].

Molecular control of early thymus organogenesis

Only very few genes are known to be critical in TEC development. Hoxa3, a member of the Hox family of transcription factors is involved in positioning of the early thymic anlage. This effect may in part be through downstream engagement of Pax1 and Pax9 as both transcription factors are specifically downregulated in $Hoxa3^{-/-}$ embryos [1]. Indeed, $Pax9^{-/-}$ mice

show an early failure of thymic organogenesis while $Pax1^{-/-}$ mice have a more subtle thymic phenotype. FoxN1 is detectable around E 10.5 and can be specifically detected in the ventral aspect of the 3rd pp at E 11 [1]. FoxN1 deficient TECs fail to attract lymphoid precursor cells (LPC) and therefore do not develop a thymus beyond the thymic rudiment. However, the defect is cell autonomous as signals experimentally provided by LPCs or wild-type epithelium are not sufficient to rescue thymic growth [6]. FoxN1 expression is regulated by wnt molecules [7].

Thymic organogenesis from E12-E15

Early thymic development ends with an epithelial thymic rudiment surrounded by mesenchymal cells. Further patterning depends on signals provided by LPCs since mutants with impaired lymphoid development do not form a proper thymus. The transcription factor Ikaros is required for lymphoid development [8]. Mice expressing a dominant-negative form of Ikaros do not have any lymphoid progenitors and consequently have an alymphoid fetal thymus [1]. TECs appear, however, normal, i.e. have a different aspect than the cysts observed in $FoxN1^{-/-}$ mice with clearly defective TECs. This illustrates that bi-directional signaling between lymphoid cells and TECs is required for proper thymic development. Other mutant mice with genetic defects causing blocks in the development of the thymus are mice expressing a transgenic human CD3ε and the Rag2 deficiency. Both genetic abnormalities lead to a thymocyte intrinsic defect resulting in blocks at early but distinct stages of thymocyte development ending in a growth arrest and disorganization of the whole thymus. Furthermore, TEC differentiation is dependent on wnt signals provided by thymocytes and TECs themselves acting in an auto- and paracrine way [7]. Thus, it is clear that thymic epithelial cell differentiation and thymocyte development are interdependent [9].

Vascularization is an important step in thymic organogenesis occuring at E 14, yet the mechanisms inducing and underlying vessel formation are unknown. Further development of the thymus into cortex and medulla is dependent on signals provided by thymocytes [10]. Differentation of the cortex requires thymocytes commited to the T cell lineage [11] while medullary development is essentially dependent on the presence of mature $\alpha\beta TCR^{+}$ thymocytes.

Molecular control of thymic epithelial cell development

The transcription factor NF-κB is composed of various combinations of members of the

Rel family of proteins. The NF-κB complex is formed of homo- or heterodimers from the five Rel family members that are NF-κB1 (p50), NF-κB2 (p52), p65 (RelA), RelB and c-Rel [12]. RelB is crucial for the differentiation of medullary thymic epithelial cells (mTECs) as RelB-/- mice lack proper thymic medulla formation [13]. $RelB^{-/-}$ animals do not express the autoimmune regulator (*aire*) gene and develop severe autoimmunity. Mice lacking both, p50 and p52 display severe thymic hypoplasia, peripheral lymphopenia that is not T cell intrinsic, lack medullary cells binding ulex europeus antigen-1 (UEA-1) and show a disturbed medulla formation [14].

A subpopulation of mTECs expresses aire [15-17]. These cells bind to the lectin UEA-1 and are important for shaping a self-tolerant T cell repertoire (see below). Lymphotoxin-β, secreted by thymocytes, induces aire expression in mTECs [18, 19]. Furthermore, aire expression seems to be dependent on NIK [20] and TRAF6 [21].

The thymic epithelium does not have a basement membrane

In a mature thymus, the organisation of the thymic epithelium differs from all other epithelial organs in the body. Rather than forming a sheet of cells positioned on a basement membrane, thymic epithelial cells form a three-dimensional meshwork. Importantly, this architectural organisation is induced during fetal development in response to thymocytes at distinct maturational stages. This particularity has been viewed as a prerequisite for proper thymocyte development. A series of recent papers, summarized in [22] challenges this observation in part though as a single cell layer system seems sufficient for T cell lineage commitment and early thymocyte development. However, T cells produced with this method can only be generated with low efficiency.

Conserved signaling pathways important for organ development, TGF-β and BMP signaling

Many developmental processes depend on secreted morphogens. Various conserved families of signaling molecules have been described in organisms such as C. elegans, drosophila, mice and humans. Fig 1 shows an overview of the most important signaling cascades involved in developmental processes. Depicted are representative members of the extracellular, soluble morphogens, the membrane receptors and key intracellular signal transduction molecules. Identical to the formation of other organs (e.g. lung, limb, teeth, hair) in which epithelial-mesenchymal interactions play an important role, signaling pathways that

are candidates for an important role in thymus organogenesis and function include the family of Wnt molecules, fibroblast growth factors (FGF), hedgehogs (Hh), and bone morphogenic protein (BMP) of the transforming growth factor-β (TGF-β) superfamily.

Simplified interactions among the various signaling cascades are depicted in Fig 1. Of note is, that Smad4 can interact with wnt signaling which is known to be important for TEC development [7, 23].

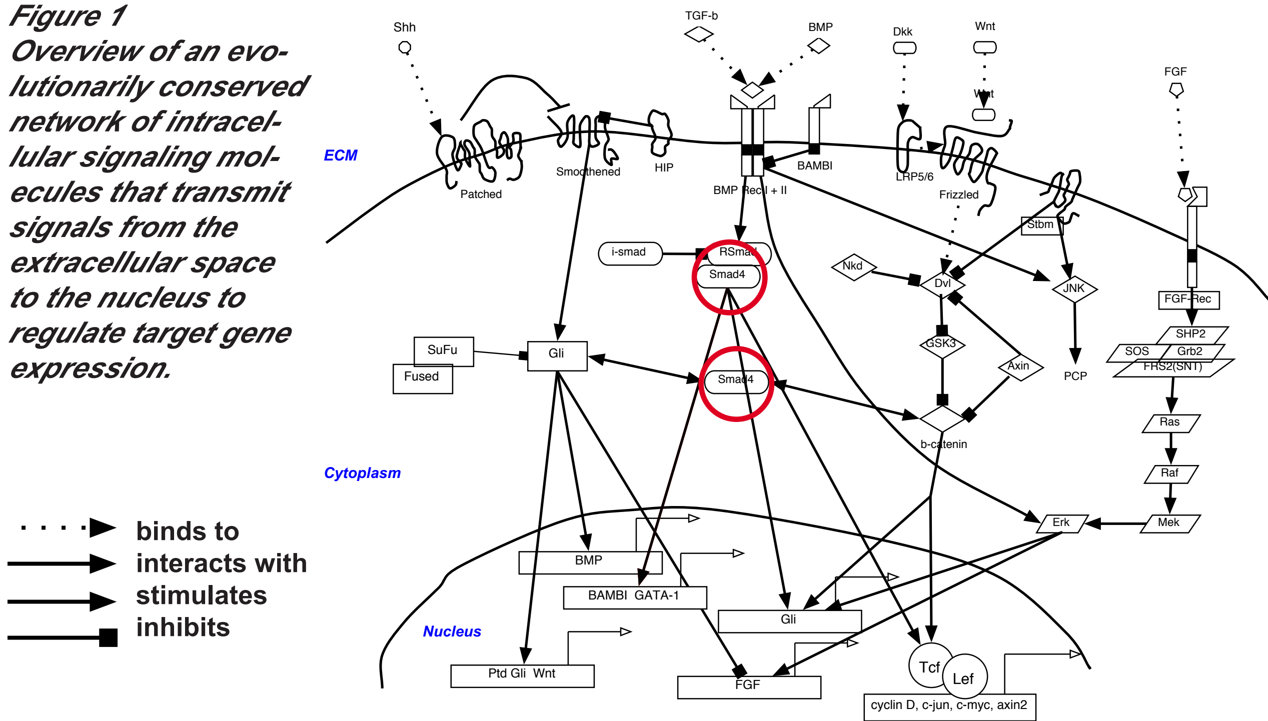

Figure 1
Overview of an evolutionarily conserved network of intracellular signaling molecules that transmit signals from the extracellular space to the nucleus to regulate target gene expression.

Shown are important intracellular molecules of the sonic hedgehog (Shh), the transforming growth factor (TGF-β) family, the wingless (wnt) and the fibroblast growth factor (FGF) families. Smad4, a molecule central for the canonical TGF-β signaling pathways is highlighted in red. Smad4 also interacts with the Shh and the wnt signaling pathways.

Thymocyte development

All known different lymphocytes are derived from the self-renewing hematopoietic stem cell (HSC) in the bone marrow (BM) and blood. Progeny of HSCs differentiate into the erythroid, myeloid and lymphoid lineage. In the latter, B and NK cells develop in the BM whereas T cells are the only hematopoietic cells that are not generated in the BM [24]. With the exception of a rare subset, T cells develop in the thymus which provides the microenvironment to generate the distinct T cell lineages: αβTCR CD4$^+$, αβTCR CD8$^+$, γδTCR, NKT and CD4$^+$CD25$^+$ regulatory (T_{Reg}) cells. During the differentiation along the hematopoietic lineages, progeny of HSC gradually loose their pluripotency [25]. HSCs first develop into multi-potent progenitors and finally are committed to a single lineage. An rapidly increasing number

of lymphoid progenitors with distinct potential to differentiate into various lineages has been described. Different studies came to somewhat contradictory conclusions and models and therefore the field remains controversial. A summary of studies describing progenitor cells can be found in [26].

The nature of the first progenitor entering the thymus remains elusive. It is known, however, that early thymocytes (DN1) have T, NK, DC and macrophage potential. Whether progenitors are committed to T cells in- or outside the thymus and whether they still have B cell potential remains controversial [24, 27]. Two different models of lymphoid commitment are discussed by Katsura [28]. It remains controversial where the T/B dichotomy occurs. Whereas Kondo et al. propose that the myeloid lineage deviates from a commom lympoid precursor (CLP), recent data by Balciunaite et al. support the model proposed by Katsura where T/B commitment occurs before myeloid commitment resulting in a B/myeloid and a T/myeloid precursor [27-29].

Within the thymus, a first TCR independent phase can be distinguished from a later, TCR dependent phase (Figure 2). Early thymocytes do not express the costimulatory molecules CD4 and CD8, they are called double negative (DN) cells and represent about 3-5% of thymic cellularity [30]. C-kit (CD117), CD25 and CD44 are used to further subdivide DN into several developmental stages called DN I – DN IV where CD117 is used to exclude committed cells from non-T-lineages [27]. The development from DN I ($CD44^{+}CD25^{-}$), via DN II ($CD44^{+}CD25^{+}$), through DN III ($CD44^{-}CD25^{+}$) to DN IV ($CD44^{-}CD25^{-}$) are functionally relevant, as the developing thymocytes gradually differentiate into specialized cells with increasing T lineage specific gene expression and step by step loose their multipotent lineage potential [27]. During mouse embryonic development, DN appear in the last trimester of gestation, DNII at E13.5, DN3 and DNIV at E14.5 and the first DP in small numbers at E 15.5. In adults it takes 2-3 days from late DN to DP, i.e. the kinetics are different in adults [1]. Another difference is the site of entry. During embryonic development, progenitors enter from outside while in adults, progenitors enter at the cortico-medullary junction [31]. Markers used to discriminate the different stages and their lineage potential are illustrated in Figure 3.

As described above, thymocyte development does not occur cell autonomously but is dependent on signals from cells of non-hematopoietic origin. In the adult thymus, precursors enter at the cortico-medullary junction and then migrate as DN I – DN III cells to the subcortical area. At this stage the recombinases RAG1 and RAG2 are active, inducing rearrangement of γ, δ and β loci. The $\gamma\delta$ TCR lineage deviates from the major T cell lineage, the $\alpha\beta$ TCR lineage at an earlier stage. Signals for this lineage choice are currently not known. However, it is known, that signals through the TCR are required for survival. The TCR of the

$\alpha\beta$ lineage is immature at this developmental stage, consisting of a successfully rearranged β TCR chain, the CD3 molecules and an invariant pre-TCR α chain (pTα). The expression of a functional β-chain is called β-selection. Signals transmitted through this receptor complex commit the cell to the $\alpha\beta$ lineage, induce proliferation and the expression of the co-receptors CD4 and CD8, an arrest of β locus rearrangement as well as rearrangement of the TCR α locus. $CD4^+CD8^+$ thymocytes are now called double positive (DP) and constitute 80-90% of thymic cellularity. Cells expressing a rearranged TCRβ chain coupled with a productively rearranged α chain that recognize MHC get a survival signal (positive selection, see below). Thymocytes that fail to recognize self-MHC die, since by default, thymocytes are prone to die. Approximately 90% of DP thymocytes die through this passive cell death [32]. Intriguingly, the very same receptor that provided the survival signal for positive selection can be detrimental for the cell as it can signal death if the TCR too strongly binds peptide presented on self-MHC (negative selection, see below).

At the time of positive selection, thymocytes express both coreceptors, CD4 and CD8. At the end of intrathymic T cell maturation, cells have either chosen the CD4 or the CD8 lineage and have downregulated the other co-receptor molecule. How this lineage choice occurs remains controversial. A breakthrough has recently been achieved by He et al. [33]. The authors identified the transcription factor ThPOK to be both necessary and sufficient to specifiy the CD4 lineage. Other factors such as Notch have also been described to be implicated in the CD4/CD8 lineage choice. It is widely accepted that TCR specificity determines CD4 versus CD8 lineage choice as illustrated by TCR transgenic mice. If the transgene recognizes MHC I, the T cells will become CD8 SP, if it recognizes MHC II, they will become CD4 SP. Co-receptor signals mediated by lck are also involved in lineage decision. DP thymocytes undergoing positive selection downregulate CD4 and CD8 and then reexpress CD4. If the TCR recognizes MHC II, the sustained signal will lead to further upregulation of CD8 and downregulation of CD8. If the TCR recognizes MHC I, CD4 will not lead to any further lck mediated signaling and this weak co-receptor signal determines CD8 commitment (Fig. 3). CD24 is highly expressed on DP cells and is subsequently downregulated during maturation towards SP cells [34]. On the other hand, CD69 is upregulated after positive selection. CD103 is upregulated in the CD8 lineage during maturation and has been used to determine recent thymic emigrants both in mice and humans [35, 36]. In the periphery, T cell matura-

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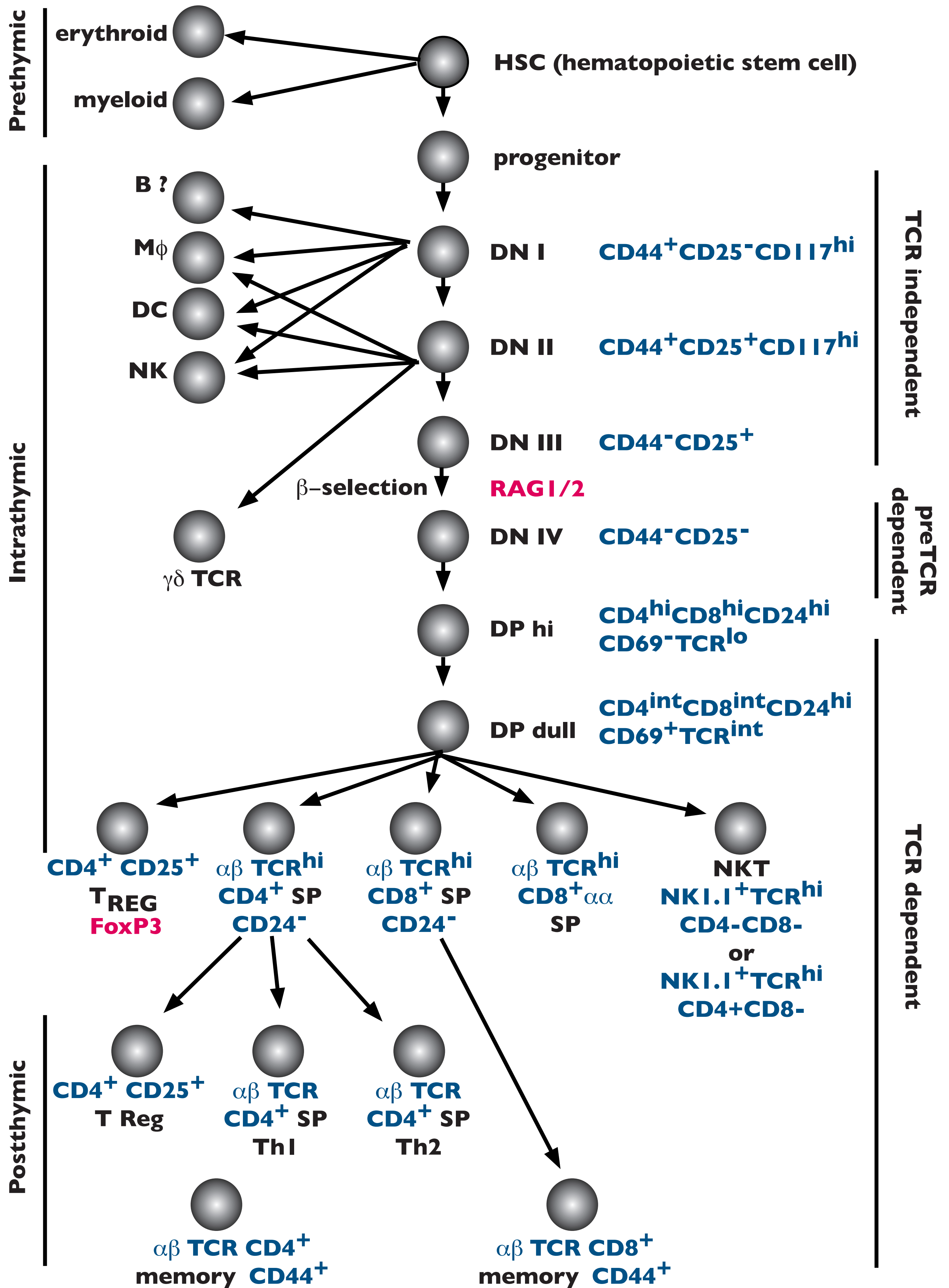
Figure 2
Distinct stages in the murine development of T cell lineages.
Black: cellular stages. Blue: Surface molecules. Red: Genetic markers.
Adapted from Rothenberg and Taghon, Annu. Rev. immunol., 2005



Prethymic
erythroid
myeloid
HSC (hematopoietic stem cell)
progenitor
Intrathymic
B ?
Mφ
DC
NK
DN I
CD44+CD25-CD117hi
DN II
CD44+CD25+CD117hi
DN III
CD44-CD25+
β–selection
RAG1/2
DN IV
CD44-CD25-
γδ TCR
DP hi
CD4hiCD8hiCD24hi
CD69-TCRlo
DP dull
CD4intCD8intCD24hi
CD69+TCRint
TCR independent
preTCR dependent
TCR dependent
CD4+ CD25+
TREG
FoxP3
αβ TCRhi
CD4+ SP
CD24-
αβ TCRhi
CD8+ SP
CD24-
αβ TCRhi
CD8+αα
SP
NKT
NK1.1+TCRhi
CD4-CD8-
or
NK1.1+TCRhi
CD4+CD8-
Postthymic
CD4+ CD25+
T Reg
αβ TCR
CD4+ SP
Th1
αβ TCR
CD4+ SP
Th2
αβ TCR CD4+
memory CD44+
αβ TCR CD8+
memory CD44+

tion continues, $CD4^+$ T cells can be polarized to Th1 or Th2 cells secreting distinct cytokine patterns and memory T cells develop after antigen encounter and T cell activation [37].

The various molecules that have been identified in thymocyte maturation and lineage choice are summarized in [24]. Many of the same regulatory factors and growth factor receptors are used repeatedly at various stages [24]. Notch signaling e.g. not only commits precursors to the T lineage but is also involved in β-selection, CD4/CD8 lineage choice, positive selection and in the periphery for Th1/Th2 polarization.

Signaling pathways involved in thymic development, TEC differentiation and cortex – medulla differentiation

As mentioned above, members of the wnt family of signaling molecules have been shown to regulate FoxN1 expression in thymic epithelial cells [7]. Shh has been shown to have direct effects on thymocytes [38, 39]. Members of the FGF family have also been reported to have important roles in thymic organogenesis. Mice lacking FGF10 [40] or FGFR2IIIB, the receptor for FGF7, FGF10 and FGF20, have severely disturbed thymic development [41] and proliferation of immature TECs is impaired [42]. All subpopulations of TECs express FGFR2IIIB and proliferate in response to stimulation with FGF-7. (Rossi et al., manuscript in preparation). Already two weeks after injection of FGF-7, treated animals revealed a gain in thymic weight and cellularity. The increased cellularity was found to be secondary to increased TEC proliferation (see below). This effect was sustained for at least three months and was paralleled by a normal architecture of the stromal meshwork and a regular composition of the different thymic epithelial cell subpopulations. For review, see [43].

Smad4 - a key molecule for TGF-β and BMP signaling

Members of the TGF-β superfamily comprises TGF-β, BMPs, activins and related proteins. Despite the enormous diversity of physiological effects, a disarmingly simple system of heteromeric receptors and second messengers transduce the biological effects of the TGF-β family members [44]. The canonical signaling for all these members involves type I and type II TGF-β receptors and intracellular signal transduction molecules called smad. In mammals, five type II and seven type I transmembrane serine/threonine kinase receptors have been described for 29 ligands [45]. Ligands can induce different signaling pathways depending on the composition of the receptor complex. Furthermore, additional proteins modify ligand-binding specificity and extracellular inhibitors influence signaling. Moreover, BAMBI, a

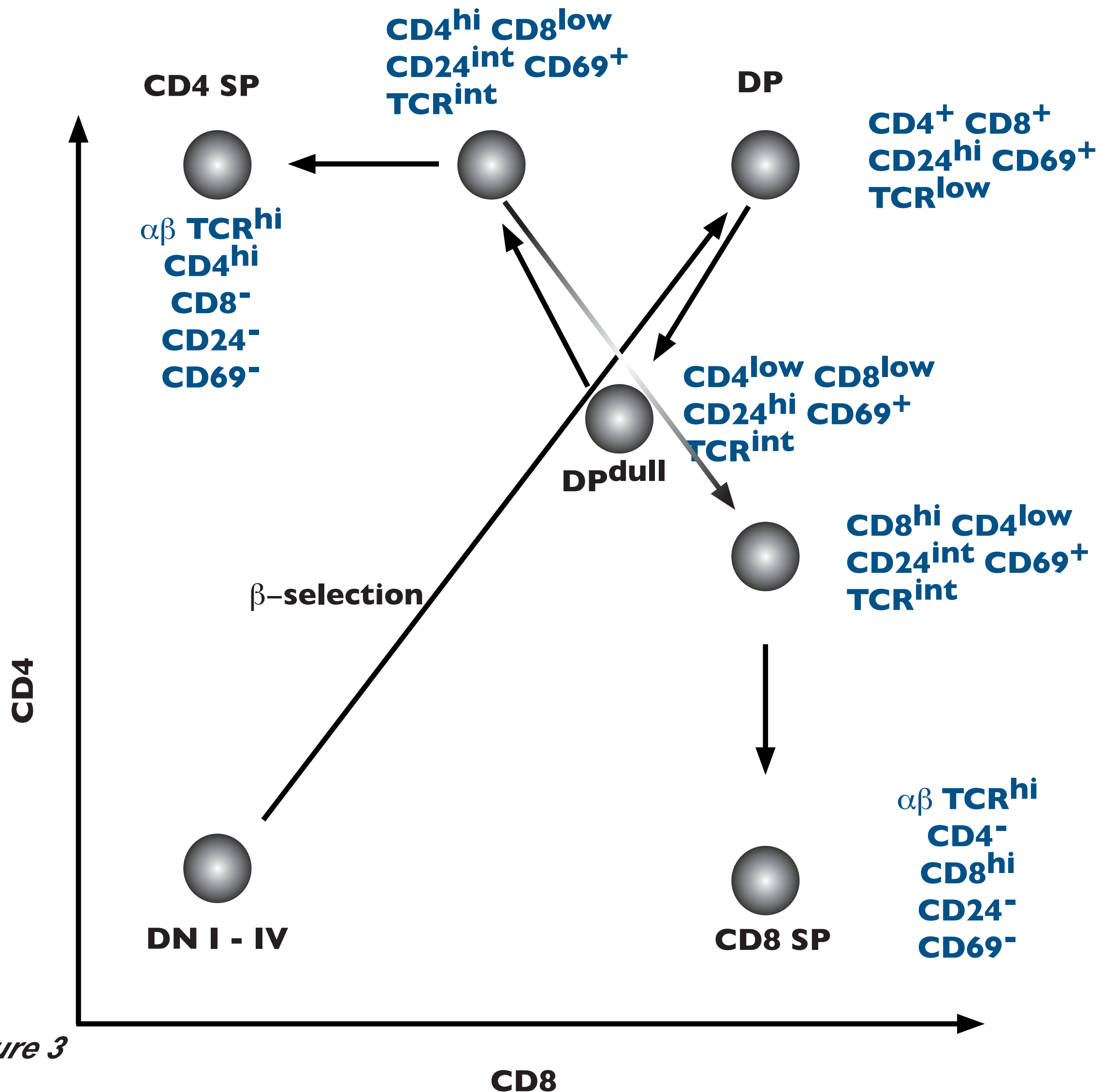


Figure 3

Maturation steps of late thymocytes defined by FACS analysis based on the expression of CD4 and CD8 co-receptors
Black: cellular stages. Blue: Surface molecules. Red: Genetic markers.

pseudoreceptor has been described [46] regulating TGF-β signaling at the receptor level.

Upon binding of an extracellular ligand at the cell surface to a complex of type I and type II receptors, the type I receptor is transphosphorylated by the type II receptor kinase. Consequently, the activated (phosphorylated) type I receptor in turn phosphorylates distinct receptor smad (R-smad) molecules. These form a complex with smad4. Activated smad complexes translocate to the nucleus, where they regulate transcription of target genes in cooperation with DNA-binding transcription factors and coactivators. Inhibitory smads (smad6 and smad7) can inhibit activation of R-smads. R-smads are ubiquinated and degraded by the E3 ubiquitin ligases smurf1 and smurf2.

Besides smad-mediated signaling, TGF-β activates other signaling cascades (for review see [45]).

Positive and negative selection

Developing T cells randomly rearrange TCR gene segments to increase the diversity of TCR specificities with a limited number of genes. Due to the randomness of this process, the generated TCRs need to be tested to be functional.

DN precursors start rearranging the TCRα locus after pre-TCR signaling. Vα/Jα joins can be detected in DN IV cells and full scale α rearrangement can be detected in DP [31]. Unlike the β chain, productive α chain rearrangment is not sufficient to terminate rearrangement of the α chain locus. Instead, only α chains that form an MHC-restricted receptor when paired with the β chain will terminate rearrangement. Since the successful generation of a MHC-restricted α/β pair is thought to occur infrequently, most DP cells express a surface TCR but are undifferentiated and still express high Rag levels. Multiple V/J recombination events can occur on the same allele which allows to test multiple productive TCRα rearrangements per cell. DP precursors expressing an MHC restricted TCR downregulate Rag, get a long-term survival signal and start migrating into the medulla for further differentiation. Thus, DP thymocytes recognizing MHC-self-peptides with low affinity receive a survival signal, a process called **positive selection**. This process occurs in the thymic cortex, takes several days to finalize and requires sustained TCR engagement [31]. The nature of self-ligands mediating positive selection remains blurry but it appears that relatively rare, low-affinity self-peptides promote positive selection. This gives rise to mature T cells having high affinity for foreign peptides that are generally structurally related to the self-peptides involved in selection. Yet, ligands for positive selection are generally not stimulatory for mature T cells [31].

TCRα, CD3δ and a motif in the TCRα chain connecting peptide are required for positive selection [31, 47]. Surprisingly, deficiency of ITAMs in the TCRζ chain did not impair positive selection but resulted in a skewed TCR repertoire. Intracellularly, several Src and Syk family members and ZAP-70 are required while negative regulators including c-Cbl, SLAP and Csk oppose positive selection.

On the other hand, random TCR specificity generation unavoidably leads to the generation of self-reactive TCRs. Therefore, these dangerous cells need to be eliminated. Thymocytes bearing a TCR that interacts with a self-peptide-MHC complex with high affinity receive a death signal to die by apoptosis, a process called **negative selection** [32]. In the absence of negative selection, the number of mature SP cells doubles. It was found that about 5% of thymocytes die through negative selection. Some T cells escape negative selection and for those there are peripheral mechanisms to induce tolerance.

The anatomical location where negative selection occurs remains controversial. There is little doubt that clonal deletion occurs at the cortico-medullary junction and in the medulla, a region enriched with DCs capable of efficiently mediating negative selection. However, it is not clear today, whether negative selection also occurs in the thymic cortex. Another controversy exists about whether positive and negative selection are sequential events or happen in parallel [32].

On a molecular level NUR77, an orphan steroid receptor appears to be involved in mediating negative selection. The role of co-stimulatory molecules is controversial where data using antibodies suggest involvement of co-stimulatory molecules while studies with genetically deficient mice do not.

An intriguing question remains: how does the TCR discriminate between signals leading to positive versus negative selection [32, 48]?

Taken together, the inability to be positively selected and negative selection lead to deletion of about 95% of all developing thymocytes. This high toll seems to be required to shape an efficient arm of the adaptive immune system, the T cells, while preventing autoreactivity.

Tolerance

Tolerance can be summarized as absence of autoreactivity irrespective of its underlying mechanism. Failure to induce tolerance, loss or breakage leads to overt immune disease with potentially fatal outcome. Autoimmune diseases vary from organ-specific to systemic manifestations and can be harmless to lethal. T cells and B cells are rendered tolerant during their development and various mechanisms keep them tolerant to self-antigens in the periphery.

Central tolerance involves all mechanisms rendering developing lymphocytes tolerant to self. I will focus on central T cell tolerance in this introduction. Peripheral tolerance results from a panel of mechanisms in the periphery aiming at controlling autoreactive T cell clones that escaped central tolerance. Of particular interest in recent years have been central T cell tolerance towards self-antigens of the periphery induced by mTECs and dominant peripheral T cell tolerance mediated by T_{Reg} cells.

a) Thymus dependent central tolerance – A role for aire in the thymic expression of tissue "specific" antigens:

The mechanisms of central tolerance leads to physical and/or functional deletion of self-reactive T cells during their intrathymic development. This process is designated negative selection (see above) and results either in apoptotic deletion of T cells or, alternatively, in a state of T cell unresponsiveness (otherwise known as anergy) [49]. In this context, thymic medullary epithelial cells and thymic stromal cells of hematopoietic origin, in particular dendritic cells, are responsible for negative thymic selection and thus shape the repertoire of T cell antigen specificities [50-52].

Thymic DCs have long been known to induce potent central tolerance. More recently, TECs have been shown to induce tolerance as well. For a long period of time, it was thought that central tolerance can only be mediated towards ubiquitously expressed antigens and blood-borne antigens that can be picked up by DCs, processed and subsequently presented to developing thymocytes. It was believed, that genes that are only actively transcribed outside the thymus in immunologically peripheral organs like the brain or pancreas, could not induce central tolerance. Several groups, however, reported the expression of so-called tissue-specific-antigens (TSA) in the thymus [53-61]. Some reports even showed the functional importance of these proteins in inducing tolerance already a decade ago [53, 62-67]. A puzzling observation was repeatedly made with the advent of transgene technology: Many "tissue-specific" promoters drove expression of transgenes in the thymus and induced tolerance [62, 68-72]. Today it seems very likely that this observation can be explained by the co-comitant expression of the autoimmune regulator (aire) gene. (see below) [73].

Only in recent years systematic analysis of the phenomenon of "ectopic" or "promiscuous" gene expression revealed the extent of tissue-specific antigen expression by mTECs [74-77]. A human monogenic autoimmune syndrome and mice deficient for aire further illustrate the functional importance of these antigens [78-81]. A link between thymic epithelial cell function and clonal deletion of self-reactive T cells has therefore been postulated. Humans expressing a defective form of the transcription factor AIRE (AutoImmune Regulator) develop the autoimmune-polyendocrinopathy- candidiasis-ectodermal dystrophy (APECED) syndrome. This disease complex encompasses hypoparathyroidism, autoimmune hepatitis, Addison's disease, thyroiditis, Type I diabetes mellitus, and other debilitating diseases mediated by the immune system [82]. The pathology of APECED seems to develop secondary to a lack in the thymic expression of self-peptides since AIRE is thought to promote ectopic expression of peripheral-tissue-restricted gene products and may thus control negative thymic T cell selec-

tion. The absence of AIRE could therefore result in the failure to express a full compliment of self-antigens and, in consequence, could by-pass the elimination of self-reactive T cells. Indeed, animals mutant for AIRE develop autoimmune diseases comparable to the profile observed in human APECED patients.

Several studies elegantly show that qualitative [83] as well as quantitative [73, 84] aspects of TSA expression are important to induce tolerance to TSA. A transgene coding for a soluble protein expressed in TECs was more potent in inducing tolerance than its membrane-bound form, indicating that the transgene might be taken up by DCs for cross-presentation of MHC II restricted peptides [85]. The necessity of such cross-presentation of a MHC II restricted transgene mimicking a TSA has been confirmed [86]. Finally, it was demonstrated directly in the thymus that self-reactive T cells fail to be centrally deleted in the absence of AIRE and that this process is responsible for the different forms of autoimmune diseases [73, 87].

An alternative hypothesis how tolerance towards TSA could be achieved has been put forth [88]. According to this model, TSA of various organs would not be expressed by a single cell but TECs would differentiate into various cell types of the body. Intriguingly, the authors show that fully differentiated specialized bronchial tissue and thyroid follicles can be found in the thymus. The tissues not only morphologically resemble end-differentiated specialized tissues but also express organ-specific genes and the respective proteins are synthesized.

b) Peripheral tolerance

A certain number of autoreactive T cells escapes the thymus which bear the danger of autoimmunity. Therefore, a panel of mechanisms in peripheral immune organs keeps these autoreactive T cells in check.

The activation threshold of the T cell receptor can be tuned by inhibitory molecules such as CD5 [89] and T cells can be negatively regulated by CTLA-4 and other molecules expressed on antigen presenting cells [90, 91]. Activation induced cell death is another way of limiting an immune response. Immune deviation is used to describe the switch of a Th1 to a Th2 type cytokine response. This mechanism is based on the dichotomy of Th1 cells secreting primarily IL-2, IFN-γ and TNF-β and Th2 cells producing IL-4, IL-5 and IL-6. These two cell types can cross-regulate each other and hence Th2 responses can dampen overreacting Th1 responses involved in autoimmune diseases. Next, anergy is a state of functional inactivation of a T cell, i.e. the cell is not physically removed but cannot be activated. This can result from TCR stimulation in the absence of signals through co-stimulatory molecules or suboptimal doses of cognate antigen. Immune-privileged sites are organs (brain, anterior

chamber of the eye and testis) that are privileged to accept grafts. The mechanisms underlying this hyporeactivity are poorly understood.

Finally, two self-reactive T cell lineages, iNKT cells, and $CD4^+FoxP3^+$ T cells have been described to bear the potential to suppress T cell responses [92]. Importantly, suppression by T_{Reg} cells is a dominant mechanism of T cell tolerance and hence bears therapeutic potential. The best studied population of regulatory cells are the $CD4^+FoxP3^+$ T cells.

Regulatory T cells (T Regs)

$CD4^+CD25^+$ regulatory T cells have gained considerable interest in the last decade since their suppressive function was demonstrated in vivo [93]. Meanwhile, in many mouse models of autoaggression, disease could be prevented by transferring $CD4^+CD25^+$ T Regs. A human $CD4^+CD25^+$ regulatory T cell counterpart has also been shown to be pathogenetically involved in human autoimmune disease. A major drawback is, however, the lack of a distinct surface marker defining T cells with regulatory activity. Only the $CD4^+CD25^+$ T Reg population has been consistently shown to display suppressive properties across species and in multiple disease models. However, T cell activation and homeostatic proliferation may lead to upregulation of CD25 making this marker cumbersome. Other markers, CTLA-4, GITR, Granzyme B, CD103 and LAG-3 have been proposed to define regulatory $CD4^+$ T cells. However, only FoxP3, a forkhead transcription factor is clearly associated with regulatory T cells and no other functions [94-96]. FoxP3 expression is highly restricted to a subtype of cells of the αβ TCR lineage, is not upregulated during T cell activation and correlates with a suppressive function, irrespective of CD25 expression [97]. $FoxP3^+$ T_{Reg} exert dominant tolerance and are selected in the thymus by TCR/MHC ligand interactions [97-100].

Their mode of action is heavily disputed. Some groups believe that the effects are contact mediated whereas other researchers have found IL-10 and TGF-β as key mediators of suppression. Further complication comes from the fact that not only $CD4^+CD25^+$ but also $CD4^+CD25^-$ T cells can be suppressive, provided that they are experimentally used in high enough doses suggesting competition as suppressive mechanism [101].

Homeostasis of αβ TCR T cells

The size of the lymphocyte pool is crucial for the adaptive immune system to be able to effectively eliminate the diverse array of potential pathogens. However, physical space is limited.

The size of T cell pool is the result of a complex balance among thymic output, T cell proliferation, T cell redistribution in tissues and the death rate [102]. Each of these factors are controlled by many mechanisms.

Evolution resulted in an elegant way to provide an immensely diverse TCR repertoire with a limited set of genes. The physical maintenance of such a diverse T cell population needs sophisticated mechanisms to preserve all specificities and avoid expansion over the space limit. How the immune system regulates and maintains the diverse T cell repertoire in the periphery is only incompletely understood. On one hand, during infections, expansion of antigen-specific T cell clones needs to be allowed and the repertoire shifts towards T cells bearing a TCR directed against the intruder. However, after elimination of the pathogen, activated T cells need to be eliminated to avoid potentially dangerous auto-aggressive clones and reduce toxic cytokine production. Moreover, the repertoire needs to be regulated to avoid predominant T cell specificites. On the other hand, the immune system needs to be able to compensate for dying T cells (due to the limited half-life of naïve T cells) or T cell lymphopenias occuring e.g. after certain viral infections [103] [104]. At least in young animals, T cells are continuously produced, mainly by the thymus.

In order to retain highly effective T cells against a certain specificity, memory T cells evolve during an infection. This allows to reduce the number of naïve T cells with this TCR specificity. However, space is limited even for memory T cells, a process called attrition [105].

Failure to eliminate T cells leads to increased T cell numbers, clinically manifest as lymphadenopathy and splenomegaly. Furthermore, persistence of activated T cells bears the risk of autoaggression. This is best illustrated by the autoimmune lymphoproliferative syndrome (ALPS) where apoptosis of T cells is impaired due to a defective apoptosis pathway [106, 107] leading to uncontrolled lymphoproliferation.

Thymic export is important for the maintenance of a diverse T cell repertoire [108]. An increase in numbers of thymi leads to increased numbers of peripheral T cells [109]. However, this may be an indirect effect due to increased IL-7 production by the grafted thymi [110]. There is little evidence for feedback from the periphery to the thymus as peripheral T cell depletion does not increase thymic export [111] whereas sublethal irradiation regenerates the activity of thymi of old mice. Moreover, highly active anti-retroviral therapy (HAART) increases thymic activity [110], yet, a direct influence of the treatment on the thymi cannot be excluded.

Once T cells exit from the thymus, they readily respond to reduced T cell numbers with lymphopenia driven proliferation. T cells that emerge in young animals enter empty secondary lymphoid organs and proliferate [103, 110]. In vivo, only a few stimuli are clearly known

to be required for naïve T cell survival. IL-7 and low affinity TCR-self-MHC interactions are required much like during thymic development. In the absence of these two signals, naïve T cells die via the Bcl-2/Bim pathway [102]. A role for TCR-self-MHC interactions is widely accepted in survival for naïve T cells and LIP, yet it is still unclear what its precise role is [103]. Upon antigen encounter, T cells become activated and require IL-2 for survival. In contrast, IL-2 is toxic for memory $CD8^+$ T cells [102]. Activated T cells die through at least two distinct pathways: activation induced cell death (AICD) and an activated T cell autonomous death pathway [102, 112]. Due to the relatively long lifespan of naïve T cells (months), proliferation of phenotypically naïve T cells is rare [103]. During homeostatic proliferation T cells upregulate surface molecules used to characterize memory T cells. Despite intensive research, however, it is still not possible to distinguish real memory T cells from memory-like T cells after homeostatic proliferation. (see below).

As mentioned previously, also memory T cell numbers are tightly controlled. Memory T cells require different stimuli for survival than naïve T cells, indicating that they occupy different niches [103]. IL-15 and IL-7 are required to keep $CD8^+$ memory T cells alive [102] while IL-2 kills $CD8^+$ memory T cells. The control of $CD4^+$ memory T cells remains poorly understood with roles reported for IL-7 and TCR mediated signals.

In summary, naïve T cells are regulated differently than activated T cells and likewise factors controlling life and death of memory cells are different. Cytokines with the common gamma chain (cγ) are required for the survival and proliferation of T cells in various activity states. Competition for particular cytokines and/or regulation of the cytokine levels contribute to the control of the T cell pool. Availability of MHC molecules through competition of interaction with APCs is another factor that may contribute to T cell homeostasis. Co-stimulatory molecules such as CD28, CD40 and 4-1BBL are not required for homeostatic proliferation but can enhance LIP [113]. CTLA-4 deficiency induces marked polyclonal T-cell proliferation, indicating a role for CTLA-4 in maintaining homeostasis. CD24 can also enhance LIP [34].

Lymphocyte activation/memory/proliferation surface markers

During the process of LIP, T cells acquire a “memory-like” phenotype including upregulation of CD44 and CD45RB without having encountered the cognate antigen and -according to some reports- CD25 [103]. In contrast, other studies report that the early activation markers CD69 and CD25 remain unchanged [103, 114, 115] in cell subjected to LIP. It is widely accepted though, that the cells bearing a memory-like phenotype indeed behave like memory cells with increased proliferation to specific antigen, direct lysis of target cells in vitro and in

vivo and faster IFN-γ secretion upon stimulation [114-116]. Moreover, the upregulated markers remain stably expressed. With intact thymic output however, the memory-like T cells are rapidly replaced by recent thymic emigrants [117].

During antigen responses the numbers of activated T cells increase rapidly and dramatically. The increase is driven by engagement of TCR with MHC presenting foreign peptides and costimulatory molecules such as CD28, OX40, 4-1BB, LFA-1 and CD2 of the surface of antigen-engaged T cells. In response to these stimuli, cells start to divide and secrete IL-2, an auto -and paracrine cytokine. In T-cell sufficient animals, TCR specificity restricts proliferation to antigen-specific T cell clones [118]. However, in lymphopenic hosts, unspecific T cells proliferate as well [110]. During activation, T cells upregulate CD44, CD69 and CD25 on the surface, while CD45RB (CD4 cells) and CD62L are downregulated. During LIP, CD25 and CD69 are not typically upregulated [103].

T cell mediated autoimmunity and autoimmune diseases

Although autoimmunity affects about 5 percent of the population in Western countries [119, 120] and represents a heavy burden to health care systems, little is presently known regarding the etiologies that result in a detrimental loss of immune tolerance [121] and specific therapies are completely lacking. A plethora of pathogenetic mechanisms has been described in mouse models and human autoimmune diseases. To present details is certainly out of the scope of this introduction. The range of mechanisms is vast, disturbance of tolerance mechanisms of central and peripheral tolerance, T cell and B cell tolerance have been described. Dysregulated T cell activation [122] and proliferation [123], T cell receptor signaling [124, 125], Antigen receptor editing [126], a lack of regulatory T cells [94-96], bystander activation [127], cytokines, molecular mimicry, lymphopenia, viral infections etc. have been made responsible for autoimmune responses. Furthermore, the cooperation among cells from the innate with cells from the adaptive immune system are important [128]. It has also been shown that dendritic cells play an important role in controlling autoimmunity. DCs can be activating or tolerizing depending on their state of activation [129]. Last but not least the pathogenesis of autoimmune disease seems to be a multistep process as most known autoimmune diseases are polygenic and triggering stimuli have been described that convert autoimmunity into overt autoimmune disease [130-132]. The list of factors, molecules and mechanisms of autoimmune pathogenesis is by far longer and more complex than this short introductory text can cover. Yet, rare autoimmune diseases have been observed that are inherited in a Mendelian fashion and that might hold the key to a better molecular understand-

ing of the pathogenesis of autoimmunity.

APECED – a monogenic autoimmune disease

Autoimmune **P**oly**e**ndocrinopathy **C**andidiasis **E**ctodermal **D**ystrophy Syndrome (APECED; OMIM #240300) constitutes such a disorder that has the potential to reveal immunological mechanisms operational to distinguish between harmless self and injurious non-self. As an autosomal recessive disease, APECED, most frequently starts in childhood, affecting foremost but not exclusively endocrine organs leading to Addison's disease, hypoparathyroidism, type 1 diabetes mellitus (T1D), ovarian failure, thyroiditis and others. Moreover, APECED patients display additional features of autoimmunity such as hepatitis, pernicious anaemia, alopecia and vitiligo [82]. The large array of autoantibodies of different specificities and the prevailing tissue infiltration with lymphocytes are eventually responsible for the destruction and thus malfunctioning of affected organs.[133]

The gene defect accounting for the APECED syndrome has initially been identified in man and the locus has been designated Autoimmune Regulator, *AIRE* [80, 81]. Following this decisive finding, the murine homologue was cloned, thus rendering it possible to study the pathogenesis of this disorder in a murine genetic model [134-136]. The AIRE protein is primarily expressed in thymic epithelium where it is thought -but not proven- to regulate the expression of organ-specific self-proteins. Such a mechanism would be in keeping with the long-standing observation that many organ specific gene products are promiscuously expressed in the thymus [53, 74, 75, 77]. For example, insulin and GAD65, are not only expressed in ß-cells of the pancreatic islets of Langerhans but are also detected in medullary thymic epithelial cells. However, the regulation and functional role of the promiscuous expression of organ-specific self-proteins in the thymus still remain highly controversial.

The identification of AIRE as the genetic cause of APECED sets now the stage to unravel the molecular basis of the immune system's capacity to induce and maintain central tolerance to self-antigens. Thus, research on AIRE will likely provide novel and important insight into the pathogenesis of APECED and may concurrently identify novel strategies for the therapy of autoimmune diseases secondary to the failure of central tolerance.

The clinical presentation of Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal dystrophy (APECED) syndrome

APECED is a rare autosomal-recessive disorder characterized by an immune-mediated destruction of several endocrine tissues, chronic candidiasis and ectodermal disorders [82]. Its typical clinical highlights are hypoparathyroidisms (87% of all APECED cases), adrenocortical failure (68%) and mucocutaneous candidiasis (80%) [137]. Other endocrine pathologies typically include gonadal failure (46%), Hashimoto's Thyroiditis (9%) and insulin-dependent diabetes (6%) [138]. Other organ specific autoimmunity affects non-endocrine tissues such as the skin (alopecia areata: 48%; vitiligo: 15%), the liver (chronic active hepatitis: 15%), and gastric parietal cells leading to pernicious anemia: (11%). The ectodermal dysplasias are highlighted by nail dystrophies, dental enamel defects and keratopathies of unspecified pathogenesis. In the majority of cases candidiasis becomes manifest in APECED individuals before the age of 5 years, while hypoparathyroidism is typically detected within the first 10 years of life and the onset of Addison's disease typically occurs when the child is younger than 15 years of age. While immunosuppressive therapy has been used to treat various components of APECED, their success has not been unequivocally witnessed. Interestingly, and in contrast to the majority of all other autoimmune disorders, APECED has been considered to be independent of an association with HLA alleles, although recent observations have suggested a potential modifying influence by the certain HLA loci on disease manifestations in Finnish and North American APECED patients [139]. Since the most frequent clinical components are mucocutaneous candidiasis, hypoparathyroidism and adrenocortical failure, the presence of any two of these pathologies in a single individual has widely been accepted to be diagnostic for APECED [82, 140].

Reflecting the broad endocrine immunopathology, the major autoantigens detected in patients with APECED are the steroidogenic P450 enzymes steroid 17-alpha-hydroxylase, 21—hydroxylase, and side chain cleavage enzyme. Other autoimmune reaction targets include antigens specific for islets of Langerhans (glutamic acid decarboxylase (GAD) 65 and 67; IA-2 and insulin), hepatocytes (P 450 species), thyroid epithelium (thyroid peroxidase, thyroglobulin) and melanocytes (SOX 9 and 10) [133].

The genetics of AIRE

APECED displays a high prevalence in distinct ethnic backgrounds (Iranian Jews 1:9000; Sardinians 1:14'400; Finns 1:25'000) and is associated with mutations in a single gene, designated autoimmune regulator (AIRE). This gene is located on chromosome 21q22.3, spans 13 kb and consists of 14 exons, which encode a predicted protein of 545 amino acids [137]. The AIRE protein is predominantly expressed in thymic epithelial cells located at the cortico-medullary junction and in the medulla proper [16]. AIRE is, however, also expressed by other cells including dendritic cells (which are also located in the thymic medulla but can equally be found in peripheral tissues) [15, 17, 136, 141].

AIRE is detected in the nucleus in a punctate pattern, a result compatible with the presence of a nuclear localization signal in the N-terminal part of the protein [142, 143]. Most interestingly, AIRE expression is not detected in target organs of APECED autoimmunity. This finding suggests that the function of AIRE may be related to the control of effector cells of the immune system rather than locally triggering the process of auto-destruction in target tissues. The AIRE protein has several structural features indicative of a function as a transcription factor such as a proline rich region (PRR), plant-homology domain (PHD) fingers and the recently described DNA-binding domain SAND [144]. These domains have typically been detected in proteins involved in transcriptional control. Supporting a direct role for AIRE as a transcription factor, transcriptional activation of several reporter gene systems with distinct minimal promoters has been reported in the presence of AIRE [145]. In keeping with these functional analyses, AIRE in either a dimeric or tetrameric form has been demonstrated to bind directly to both DNA and the nuclear CREB-binding protein CBP, which acts as a co-activator for several transcription factors [146].

The physiological role of AIRE

At least four distinct pathways of AIRE function have been envisaged. One mechanism would predict that AIRE regulates the promiscuous gene expression of peripheral-tissue specific transcripts [79]. The phenomenon that proteins such as pro-Insulin, GAD 67, proteolipid protein (PLP, a major component of myelin), the acetylcholin receptor and retinal S antigen are expressed by thymic medullary epithelial cells and dendritic cells has indeed been linked to the mechanism of central tolerance [69, 77].

The second mechanism how AIRE could be involved in thymic selection is the activation of co-stimulatory signal molecules operational in negative selection by thymic stromal cells.

These molecules would be required to induce either programmed cell death or a state of anergy among developing, autoreactive T cells. The third mechanism by which AIRE may contribute to T cell tolerance is its role in the production of regulatory T cells, a population of effector cells that suppresses the activation and expansion of autoreactive T cells. Finally, the forth mechanism foresees that AIRE is important for the development of a subpopulation of medullary epithelial cells that effects negative thymic selection independent of promiscuous gene expression.

Recent findings suggest, however, that AIRE may be important for promiscuous gene expression [79]. Analyzing the gene expression profile of medullary thymic epithelial cells from wild-type and AIRE-deficient animals revealed a loss of thymic promiscuous gene expression in the genetically mutant mice. However, these results are possibly flawed since the study investigated mRNA derived from all epithelial cells of the medulla and not just from the normally AIRE-positive subpopulation of epithelial stromal cells. This distinction is important as the possibility has not been excluded that AIRE may actually be necessary for the maturation and expansion of a subpopulation of thymic epithelial cells. In the instance of homologous AIRE gene disruption, these cells could be unable to differentiate into various cell types which in turn – but for very different reasons – would result in a lack of promiscuous gene expression [88]. Therefore, it is still unknown what the precise role of AIRE in the context of a regular thymic function is.

Experimental models

A) Aire-cre mice as a tool to activate or silence genes

In order to study the function of Aire expressing cells in vivo, it would be desirable to have a tool to genetically manipulate this rare subpopulation of TECs in vivo. As the the cre/loxP-system is a binary system, it allows both, in vivo gain (Figure 4A a) or loss of function (Figure 4A b), depending on whether the cre expressing mouse is crossed with a mouse carrying a conditional transgene or a conditional gene knockout allele, the transgene will be activated or the gene of interest deleted, respectively. Therefore, a strategy was worked out to direct Cre expression under the transcriptional control of the aire promoter. Since it was an additional aim to create a mouse model of the human APECED syndrome, cre was designed to replace the endogenous *aire* gene by gene targeting in order to inactivate aire.

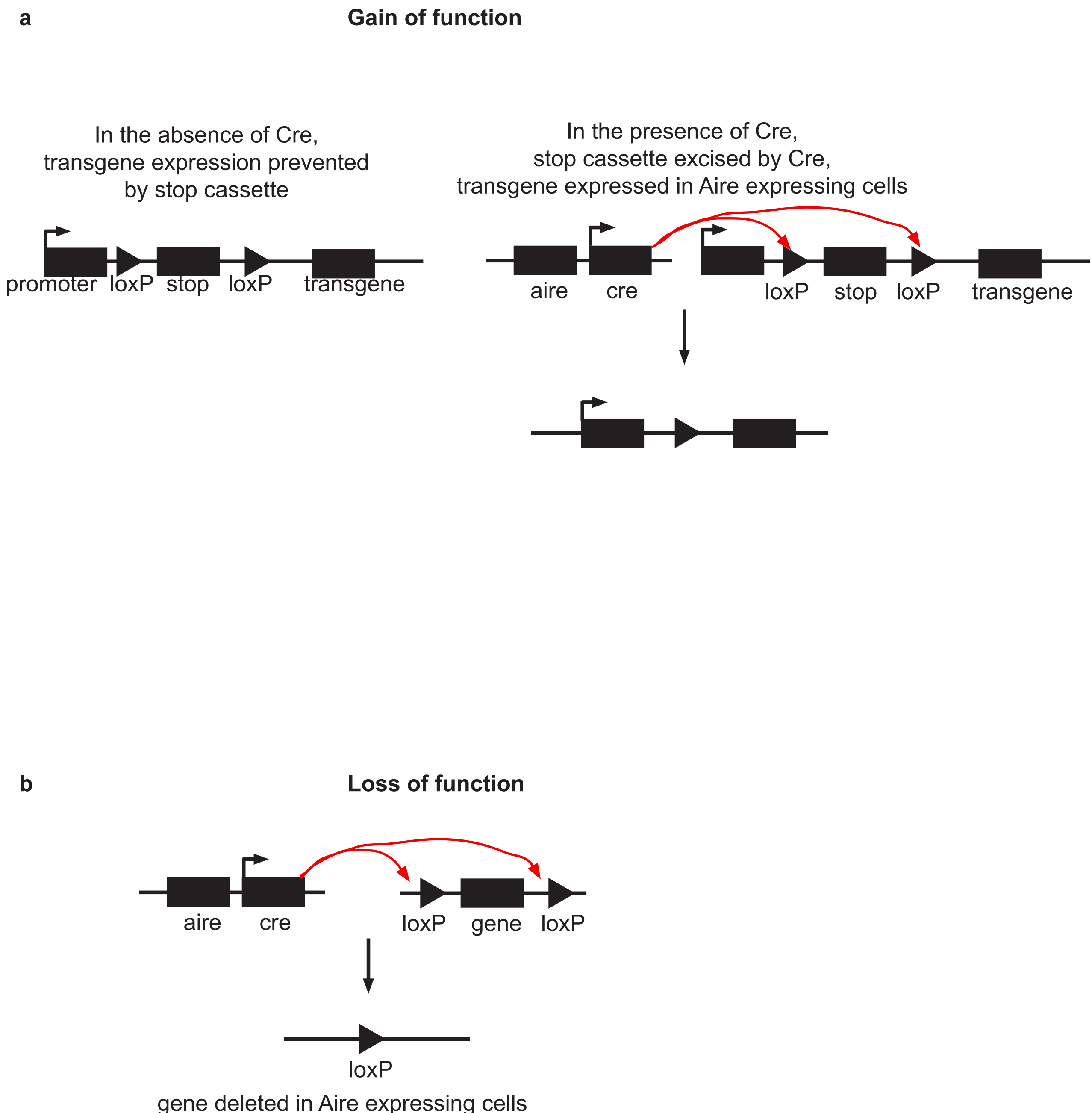


Figure 4A
Schematic representation of the tissue specific activation (a) or inactivation (b) of genes by gene-targeted aire-cre mice
a) Aire-cre mice crossed to mice carrying a conditional transgene:
A subpopulation of thymic epithelial cells expresses Cre under the transcriptional control of the aire promoter. The Cre enzyme will recombine the two loxP sites of the conditional transgene resulting in activation of the transgene. This mutation renders the smad4 gene non-functional (a).
b) Aire-cre mice crossed to conditional gene knockout mice:
Again, a subpopulation of TECs expresses Cre under the transcriptional control of aire. In contrast to a, when aire-cre mice are crossed to a mouse carrying a conditional gene knockout allele, the gene is inactivated rather than activated.

B) Thymic epithelial-specific Smad4 ablation

FoxN1 is a transcritpion factor that is specifically expressed in TEC and keratinocytes. Therefore it was decided to take advantage of the restricted foxN1 expression to direct TEC specific Cre-mediated gene ablation by creating a transgenic mouse harboring a cre recombinase under the transcriptional control of foxN1 (Zuklys S., unpublished). Crossing the FoxN1-cre mouse to a mouse harboring a conditional smad4 allele should allow to inactivate smad4 specifically in TECs while leaving thymocyte development and T cell function intact (Figure 4B).

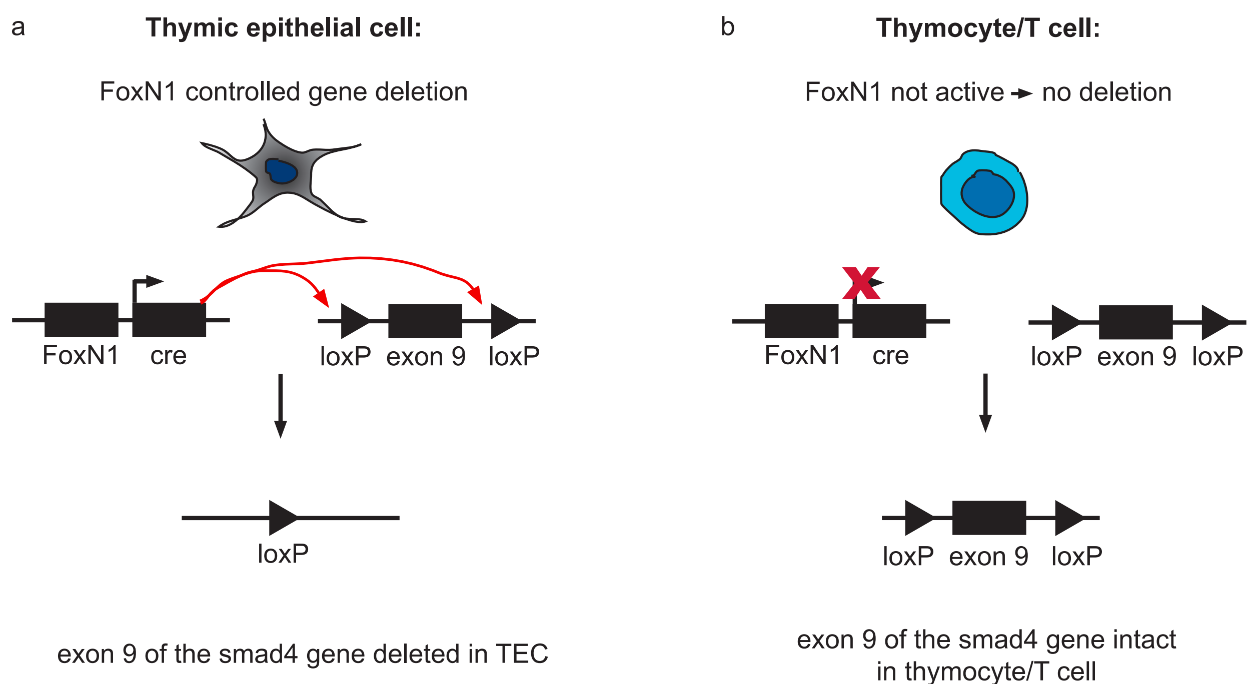


Figure 4B
Schematic representation of the tissue specific smad4 deletion in TEC$^{smad4-/-}$ mice
Thymic epithelial cells express Cre under the transcriptional control of the FoxN1 promoter. The Cre enzyme will recombine the two loxP sites of the conditional smad4 allele resulting in deletion of exon 9 of the smad4 gene. This mutation renders the smad4 gene non-functional (a). Other cell types, including thymocytes and mature T cells do not express FoxN1. As a consequence, Cre is not expressed preventing excision of exon 9 of the conditional smad4 allele. Thus, smad4 remains functional in all cells that do not express FoxN1, including thymocytes and T cells (b).

Nomenclature of Mice

Aire gene targeted mice

Gene targeted mice were collectively designated aire-cre independent of a particular genotype. To describe particular genotypes, the designations aire$^{CN/wt}$ (heterozygous for **C**re **N**eo (=CN)) or aire$^{CN/CN}$ (homozygous) were defined. After crossing to Flp-Deleter mice [147], offspring were designated aire$^{CNDF/wt}$ (for **C**re **N**eo **D**eleted **F**lipase) and aire$^{CNDF/CNDF}$, respectively. After further backcrossing -when the Flipase transgene was lost- mice were called aire$^{CND/wt}$ and aire $^{CND/CND}$. The three independent lines (103, 292 and 336) were maintained as separate mouse lines. Accordingly the ES clone number was added to the name. In addition, the generation of backcrossing to C57bl/6 and Balb/c mice was included in the name.

For details see the Materials & Methods section.

Mice used to study the role of Smad4 in thymic epithelial cells

All mice studied in part B were homozygous for the conditional *smad4* allele [148], i.e. *smad4*$^{loxP/loxP}$ and were either positive for the FoxN1-cre transgene (Zuklys et al., unpublished) or not. Accordingly they will be called

TEC$^{smad4-/-}$ (or cre^{+} in figures) for *smad4*$^{loxP/loxP}$ FoxN1-cre

and

control (or cre^{-} in figures) for *smad4*$^{loxP/loxP}$

control littermates if not indicated otherwise. This nomenclature was chosen to simplify figures and to improve readability of the text. It tries to reflect the fact that the *smad4* gene is inactivated exclusively in thymic epithelial cells.

Aim of the Thesis

- To generate a mouse model of the human APECED syndrome
- To generate a tool to genetically manipulate *in vivo* Aire expressing medullary thymic epithelial cells
- To investigate the role of Smad4 in thymic epithelial cells

Results

PART A

A.3.1 Generation of mice expressing the Cre recombinase under the transcriptional control of the aire promoter

A.3.1.1 Cloning of the aire-cre targeting construct

The bacteriophage recombinase *cre* cDNA preceded by a nuclear localization signal (NLS) was inserted 3' of a 3.5kb arm of *aire* homology followed by an IRES-hrGFP sequence and a SV 40 poly A signal. 3' of the GFP followed a neomycine resistance cassette flanked by FRT sites for conditional removal of the potentially interfering sequence. The very 3' end of the targeting vector consisted of a 4.2kb arm of *aire* homology. A plasmid map of the final targeting vector p79 is shown in Annex I. Details how the aire-cre targeting construct was synthesized are described in Materials & Methods.

A.3.1.2 Testing the aire-cre targeting construct

In order to test the functionality of the final targeting vector, cre-IRES-hrGFP-SV40 polyA was subcloned into a vector with a CMV promoter that is ubiquitously active in mammalian cells. This additional step was necessary since the *aire* promoter is only active in specialized cells and hence cannot be used to test the final construct directly. HEK293 cells were transfected with plasmid p67 (see annex I). 24h after transfection, Cre^+ nuclei (Figure 5a) and GFP^+ cytoplasm (Figure 5b) could be detected in transfected but not untransfected HEK293 cells (data not shown).

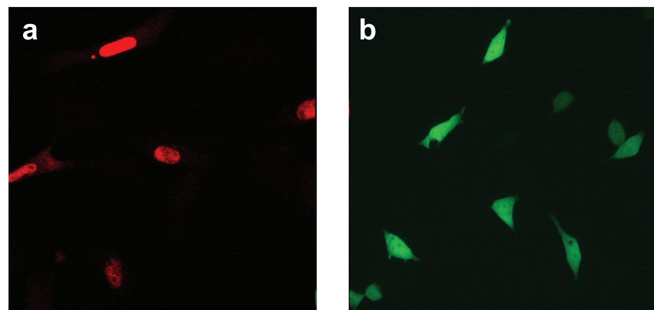
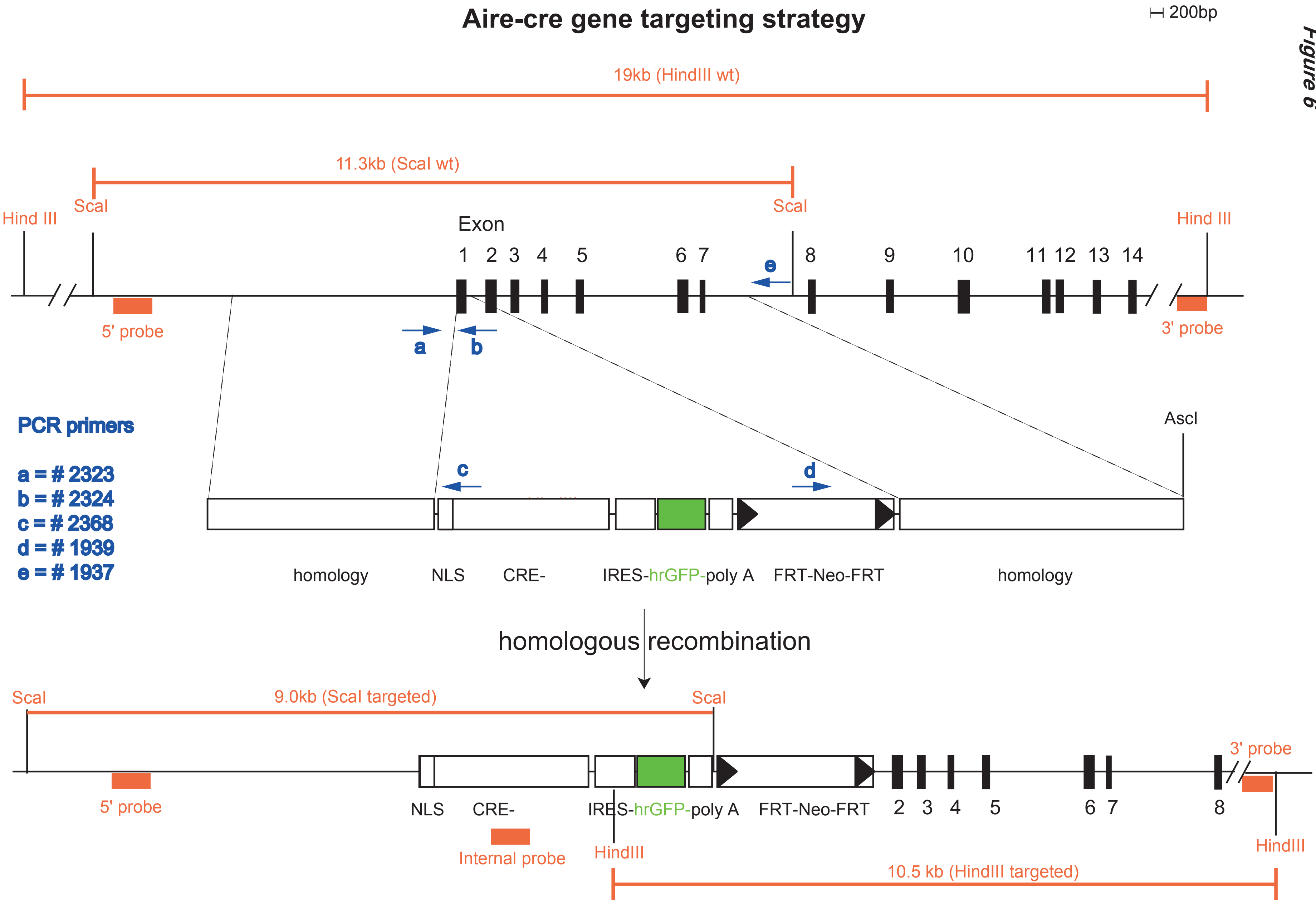


Figure 5
Cre and IRES-GFP expression work in vitro
HEK 293 cells were transiently transfected with p67 and stained with anti-cre antibody revealing a clear nuclear signal (a) while GFP localization is cytoplasmic (b). The original magnification in a was higher than in b.

Figure 6



A.3.1.3 Gene targeting

100µg of linearized plasmid p79 was electroporated into $5x10^7$ 129/Sv ES cells in single cell suspension. Electroporated ES cells were plated on a monolayer of neomycine resistant transgenic embryonic feeder (EF) cells. Neomycine resistant ES clones harboring a target construct integration were selected with 500µg/ml G418 based on their antibiotic resistance. Individual ES clones were hand-picked and split for freezing and expansion to amplify DNA for screening of correct *aire* gene targeting. Details are described in Materials & Methods.

A.3.1.4 Southern blot screening of DNA extracted from targeted ES clones

DNA from 360 targeted ES clones was extracted for the screening of integration of the targeting construct. Initial screening was based on southern blotting (Figure 6). ScaI digested genomic DNA from single ES clones was subjected to southern blot screening using the 5' probe (Figure 7a) with primers #1925/#1926 (see Annex III) yielding a 532 bp probe. 9/102 (9%) clones were wildtype and 93/102 (91%) were heterozygously targeted. DNA from ES cell clones with 5' correctly targeted *aire* were HindIII digested and analyzed with the 3' probe (Figure 7b), produced by primers #2127/#2128, resulting in a 535 bp probe. ES clones displaying correct homologous recombination at both ends of the targeting construct were subjected to screening with an internal probe to exclude ES cells with random target construct integration (primers #2055/#2056). Clones with several integrations were excluded (data not shown).

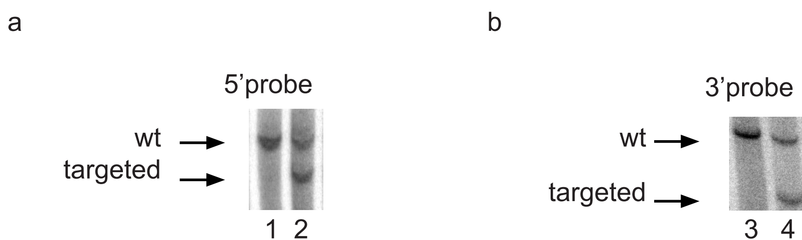


Figure 7
Correct targeting of ES cells.
DNA from aire-cre targeted ES cells were probed with a radioactively labelled 5' (lanes 1 and 2) and a 3' probe (lanes 3 and 4). Lanes 1+3 show a wildtype ES clone, lanes 2+4 show DNA from a heterozygously targeted ES clone.

A.3.1.5 PCR screening of DNA extracted from targeted ES clones

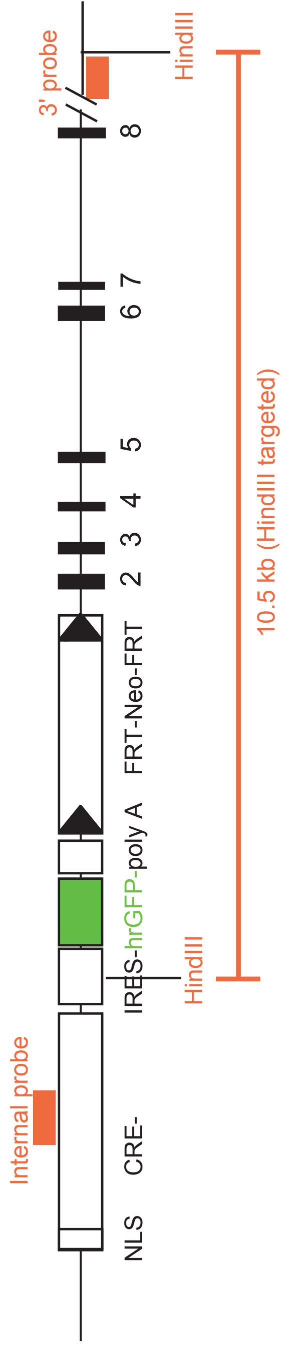
Since the targeting efficiency as determined by southern blotting was much higher (91%) than expected [149] PCR screening was performed to confirm the observation. A 3’ primer pair (#1939/#1937) confirmed the results (data not shown).

A.3.1.6 Expansion of correctly targeted ES clones and blastocyst injection

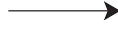
Five correctly targeted ES cell clones were thawed and expanded and 3 different ES cell clones (clones 103, 292 and 336) were injected into blastocysts as described in Materials & Methods. As only 8-15 cells were injected per ES clone, DNA from the remaining cells of clones 103, 292 and 336 was extracted and verified by southern blotting to be the correctly targeted clones (data not shown).

Figure 8

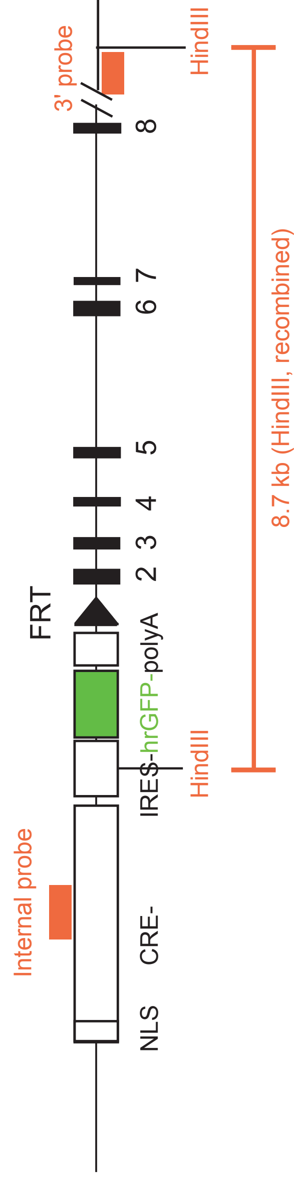
In vivo removal of the neomycine resistance cassette



Removal of Neomycine resistance cassette:



Cross aire-cre-neo to Flipase (Flp) deleter mice



A.3.1.7 Germline transmission and removal of the neomycine resistance cassette

Chimeras were obtained from all 3 injected ES clones. Chimeras were bred to C57bl/6 mice to obtain germline transmission (data not shown). Offspring was genotyped by PCR for the *cre* transgene using oligonucleotides #2323/#2324/#2368 (see annex III). Cre^{+} mice were crossed to homozygosity (Figure 9a) in order to inactivate the *aire* gene. In addition, Cre^{+} mice were crossed to Flipase-Deleter (Flp) mice [147] to remove the FRT-flanked neomycine resistance cassette as it has been reported [150] that such a resistance cassette can influence *cre* transgene expression even with a “knock-in” i.e. gene targeting approach (Figure 8). Correct removal of the neo cassette was monitored by PCR and revealed substantial mosaicism (data not shown). Mice with complete removal were selected to continue breeding and removal of the neomycine resistance cassette was verified in F2 offspring (Figure 9b).

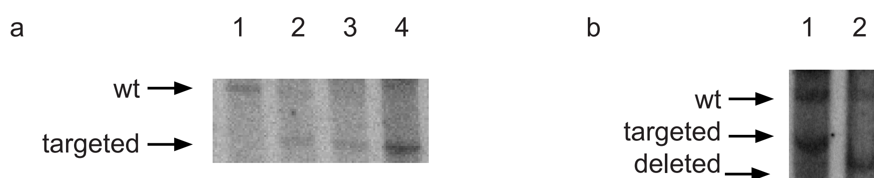


Figure 9
Confirmation of correct targeting and neomycine resistance cassette deletion in mice
a) Southern blot of aire$^{wt/wt}$ (lane 1), aire$^{wt/CN}$ (lanes 2 and 3) and aire$^{CN/CN}$ mice (lane 4) using the 3’ probe for the detection of correct targeting of the aire locus.
b) Southern blot from Aire$^{wt/CN}$ (lane 1) and Aire$^{wt/CN}$x Flp-Deleter mice (lane 2) assayed with the 3’ probe for the detection of correct deletion of the neomycine resistance cassette.
For a cartoon of the targeting and southern blot strategy see Annex I.

A.3.2 Spontaneous infiltration of peripheral organs in aire-cre mice

Aire deficient mice spontaneously develop multiorgan lymphocytic infiltration [78, 79]. In order to test whether our gene targeting strategy inactivated the *aire* gene, we histologically examined the most frequently infiltrated organs liver, salivary gland and pancreas (Figure 10). Aire$^{CN/CN}$ and aire$^{CNDF/CNDF}$ mice spontaneously developed lymphocytic multi-organ infiltration of liver (Figure 10, panel 10d), pancreas (Figure 10, panel 10e) and submandibular salivary gland (Figure 10, panel 10f). Aire$^{wt/wt}$ mice did not show organ infiltration (data not shown) while aire$^{CN/wt}$ heterozygous mice also developed a limited extent of infiltration at a lower frequency (Tetsuya and Shikama-Dorn, unpublished) in contrast to previous reports [78, 79].

Furthermore, the severity of infiltration varied substantially interindividually in aire$^{CN/CN}$ and aire$^{CNDF/CNDF}$ individuals, some animals displayed only restricted infiltrations while others displayed a massive tissue destruction (data not shown). However, no correlation between infiltration and the presence or absence of the neomycine resistance cassette could be found. Mice of clones 292 and 336 were bred to homozygosity and both developed comparable organ infiltration.

In general, despite extensive organ infiltration, mice appeared clinically healthy with the exception of the mouse with the heavily destroyed pancreas shown in Figure 10, panel 10e that appeared clinically sick, was smaller than control littermates (data not shown) and had multiorgan infiltration.

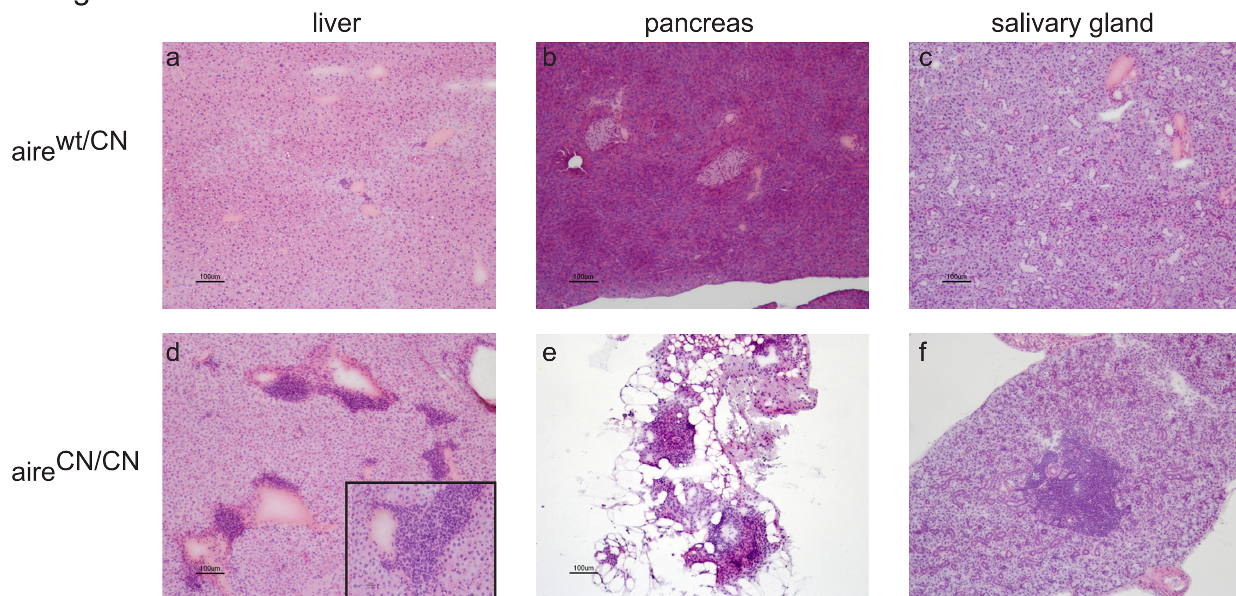


Figure 10
Spontanous lymphocytic infiltration of peripheral organs in aire deficient mice.
HE staining of liver (a+d), pancreas (b+e) and salivary gland (c+f) cryosections from 4 months old aire$^{CN/CN}$ and aire$^{wt/CN}$ control mice. Panel d additionally shows infiltrating cells at a higher magnification.

A.3.3 Characterization of Cre expression in aire-cre gene targeted mice

A.3.3.1 Highly specific Cre expression in postnatal medullary thymic epithelial cells

Aire is physiologically expressed in a rare subtype of mTECs [15, 16, 141, 151-153]. In order to determine the specificity of Cre expression in aire-cre mice, immunohistochemical analysis of Aire (green) and Cre (red) expression were performed in postnatal thymus (Figure 11). Thymic size and the demarcation into cortex and medulla were normal in *aire*-deficient aire$^{CNDF/CNDF}$ mice. Cytokeratin 18 (blue) was used as a marker for cortical thymic epithelial cells.

Panel a shows the expression of Aire in thymic epithelial cells of wildtype mice. Positive cells were found in the medulla but not in the cortex (panels c+d). Background staining for Cre was not detected when using the Cre-specific antibody on tissue from wildtype mice (panel c). TECs from heterozygous aire$^{CNDF/wt}$ mice showed a regular aire expression pattern (panels e, g+h) and display Cre expression in the medulla (panels f+h) where most Cre^{+} cells are also Aire^{+} (panel h; yellow). However, some cells were only Aire^{+} (panel h; green) or, alternatively, only Cre^{+} (panel h; red). Thymic tissue from mice homozygous for the loss of *aire* (aire$^{CNDF/CNDF}$) did not stain with Aire-specific antibodies (panel i) while Cre expression could be detected in some but not all cells of the medulla (panels k-m).

These results illustrate the specificity of Cre expression in a subtype of postnatal medullary thymic epithelial cells in aire-cre gene targeted mice. Furthermore, panel i demonstrates that the Aire protein is not present in aire$^{CNDF/CNDF}$ mice, underlying the basis for the observed lymphocytic organ infiltration (Figure 10).

A 3.3.2 LacZ reporter mice suggest Cre expression in aire-cre mice prior to the thymus formation

In order to assess the function of Cre in aire-cre mice, we crossed aire$^{CNDF/wt}$ mice to ROSA26 lacZ reporter mice [154]. These indicator mice express the β-galactosidase gene only upon Cre-mediated removal of a loxP flanked transcriptional stop cassette and are therefore suitable to test tissue specificity of Cre expression in *cre* transgenic mouse lines. Postnatal thymic cryosections of [aire$^{CNDF/wt}$ x ROSA26 lacZ] mice revealed two types of blue cells (Figure 12). Most prominently, large cells and clusters of cells located in thymic medulla (panels b+c) were found in a pattern reminiscent of the physiologic Aire expression (see Figure 11). A second type of cells stained as single, punctuate blue dots in the medulla

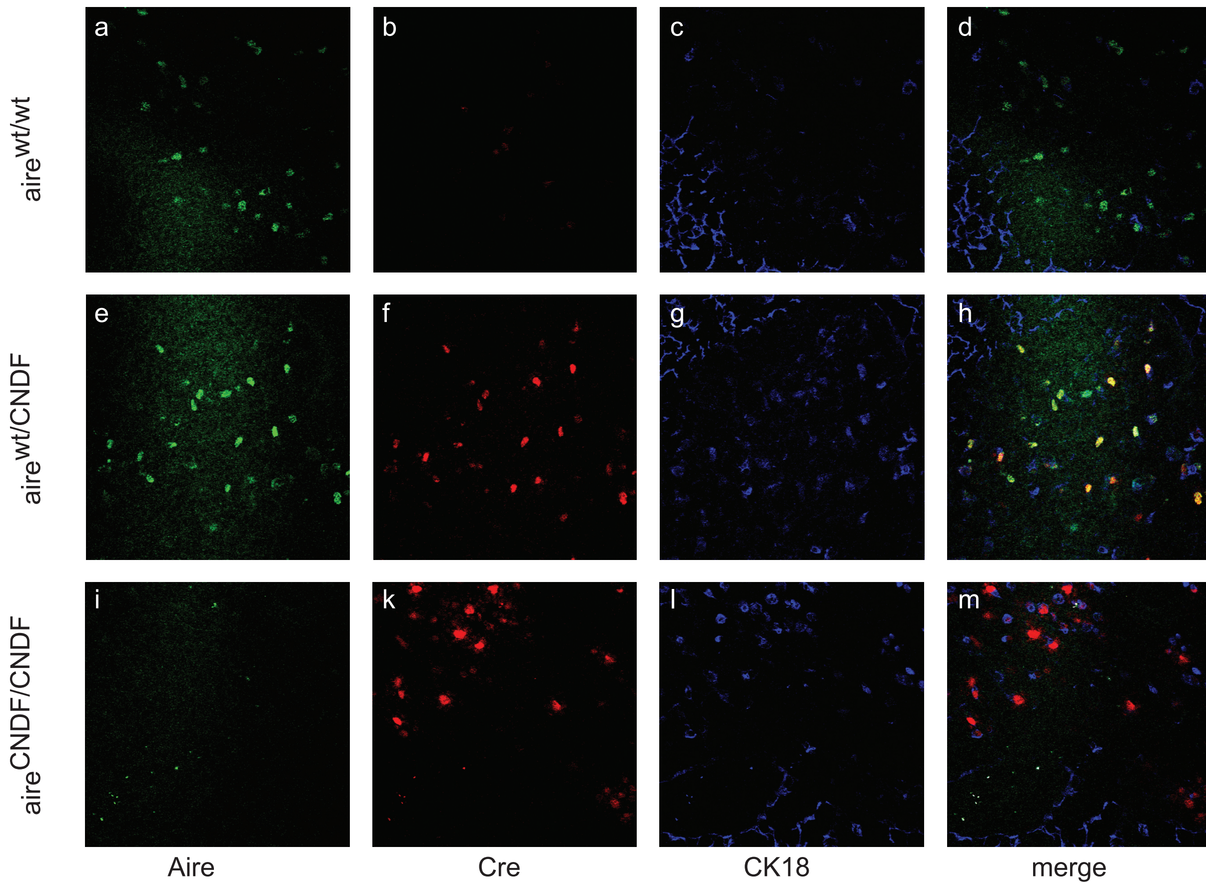


Figure 11
Cre expression in aire gene targeted mice faithfully matches aire expression in postnatal thymus
Immunofluorescence confocal microscopy of frozen thymus sections of aire$^{wt/wt}$ *(a-d),* aire$^{wt/CNDF}$ *(e-h) and* aire$^{CNDF/CNDF}$ *(i-m) mice.*
Stainings were done for endogenous aire (green), transgenic cre (red) and the cortical TEC marker cytokeratin 18 (blue). The panels d, h and m display an overlay of all signals from all fluorochromes. Aire and cre colocalization appears as yellow.
Pictures are a kind courtesy of Dr. N. Shikama-Dorn.

and -surprisingly- the cortex. Thymic tissue from aire$^{wt/wt}$ mice did not stain blue, excluding unspecific endogenous β-galactosidase activity (panel a).

To discriminate between staining of TECs and thymocytes and to define lacZ^{+} TEC subtypes, lacZ stainings were performed with subsequent immunohistochemical analysis of markers defining subtypes of TECs (panels d-i). Some blue cells colocalized with cytokeratin18^{+} (CK18) cTECs (panel d+g) and some but not all cytokeratin5^{+} (CK5) mTECs were also lacZ^{+} (panels e+h). TECs were further subdivided into major (CK18^{-}) and minor (CK18^{+}) mTECs by the use of CK18 and the lectin UEA-1 [155]. As expected, all minor medullary CK18^{+} UEA-1-binding TECs that are known to express Aire [74] were lacZ^{+} (panels g+i).

However, not all lacZ$^+$ cells were costaining for CK18 (panel g) or binding UEA-1 (panel i).

Since Cre-mediated recombination is an irreversible system, these results suggested that Cre had been expressed at an earlier time point in a presumable precursor of cTECs and mTECs. Despite the unexpectedly high number of lacZ$^+$ cells observed, the obvious absence of staining of thymocytes excludes that aire-cre mice express Aire in every single cell. To further assess if β-galactosidase expression was restricted to thymic tissue or could also be found in secondary lymphoid and non-lymphoid tissues, spleen, liver, kidney, skin, brain and adrenal glands of [aire$^{CNDF/wt}$ x ROSA26 lacZ] mice were analyzed for β-galactosidase activity. Surprisingly, and in contrast to previous reports that could not detect the Aire protein outside the thymus [15, 156] all organs contained lacZ$^+$ cells. However, the extent of cells staining positive ranged from 80-95% in the liver to very few cells in the kidney (data not shown) illustrating that Cre expression in Aire-cre mice is not ubiquitous.

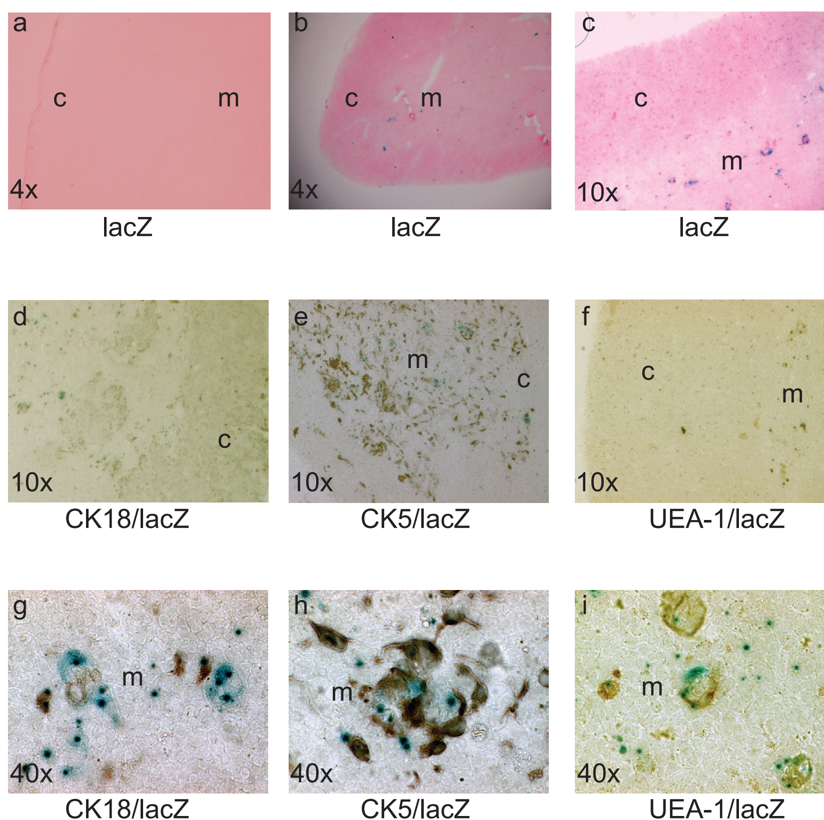


Figure 12
Thymic cryosections of lacZ reporter mice crossed to aire$^{wt/wt}$ and aire$^{cre/wt}$ mice reveal unspecific recombination in aire$^{cre/wt}$ mice

a) Absence of lacZ staining in Aire$^{wt/wt}$ mice.
b+c) lacZ (blue) staining of aire$^{cre/wt}$ mice reveals cortical and medullary staining.
d+g) Cytokeratin 18/lacZ costaining confirms that some CK18$^+$ TECs are lacZ$^+$. All minor medullary CK18$^+$ TECs are lacZ$^+$.
e+h) Some cytokeratin 5$^+$ TECs costain with lacZ while some lacZ$^+$ cells are CK5$^-$.
f+j) All UEA-1 binding TECs are also lacZ$^+$. Yet there are lacZ$^+$ UEA-1$^-$ cells.
c= cortex
m= medulla

All mice were heterozygous for the conditional ROSA26 lacZ reporter allele.
The original magnifications are indicated.
a-c were counterstained with nuclear fast red.

A.3.3.3 Widespread Aire expression in multiple lymphoid and non-lymphoid organs

To dissect the contradicting results from the analysis of Cre expression using immunohistochemistry and lacZ reporter mice, the *aire* mRNA expression in an array of organs of C57bl/6 mice was determined to test whether *aire* expression was broader than reported. Three independent primer pairs were used to amplify cDNA's generated from *aire* mRNA since several *aire* splice variants are known [136]. To exclude genomic contamination in the cDNA preparation and subsequent amplification of genomic *aire* sequences rather than *aire*-specific transcripts, all oligonucleotides were designed as intron-spanning primers spanning introns 2, 7 and 8, respectively (see Annex III, primer pair 2107/2108 (spanning exon 2), 2144/2145 (spanning exon 7) and 490/491 (spanning exon 8)). All primer pairs yielded identical results summarized in table I. Astonishingly, except for lactating mammary gland and skeletal muscle, *aire* mRNA transcripts were found in any organ analyzed (Table I). This result questioned the restriction of *aire* expression to lymphoid organs.

Organ	Signal
Bone marrow	+
Brain	++
ES cells	+++
Eyes E 15	+
Gut	+++
Kidney	+++
Lactating mammary gland	-
Liver	+
Lymph Node	+++
Lung E14	+
Salivary gland	+
Sceletal muscle	-
Skin	+
Spleen	++
Testis	+++
Thymus E 14	+
Thymus E 17.5	+++
Thymus postnatal day 1	+++
Thymus adult	+++

Table I
Aire mRNA expression in C57bl/6 mouse organs detected by 36 cycles of end-point RT-PCR amplification. -, +, ++ and +++ stand for estimated relative amplification signals.

A.3.3.4 Proof of Cre activity during mouse embryonic development

To test the hypothesis that Cre is active in aire-cre mice prior to thymic development, [aire$^{CNDF/wt}$ x ROSA26 lacZ] embryos were generated (Figure 13). As expected, E 6.5 (data not shown) and E 7.5 [aire$^{CNDF/wt}$ x ROSA26 lacZ] embryos already showed substantial β-galactosidase activity (panel a) while [aire$^{wt/wt}$ x ROSA26 lacZ] littermates were lacZ$^-$. Interestingly, the embryos at E 6.5 and E 7.5 were only incompletely blue, suggesting that Cre had been active only after certain steps of differentiation. From E 9.5 on, [aire$^{CNDF/wt}$ x ROSA26 lacZ] appear to be ubiquitously blue (panel b). Sections of E 12.5 whole mount lacZ stained embryos revealed, however, that not every single cell was lacZ$^+$ (data not shown). Yet, sections through the developing thymus at E 12.5, a time point when aire mRNA cannot be detected by RT-PCR yet (Table I), demonstrated clearly, that the cells in thymi from [aire$^{CNDF/wt}$ x ROSA26 lacZ] are chiefly lacZ$^+$ while their [aire$^{wt/wt}$ x ROSA26 lacZ] littermates have thymi with virtual absence of β-galactosidase activity (panel c).

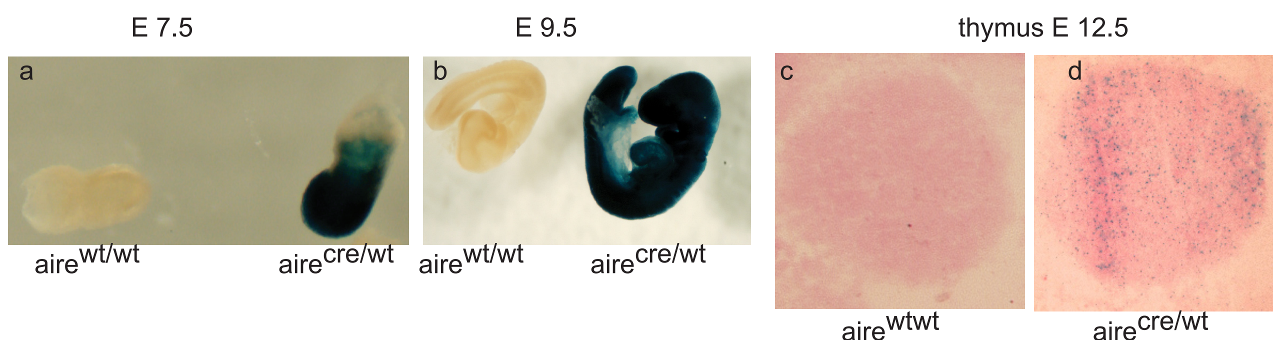


Figure 13
lacZ reporter mice reveal cre expression in aire$^{cre/wt}$ ROSA26$^{lacZ/lacZ}$ mice prior to E 7.5
Aire$^{cre/wt}$ mice were crossed to conditional ROSA26-lacZ indicator mice to analyze functionally cre expression during embryonic development.
a) E 7.5 aire$^{cre/wt}$ ROSA26$^{lacZ/lacZ}$ embryos are partially positive for lacZ while an aire$^{wt/wt}$ ROSA26$^{lacZ/lacZ}$ control littermate does not show any background lacZ staining.
b) Aire$^{wt/wt}$ ROSA26$^{lacZ/lacZ}$ E 9.5 embryos are devoid of lacZ staining while aire$^{cre/wt}$ ROSA26$^{lacZ/lacZ}$ embryos appear totally blue.
c) Frozen sections of E 12.5 embryos clearly demonstrate lacZ expression in a majority of cells in aire$^{cre/wt}$ ROSA26$^{lacZ/lacZ}$ but not aire$^{wt/wt}$ ROSA26$^{lacZ/lacZ}$ mice in the early thymus.

In summary, aire-cre mice faithfully expressed Cre in Aire-expressing medullary thymic epithelial cells in postnatal thymi. However, Cre was expressed already during embryonic development. This early recombination overrides the specificity achieved later in life. It remains to be determined whether the embryonic Cre activity is physiologic or occurs as a consequence of the transgenic targeting construct.

Part B

B.3 Effects of thymic epithelial-specific *smad4* deficiency on thymopoiesis and peripheral T lymphocytes

B.3.1 Deciphering thymic expression of mRNA transcripts of the TGF-β family of signaling transduction molecules

To test which signal transduction pathways that are known to be involved in embryonic development are expressed during thymic development, the presence of mRNA transcripts of selected secreted molecules, receptors and intracellular signal transduction molecules was analyzed by RT-PCR on thymic tissue. cDNA was generated from embryonic and post-natal whole thymus, thymic epithelial cell lines and thymocytes (Table I). Oligonucleotide sequences for each primer pair can be found in Annex III. Transcripts of *bmp2*, *bmp4* and their inhibitor *noggin* were found in whole thymus of embryonic and postnatal tissue (panel a). To discriminate between expression by stromal cells or thymocytes RT-PCR analysis was performed on thymic epithelial cell lines (panel b) and purified thymocytes (panel c). mRNA for *bmp2*, *bmp4* and *noggin* could be found in some but not all TEC lines while no transcripts were found in thymocytes. Next, the expression of receptors of the TGF- family was determined to test on what cells *bmp2* and *bmp4* might act. Transcripts for all the receptors analyzed (*bmpr Ia, bmpr Ib, bmpr II, tgf-β r II, activin r II and alk2)* could readily be found in whole thymus and TEC lines while a much more restricted pattern of receptor transcripts was found in thymocytes. In accordance with these results, transcripts of the intracellular signal transduction molecules *smad1*, *smad3*, *smad4* and *smad5* were strongly expressed in TEC lines. Transcripts for *smad3* and *smad5* could also be found in thymocytes. Based on the presence of mRNA of several receptors and intracellular signal transduction molecules of the TGF-β family in TEC lines it was hypothesized that TGF-β family signaling is involved in thymic epithelial cell development.

a whole thymus

	thymus E 14	thymus newborn	thymus adult
bmp2	xx	x	x
bmp4	xxx	x	x
noggin	x	x	x
bmpr Ia	xxx	xxx	xx
bmpr Ib	xxx	x	0
bmpr II	x-xx	x	x
tgf-brII	xxx	xxx	x
activinRIIA	xxx	xxx	xx
alk2	0	xx	
bambi	xxx		
smad1	xxx	xxx	
smad3	x	xx	x
smad4	xxx	xxx	
smad5	xx	xxx	xxx

Table II
mRNA expression pattern of secreted TGF-β family signaling molecules, receptors and intracellular signal transduction molecules in whole thymus (a), TEC lines (b) and purified thymocytes (c). 36 cycles of end-point RT-PCR amplification. 0 = not expressed, +, ++ and +++ stand for estimated relative amplification signals.

b thymic epithelial cell lines

	TEC 1.2	TEC 1.4	TEM 2.3	TEM C6
bmp2	x	0	0	
bmp4	xx	0	0	
noggin	xx	xx	xxx	xxx
bmpr Ia	xxx	xxx	xxx	0
bmpr Ib	xx	0	xxx	xxx
bmpr II	xx	x	xx	x
tgf-brII	xxx	xx	xxx	xxx
activinRIIA	xxx	xx	xxx	0
alk2	xxx	xx	xx	xx
bambi	0	0	0	0
smad3	xxx	xx	xxx	xxx
smad4	xxx			
smad5	xx	x	xx	xx

c purified thymocytes

DN	DP	SP $CD4^+$	SP $CD8^+$
0	0	0	0
0	0	0	0
	0	x	0
	0	0	0
	0	x	0
	x	xxx	0
	x	x	x
	x	xx	x
	xx	xx	x

B.3.2 Effects of the experimental system are restricted to the immune system

B.3.2.1 Normal development and fertility of TEC$^{smad4-/-}$ mice

TEC$^{smad4-/-}$ mice were born with a Mendelian frequency, showed normal weight gain, had a normal life span (assayed up to 11 months) and were fertile, indicating that FoxN1-cre mediated *smad4* deficiency did not affect viability of embryos or postnatal mice (data not shown). However, as expected, TEC$^{smad4-/-}$ mice had increased hair loss and malformations of the nails (data not shown) compared to control littermates.

B.3.2.2 Thymic hypoplasia in TEC$^{smad4-/-}$ mice

The thymic size was substantially reduced in TEC$^{smad4-/-}$ mice when compared to control littermates (Figure 14a). This difference, reflecting the total thymic cellularity, was observed as early as E18.5 and persisted throughout adulthood (Figure 14b). Both, females and males showed a comparable reduction to 10–20% of the cellularity of control littermates (data not shown). Both control and TEC$^{smad4-/-}$ mice showed a physiological increase in thymocyte cellularity that peaked around 5-6 weeks of life (Figure 14b). There were, however, some sex specific differences in mice older than 6 weeks. Subsequently, thymic involution ensued in TEC$^{smad4-/-}$ as well as in control littermate mice. Changes in thymic cellularity of Smad4 deficient animals paralleled the physiological growth and involution with regards to timing but not extent (Figure 14b).

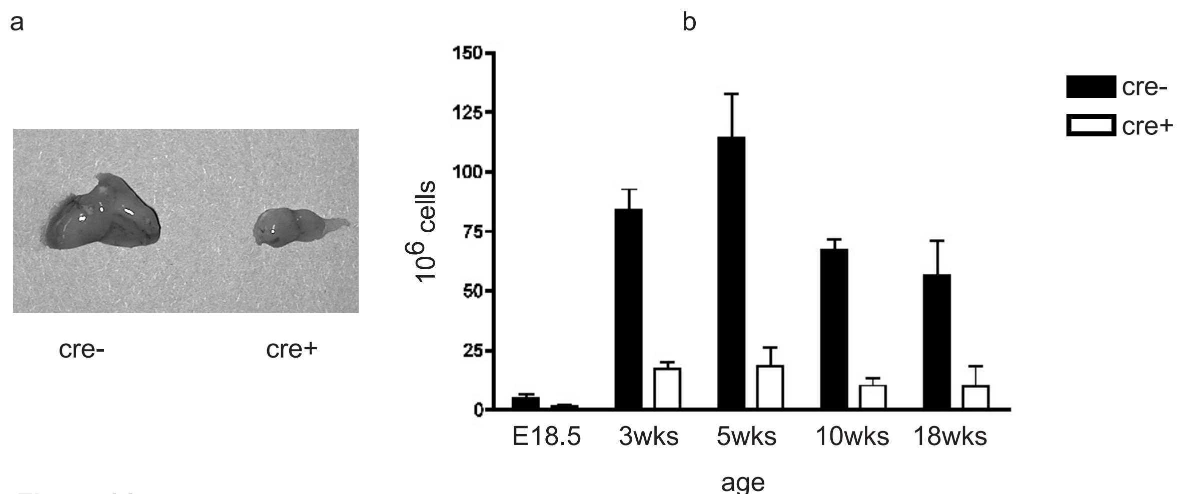


Figure 14
Thymic hypoplasia in cre+ mice
a) Thymus size of female mice of 8 weeks of life
b) Comparative analysis of thymic cellularity in female mice as a function of age. wks stands for weeks

B.3.3 Validity of the experimental system

B.3.3.1 Genomic deletion of the conditional *smad4* allele in thymic epithelial cells from TEC$^{smad4-/-}$ mice

To determine the efficiency of FoxN1-cre mediated recombination of the conditional *smad4* locus in thymic epithelial cells of TEC$^{smad4-/-}$ mice, PCR amplification of genomic DNA extracted from TECs purified by FACS was performed (Figure 14B). Primers were flanking the two loxP sites amplifying a longer wildtype from the non-recombined (panel a) and a shorter DNA fragment from the recombined locus (panel b). TECs from TEC$^{smad4-/-}$ contain DNA with a mixture of recombined and non-recombined *smad4* locus while control mice only display the non-recombined locus (panel c). Quantification of the deletion efficiency would be premature as this is a preliminary result.

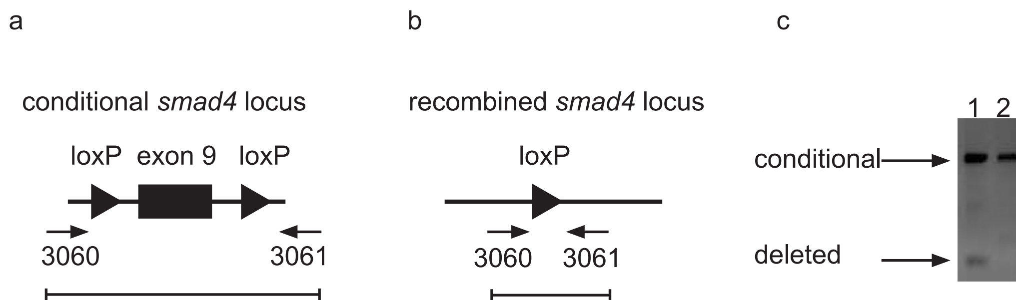


Figure 14B
Efficiency of genomic deletion of exon 9 of the conditional smad4 gene
a+b) Schematic representation of the conditional smad4 locus. Exon 9 is flanked by loxP sites. Arrows indicate PCR primers used to determine the efficiency of cre-mediated removal of exon 9.
c) Genomic DNA was extracted from purified thymic epithelial cells from TEC$^{smad4-/-}$ (lane1) and control mice (lane 2) and subsequently amplified by PCR using primers #3060 + #3061.

B.3.3.2 Effect of the FoxN1-cre transgene and the conditional *smad4* allele on thymic and splenic cellularity, thymocyte maturation and T cells

The FoxN1-cre transgene alone or the conditional *smad4* allele alone did neither alter thymic nor splenic cellularity (Figure 15 a+b). In addition, the frequencies and absolute numbers of thymocytes and peripheral T cell subpopulations were independent of the presence of the conditional allele or the FoxN1-transgene (data not shown).

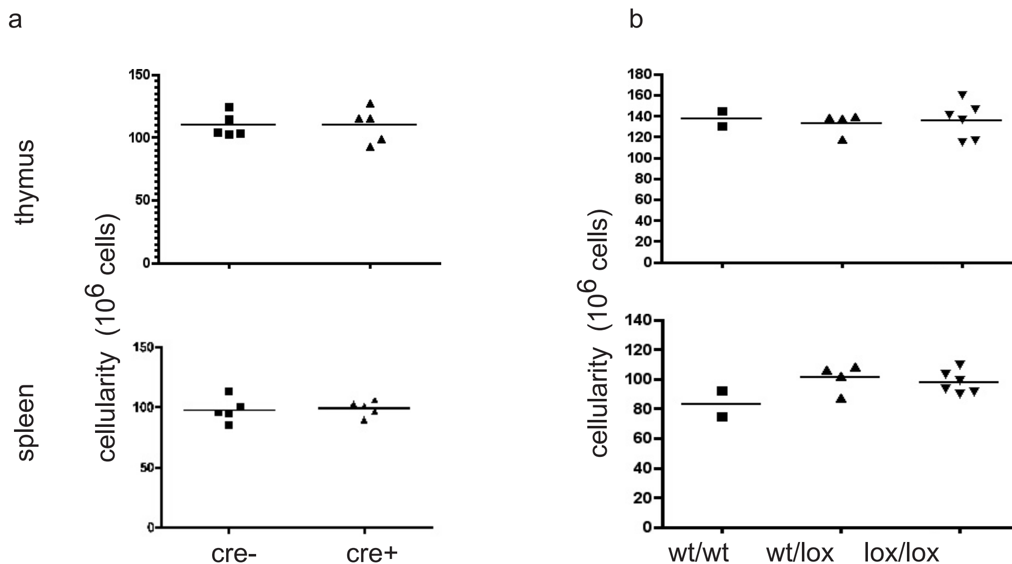


Figure 15
Influence of the conditional smad4 allele or the FoxN1-cre transgene on thymic or splenic cellularity
a) Effect of the FoxN1-cre transgene on thymic and splenic cellularity in 3 weeks old cre- and cre+ mice. Both, cre- and cre+ mice were smad4$^{wt/wt}$.
b) The presence of the loxP sites on one or both smad4 alleles in the absence of Cre does not affect thymic or splenic cellularity in 3 weeks old female mice.

B.3.3.3 Absence of a T cell intrinsic defect in TEC$^{smad4-/-}$ mice

To exclude the possibility that transgene integration or expression impact on thymocyte maturation, expansion and function, chimeras were generated by adoptive transfer of T cell depleted bone marrow (BM) from smad4$^{loxP/loxP}$ or TEC$^{smad4-/-}$ donors into lethally irradiated wildtype recipients. Donor cells were congenic for CD45.2 while the recipient hematopoietic cells expressed CD45.1. At the time of analysis, 8 weeks after transplantation, donor chimerism reached 93.0 +/- 1.6 % (data not shown).

The capacity to reconstitute the lymphohematopoietic system was unaffected by the FoxN1-cre transgene as there was no difference of the total cellularity of the thymus (Figure 16a) or spleen (Figure 17a) when comparing the two experimental groups. Specifically, thymocyte maturation was equal in recipients of BM from both genotypes with identical absolute (data not shown) and relative numbers of DN, DP, CD4 SP and CD8 SP thymocytes (Figure 16b). Late thymocyte maturation of CD4 SP and CD8 SP thymocytes was normal (Figure 16c+d) in both groups.

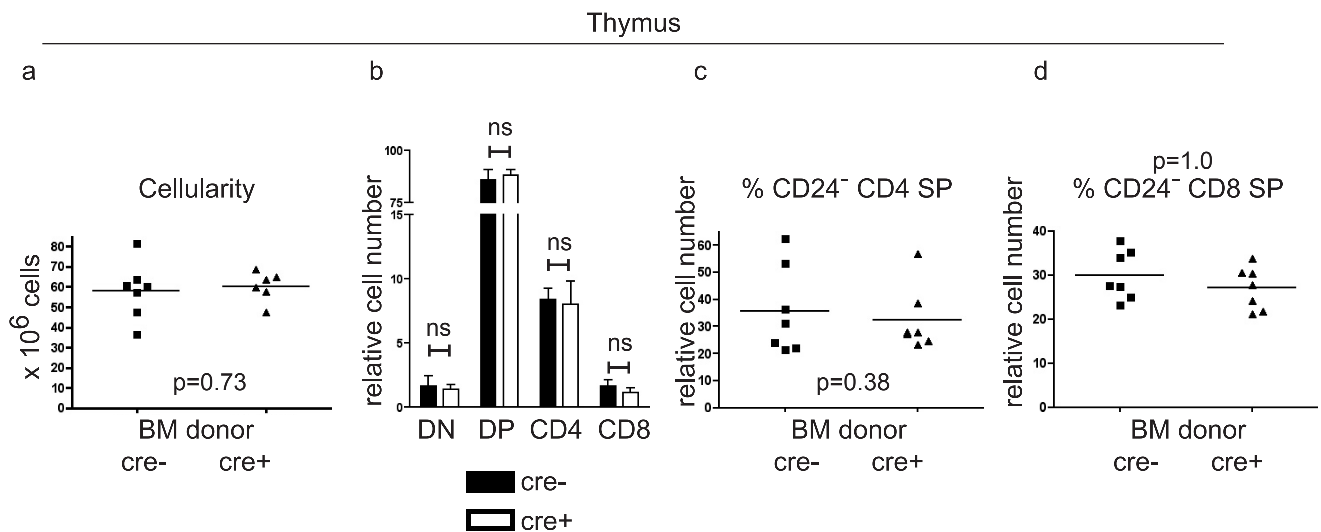


Figure 16
Thymic cellularity and maturation of thymocytes is identical in recipients of bone marrow cells from cre- and cre+ donors excluding a thymocyte/T cell intrinsic defect.
Lethally irradiated recipient female mice were transplanted with bone marrow from either female cre- or cre+ donors. 8 weeks after transplantation, chimerism of hematopoietic cells was 93 +/- 1.6 % of donor origin.
a) Thymic cellularity is equal in recipients of cre- and cre+ donors
b) Relative and absolute numbers of DN, DP, CD4 SP and CD8 SP are comparable in hosts transplanted with cre- or cre+ donors.
c+d) Equal maturation of SP thymocytes in hosts transplanted with BM from cre- or cre+ donors.
ns = not significant
For both groups, BM cells from 3 donor females were prepared as described in materials & methods. Samples were prepared separately to inject 2 or 3 recipients with BM cells from the same donor, i.e. recipients received BM cells derived from independent mice rather than pooled BM.

In the periphery, both the relative and absolute numbers of $CD4^+$ and $CD8^+$ T cells in lymph nodes (LN) were interchangeable between hosts transplanted with BM from $smad4^{loxP/loxP}$ or $TEC^{smad4-/-}$ donors (Figure 17b). Furthermore, the frequency of $CD4^+$ and $CD8^+$ T cells with an activated/memory phenotype was indistinguishable in both experimental groups (Figure 17c). $CD4^+CD25^+$ T cells were equally abundant in mice transplanted with control or

$TEC^{smad4-/-}$ bone marrow (Figure 17d).

Taken together, these control experiments proved that neither of the genetic modifications alone affected thymocyte or T cell biology. Furthermore, the bone marrow chimeras ruled out that any observed phenotype in $TEC^{smad4-/-}$ mice was due to a thymocyte or T cell intrinsic defect.

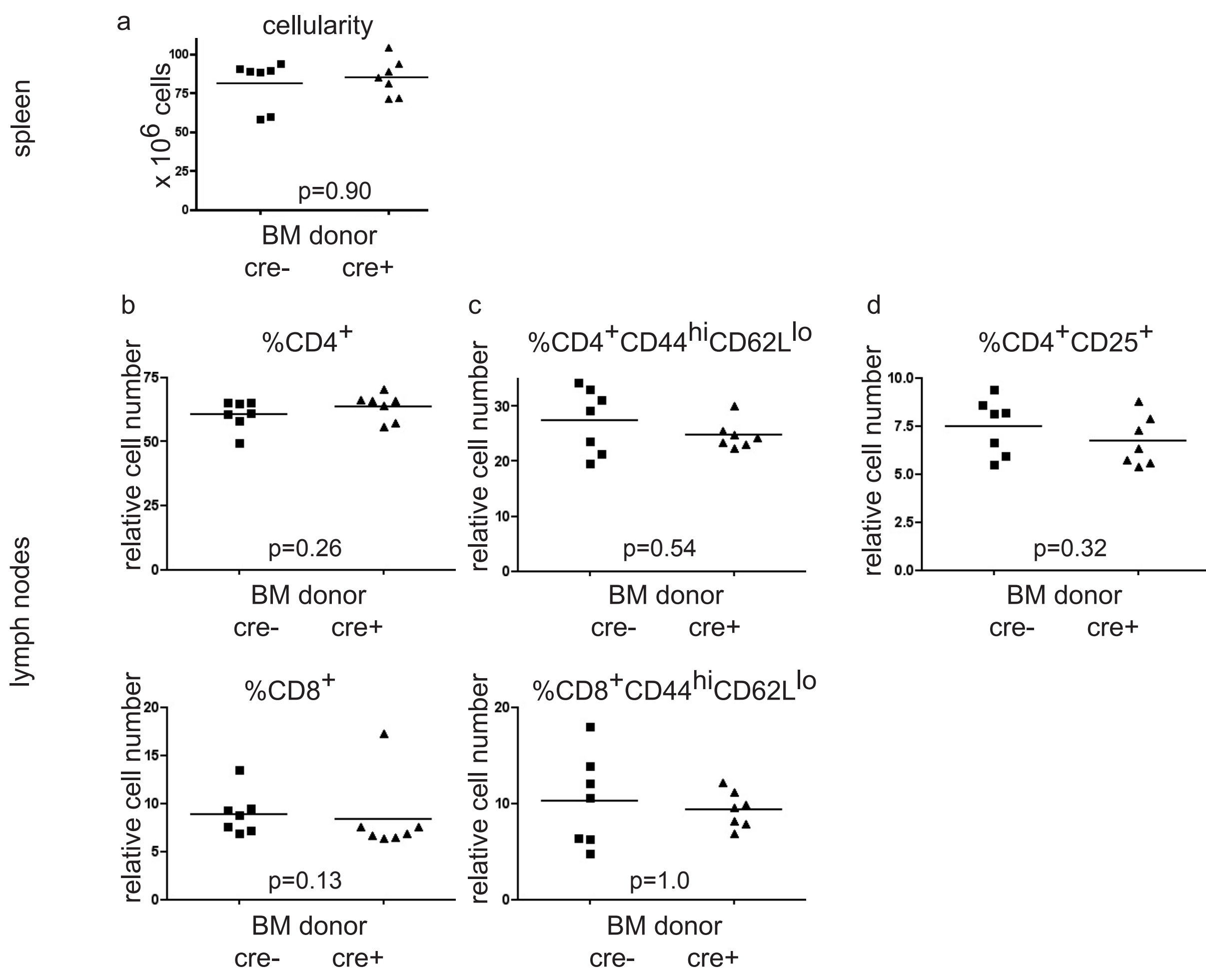


Figure 17
Normal splenic cellularity, T cell number and T cell phenotype in the periphery of recipients of bone marrow cells from cre- or cre+ donors
Analysis of peripheral lyphoid organs of the same experiment as in Fig. 16 .
a) Identical splenic cellularity in hosts transplanted with cre- or cre+ donor BM.
b) Comparable frequency of live (PI^-) $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T cells in lymph nodes of hosts transplanted with either donor genotype.
c) Similar frequency of $CD4^+$ (upper panel) or $CD8^+$ (lower panel) T cells with an activated/memory phenotype in recipients or cre- or cre+ donor BM.
d) Relative abundance of $CD4^+CD25^+$ T cells within live $CD4^+$ T cells is equal in mice transplanted with cre- or cre+ donor BM.

B.3.4 Thymic epithelial cell specific *smad4* deficiency leads to subtle thymocyte developmental defects

B.3.4.1 Normal thymic stromal architecture in TEC$^{smad4-/-}$ mice

The overall architecture of the thymus with *smad4*-deficient TECs remained preserved with a clear demarcation of cortex and medulla. (Figure 18, panels a, b, d, e). Normal expression of CK 18, a marker for cortex and CK 5 and MTS-10, markers for the medulla (see also Figure 11) exemplified the regular differentiation of TECs in control and TEC$^{smad4-/-}$ mice. Finally, MTS-24^{+} cells, putative TEC precursor cells, could be detected in mice of both genotypes.

Thus, histologically the thymic architecture and stromal composition appeared normal in TEC$^{smad4-/-}$ mice.

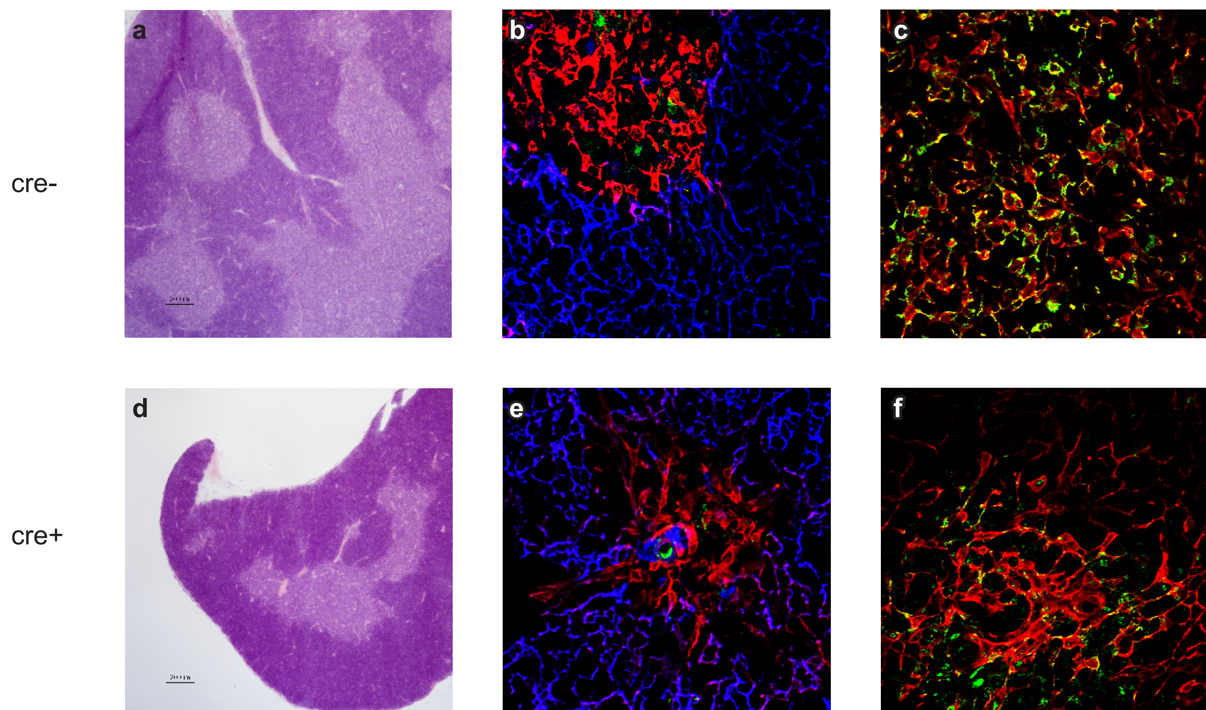


Figure 18
Thymic compartimentalization, architecture and stromal composition of cre- (a-c) and cre+ (d-f) mice
a + d) HE staining
b + e) Immunohistochemistry for cytokeratin 18 (blue), cytokeratin 5 (red) and MTS24 (green)
c+ f) Immunohistochemistry for cytokeratin 5 (red) and MTS10 (green)

B.3.4.2 Cellular composition of TEC$^{smad4-/-}$ thymi

FACS analysis of thymocyte subpopulations (Figure 19) revealed similar relative numbers of CD4$^-$CD8$^-$ double negative (DN) and a minor reduction of double positive (DP) thymocytes in E 18.5 embryos (panels a-c) in TEC$^{smad4-/-}$ mice when compared to control littermates. As a consequence of the overall reduced thymic cellularity the total number of both, DN and DP thymocytes was significantly reduced in TEC$^{smad4-/-}$ E 18.5 embryos (panel d). Further subdivision of the DN cells revealed equal relative and absolute numbers of DNI-DNIV in mice of both genotypes (panels e+f).

Similar results -including SP thymocytes that are not yet present in embryonic mice- were obtained in postnatal mice (panels g-m).

Apart from αβTCR thymocytes, B, NK and γδTCR lymphocytes could be found at normal frequencies (data not shown).

B.3.4.3 Minor maturational defect of CD4 and CD8 single positive thymocytes in TEC$^{smad4-/-}$ mice

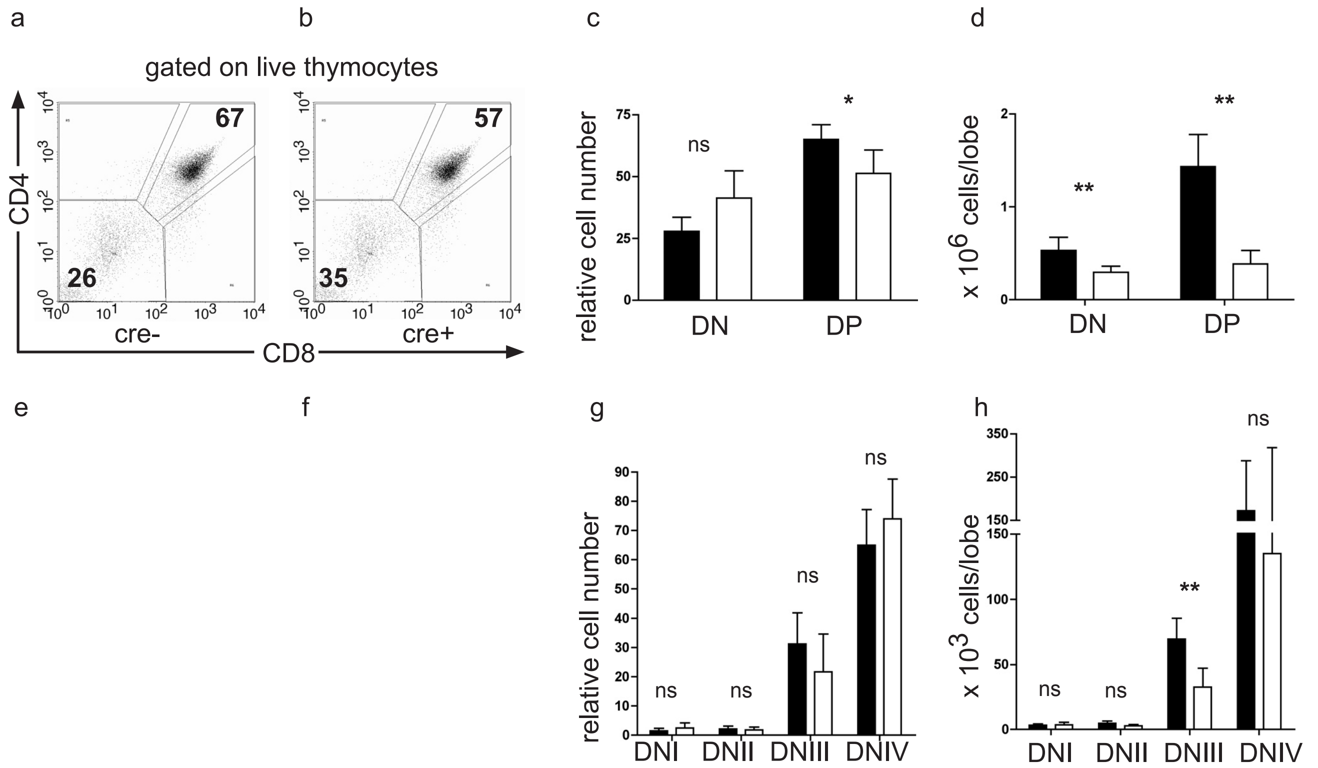
Despite the regular relative number of SP thymocytes, it appeared that thymi from TEC$^{smad4-/-}$ mice contain less fully mature CD8$^+$CD4$^-$ thymocytes. Therefore, CD4 expression levels were analyzed in CD8 SP to quantitate the relative number of CD8 SP thymocytes that have undergone complete downregulation of the coreceptor CD4 (Figure 20a+b). Mice lacking Smad4 in TECs had a reduced number of CD8$^+$CD4$^-$ SP thymocytes (Figure 20b, lower panel). In analogy, CD4 SP thymocytes were analyzed for CD8 downregulation revealing a minor relative reduction in fully mature CD4 SP in TEC$^{smad4-/-}$ mice compared to control littermates.

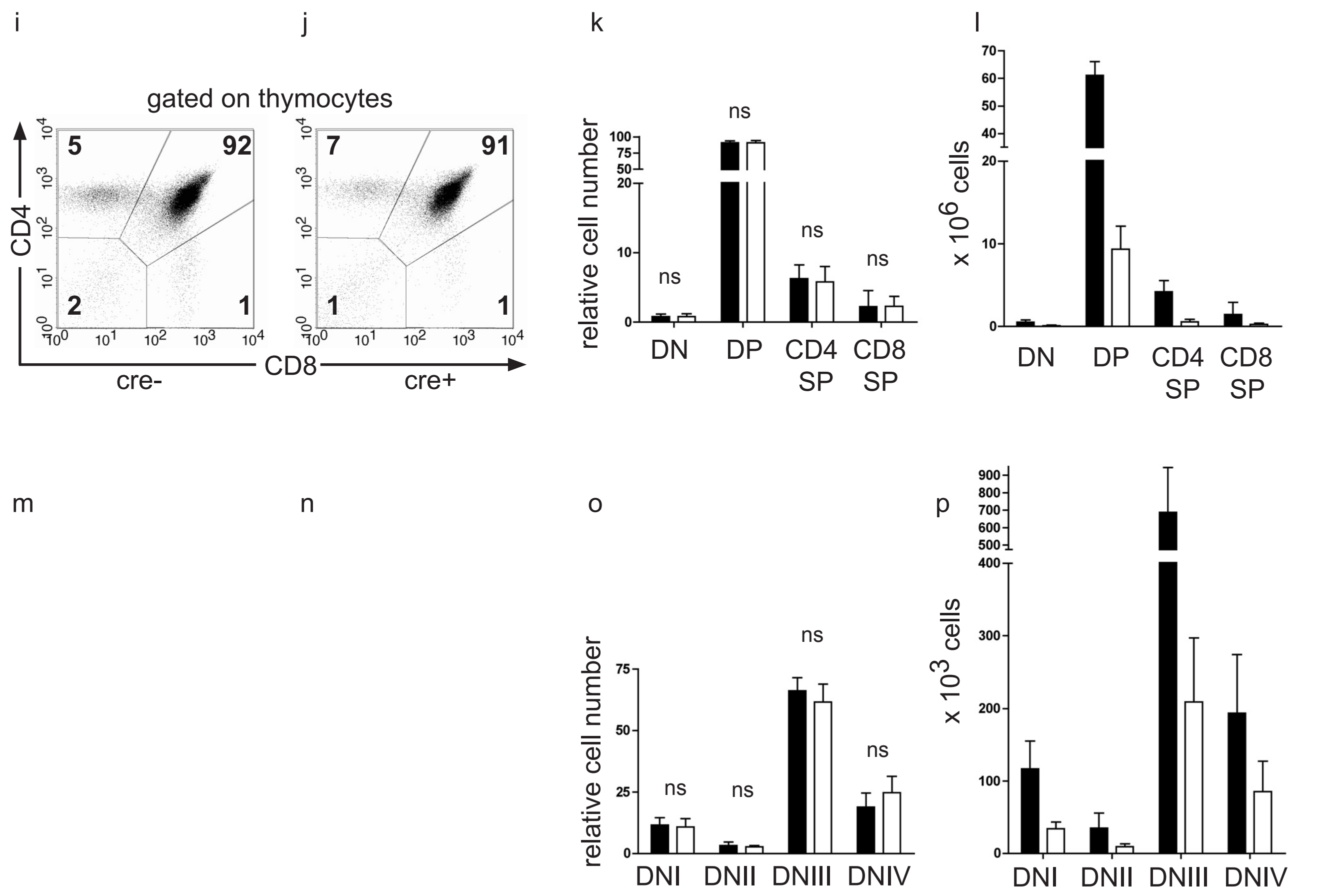
Next page: Figure 19

Thymocyte development in embryonic (E18.5, upper panel) and postnatal mice (lower panel).
** indicates p<0.05, ** indicates p<0.01, ns= not significant*

■ cre-

□ cre+


embryonic
a
b
c
d
gated on live thymocytes
CD4
CD8
cre-
cre+
67
26
57
35
relative cell number
ns
*
DN
DP
x 10^6 cells/lobe
**
**
e
f
g
h
DNI
DNII
DNIII
DNIV
x 10^3 cells/lobe


postnatal
i
j
k
l
gated on thymocytes
CD4
CD8
cre-
cre+
5
92
2
1
7
91
1
1
relative cell number
ns
DN
DP
CD4 SP
CD8 SP
x 10^6 cells
m
n
o
p
DNI
DNII
DNIII
DNIV
x 10^3 cells

In order to study thymocyte maturation more in detail, expression of the cell surface markers CD24 and CD69 (see Figure 3) was determined. Both proteins are being used to characterize thymocyte maturation although their functions are unknown [34, 157]. The relative number of CD24 low to negative (mature) cells among SP thymocytes was reduced in TEC$^{smad4-/-}$ mice compared to controls (Figure 21 a+b). CD69 surface expression increases steadily during thymocyte development [158]. Gating on immature CD69^{int} TCRβ^{int} thymocytes revealed a relative reduction of immature CD4 SP thymocytes (Figure 21c+d) in TEC$^{smad4-/-}$ mice when compared to control littermates. Taken together, these results suggested minor developmental defects or delays in mice with a thymic epithelial-specific Smad4 deficiency.

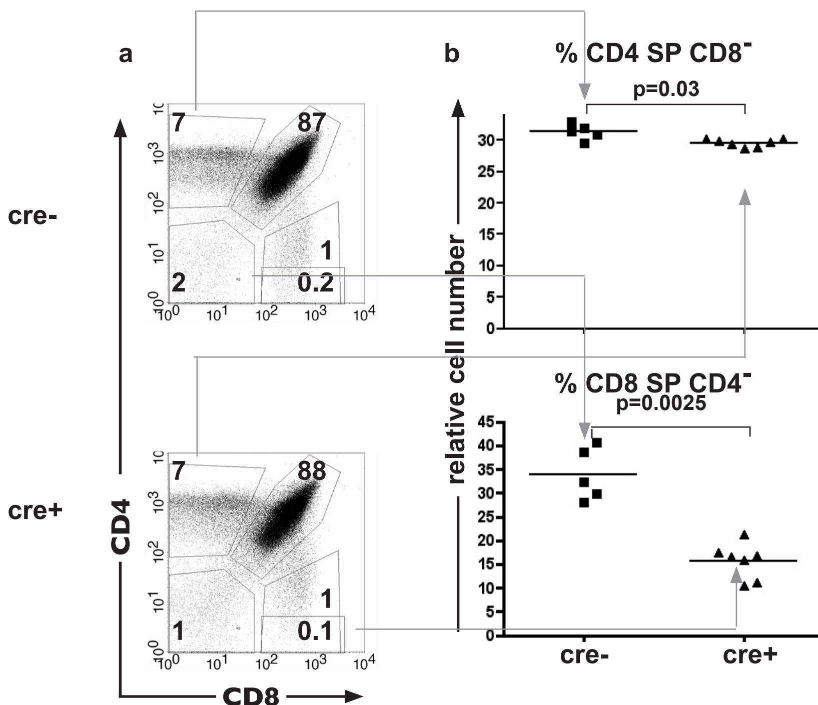


Figure 20
Maturational defect of CD8 SP thymocytes in the absence of Smad4 in thymic epithelial cells.
a) Relative numbers of CD8 SP thymocytes are comparable between cre- and cre+ mice. (Gated on thymocytes; Numbers in gates indicate the percentage of cells within that gate)
b) Reduced relative numbers of CD4SP with CD8⁻ and CD8 SP thymocytes with completely downregulated CD4 in cre+ animals (gated on CD8 SP).

B.3.4.4 Regular usage of most TCR Vβ chains by single positive thymocytes in TEC$^{smad4-/-}$ mice

To assess whether the observed maturational defects affected the TCR Vβ usage in TEC$^{smad4-/-}$ mice, a panel of antibodies directed against various TCR β chains was used to determine TCR Vβ diversity. The frequencies of Vβ3, Vβ4, Vβ5.1/5.2, Vβ6, Vβ7, Vβ8.1/8.2, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13, Vβ14 and Vβ17a among the TCRs on SP thymocytes were compared between control and TEC$^{smad4-/-}$ mice. The sum of the relative usage of these Vβ genes covers 2/3 of all TCRs present on CD4 SP and 3/4 of all TCRs expressed by CD8 SP thymocytes in the mixed genetic background studied. The relative usage of the various Vβ genes was interchangeable for most Vβ genes analyzed except for Vβ6 in CD4 SP and Vβ12 in CD8 SP thymocytes between TEC$^{smad4-/-}$ and control littermates (Figure 22).CD4 SP

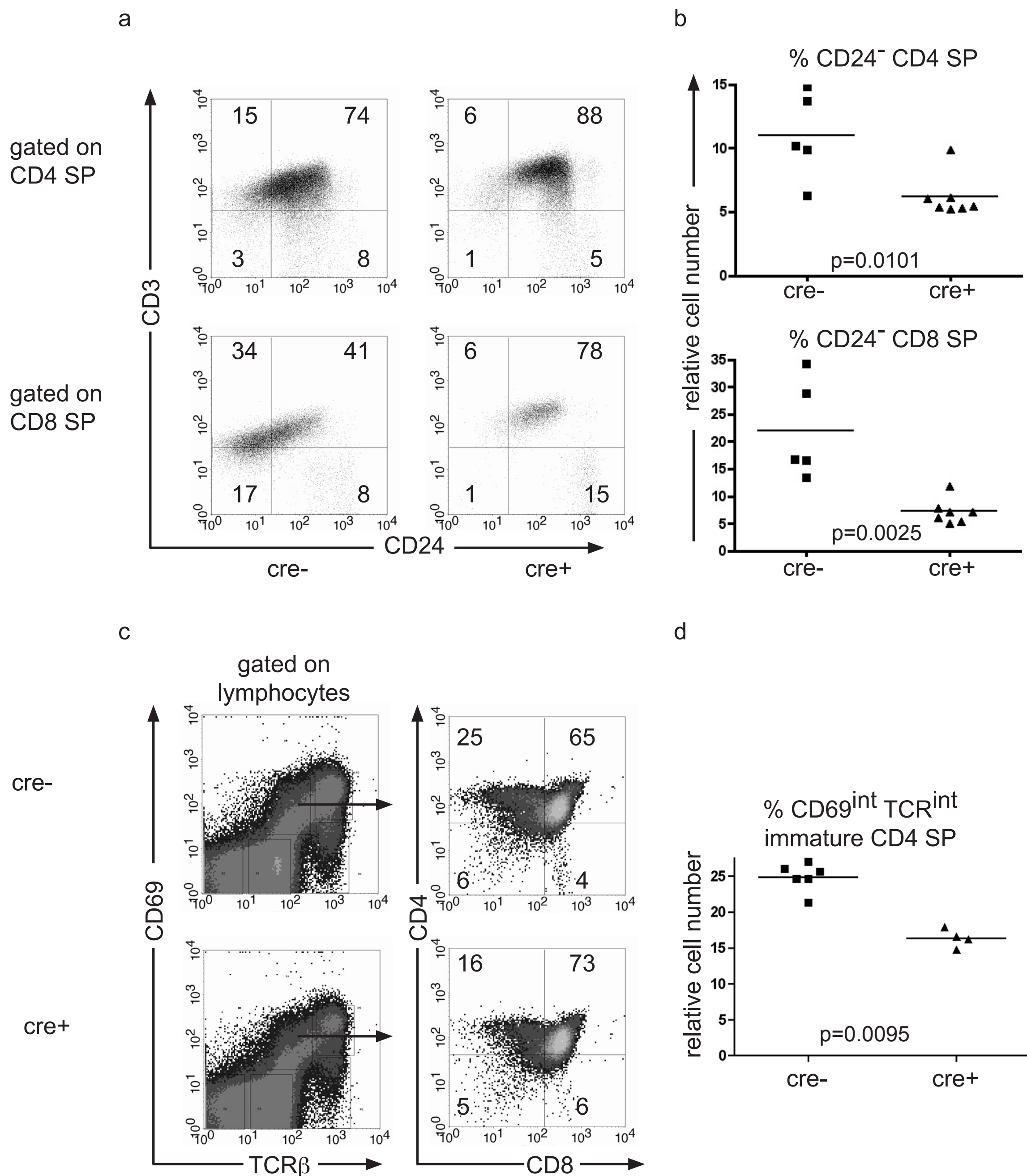


Figure 21

Late thymocyte development in the absence of smad4 in thymic epithelial cells is altered.

a) CD24- mature SP (both CD4 and CD8) thymocytes are reduced in cre+ animals compared to cre- littermates. Numbers indicate the percentage of thymocytes within that gate.

b) Frequency of mature CD4 SP and CD8 SP thymocytes devoid of CD24 expression, a marker for maturation among single positive thymocytes.

c) Analysis of thymocyte maturation as a function of CD69 and TCRβ expression. CD69^{int}TCR β^{int} thymocytes contain a reduced relative number of immature CD4 SP thymocytes in cre+ mice.

d) Frequency of immature CD4 SP thymocytes as gated in panel c

thymocytes of TEC$^{smad4-/-}$ mice had a small but statistically significant relative decrease of Vβ6^{+} usage (panel c) while a statistically significant larger proportion of CD8 SP used Vβ12 in TEC$^{smad4-/-}$ mice when compared to control mice (panel d). Yet, overall, the maturational defect of thymocytes in TEC$^{smad4-/-}$ mice did not influence TCR Vβ diversity and usage.

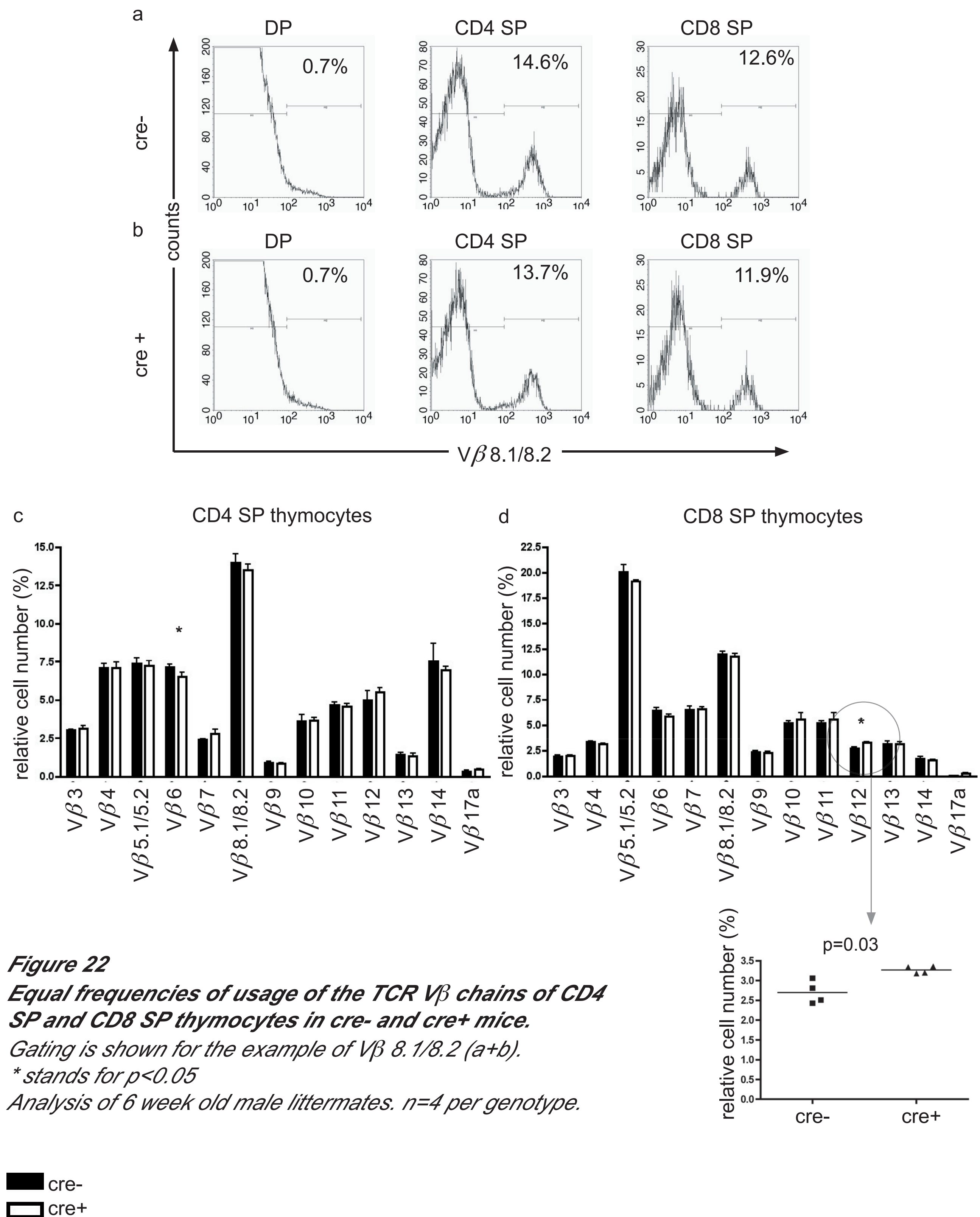


Figure 22
Equal frequencies of usage of the TCR Vβ chains of CD4 SP and CD8 SP thymocytes in cre- and cre+ mice.
Gating is shown for the example of Vβ 8.1/8.2 (a+b).
** stands for $p<0.05$*
Analysis of 6 week old male littermates. n=4 per genotype.

B.3.4.4b Smad4 deficient thymic epithelial cells fail to respond to KGF

KGF induces proliferation of thymic epithelial cells and regulates the expression of members of the TGF-β family of signaling molecules (Rossi et al., unpublished observation). To test whether Smad4 was involved in the effect of KGF on TEC proliferation, $TEC^{smad4-/-}$ mice and control littermates were injected i.p. with KGF (5mg/kg/mouse) or HBSS on 3 consecutive days at the age of 6 weeks. The thymic cellularity was analyzed 14 days after the last injection. Control mice injected with KGF showed a significant increase in thymic size (Figure 22B a) and cellularity (Figure 22B b) when compared to control mice injected with HBSS. In contrast, thymi with an epithelial Smad4-deficiency failed to significantly increase the mean cellularity in response to KGF. This result implied that Smad4-deficient TECs cannot proliferate in response to KGF.

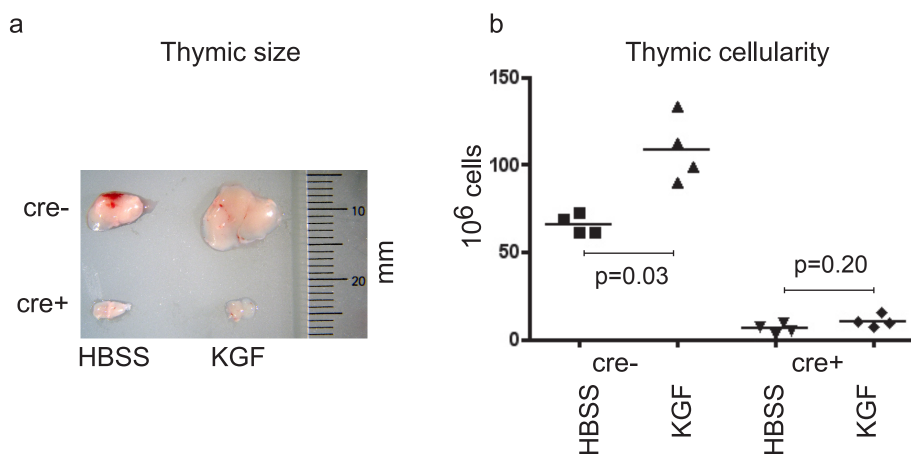


Figure 22B
KGF-mediated effect on increased thymic cellularity is dependent on Smad4
Thymic size (panel a) and cellularity (panel b) of treated (KGF) and untreated (HBSS) cre- and cre+ 6 weeks old mice.

B.3.5 Thymic epithelial-specific *smad4* deficiency significantly alters the peripheral T cell pool

B.3.5.1 Splenic cellularity and composition of lymphocytes in secondary lymphoid organs of TEC$^{smad4-/-}$ control mice

Splenic size and cellularity was comparable between mice with a TEC specific Smad4 deficiency and control littermates over the entire course of observations of 3-18 weeks of age (Figure 23). However, the relative and absolute numbers of T cells were reduced in TEC$^{smad4-/-}$ mice aged 5-9 weeks (Figure 24 a+b) in comparison to control mice. Both, CD4^{+} and CD8^{+} T cells were reduced at all time points examined, both in spleen (data not shown) as well as in lymph nodes (Figure 24 c+d). While lymphopenia was most pronounced in young TEC$^{smad4-/-}$ mice, aging incompletely corrected the reduced numbers of T cells. The CD8^{+} T cells were more reduced than CD4^{+} T cells.

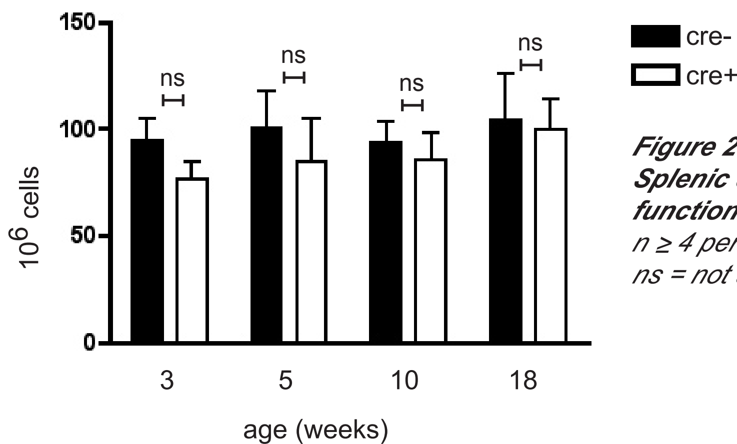
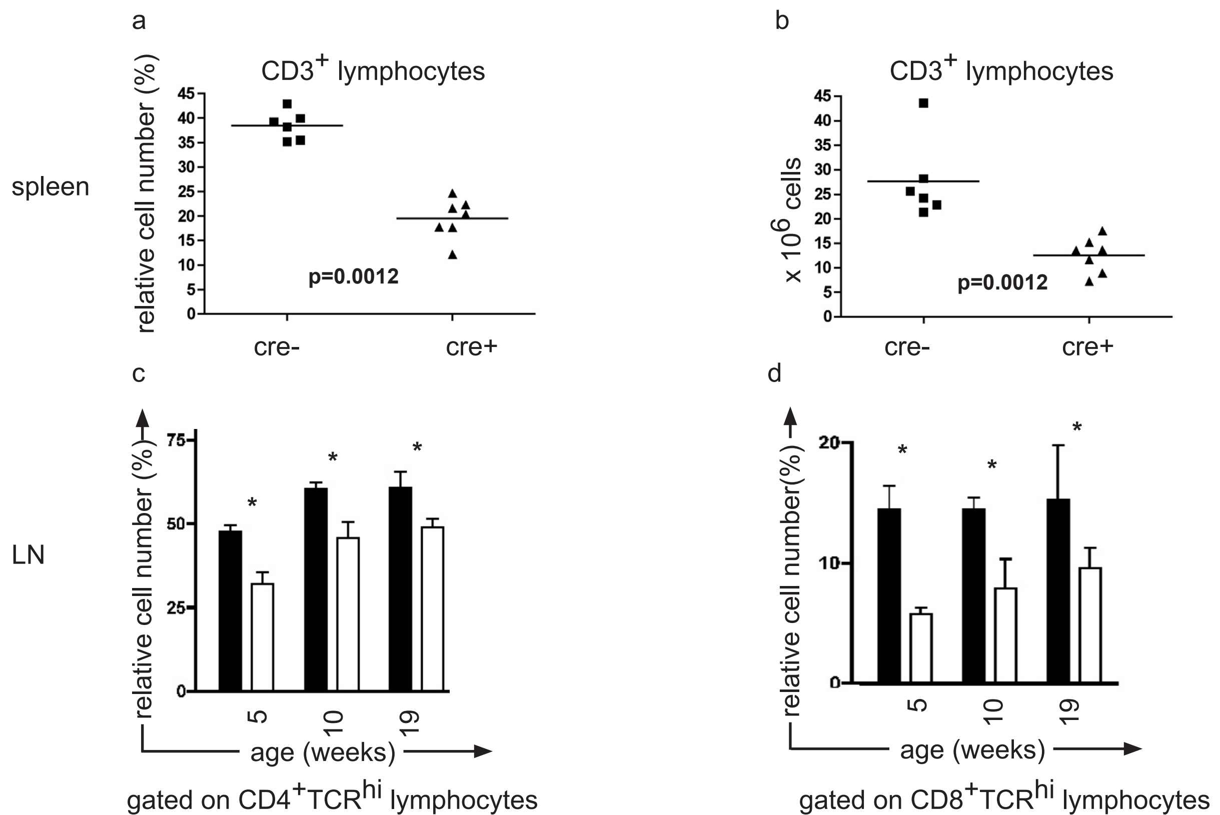


Figure 23
Splenic cellularity in cre- and cre+ mice as a function of age
n ≥ 4 per group
ns = not statistically significant

Next page: Figure 24
Cre+ mice display chronic lymphopenia.
Relative frequency (panel a) and absolute numbers of T cells (panel b) in the spleen of cre+ mice are reduced by about 50% when compared to cre- littermates at 5-9 weeks of life. Differences in peripheral CD4^{+} T cells (panel c) or CD8^{+} T cells (panel d) remain at least up to 19 weeks after birth.
c+d) n = 4 or more per genotype and time point
** stands for $p<0.05$*
cre-
cre+



B.3.5.2 Increased frequency of T cells displaying an activated/memory phenotype in TEC$^{smad4-/-}$ mice compared to control mice

T cell lymphopenia is known to be counteracted by lymphopenia induced proliferation (LIP) [103], a process leading to a change in the expression of cell surface molecules. To analyze whether T cells in lymphopenic TEC$^{smad4-/-}$ mice display signs of LIP, expression levels of CD44, CD62L and CD45RB were determined as marker molecules for activated/memory-like T cells. Both, CD4^{+} and CD8^{+} T cells of TEC$^{smad4-/-}$ animals displayed an almost two-fold relative increase of a CD44^{hi}CD62L^{lo} activated/memory-like phenotype when compared to control littermates (Figure 25A a+b). This phenotype could be observed from 3-19 weeks of age and was confirmed by assessing expression levels of CD45RB, another marker which is downregulated on activated/memory-like T cells. In concert with the elevated frequency of CD44^{hi}CD62L^{lo} T cells, the relative number of CD45RB^{lo} T cells was increased in CD4^{+} and CD8^{+} T cells of TEC$^{smad4-/-}$ mice (data not shown).

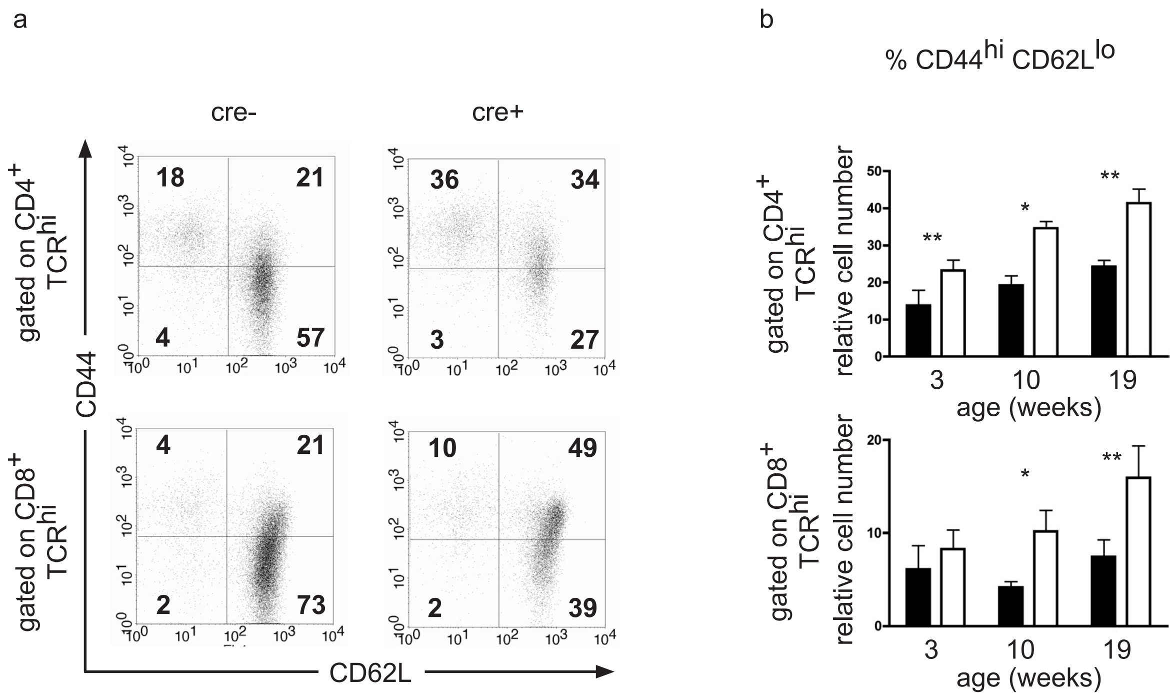


Figure 25A

Increased frequency of peripheral T cells with a memory-like phenotype in cre+ mice when compared to cre- mice

The relative number of naive $CD44^{lo}CD62L^{hi}CD4^{+}$ (panel a, upper plots) and naive $CD8^{+}$ (panel a, lower plots) T cells is reduced in cre+ compared to cre- mice while activated/memory-like $CD44^{hi}CD62L^{lo}$ T cells, both, $CD4^{+}$ and $CD8^{+}$ are increased at 11 weeks of age. These differences can be observed from 3 weeks of age up to 19 weeks (panel b).

a) Numbers indiate percentage in each quadrant.

** stands for $p < 0.05$, ** stands for $p < 0.01$ (see also comments in Materials & Methods)*

cre-

cre+

B.3.5.3 Possible mechanisms for the incomplete correction of T cell numbers in TEC$^{smad4-/-}$ mice

Lymphopenia and the observed increased frequency of T cells with a memory-like surface phenotype suggested ongoing LIP in TEC$^{smad4-/-}$ mice. However, the persistent reduction of T cells even in mice aged 19 weeks was unexpected as LIP can efficiently “fill-up” empty hosts [159]. Therefore, a number of hypotheses were formulated that explain mechanisms potentially accounting for the incomplete correction of T cell numbers in TEC$^{smad4-/-}$ mice.

1. Reduced thymic output
2. Absence of signals inducing lymphopenia induced proliferation in TEC$^{smad4-/-}$ mice
3. A proliferation defect of T cells “imprinted” during development and selection in a Smad4-deficient thymic environment
4. Increased anti-proliferative suppression by T_{Reg} cells
5. Inability to respond to homeostatic signals
6. Increased cell death of peripheral T cells in TEC$^{smad4-/-}$ mice

The results of the experiments performed to verify these points are shown below. As a reminder, for each experiment the corresponding hypothesis will be displayed in **grey**.

B.3.5.3.1 Hypothesis 1

TEC$^{smad4-/-}$ mice have a reduced thymic output

B.3.5.3. 1 TEC$^{smad4-/-}$ mice have a reduced thymic output

The reduced thymic size in TEC$^{smad4-/-}$ mice was indicative of a reduced thymic output. To quantitate the thymic T cell production rate, thymocytes were labeled by intrathymic FITC injection. Splenic recent thymic emigrants (RTE) were measured 17h post injection. As expected, significantly reduced numbers of RTEs were found in the spleens of TEC$^{smad4-/-}$ mice when compared to the number of RTEs in control spleens (Figure 25B).

Table III

Individual mice	Measured CD4^{+} FITC^{+} splenic T cells	Thymic labelling efficiency (%)	Corrected number of CD4^{+} splenic RTEs
control	861	19.8	4348
control	300	16.7	1796
control	673	15.5	4356
control	365	15.2	2397
control	208	9.1	2286
control	202	8.6	2349
TEC$^{smad4-/-}$	131	39.7	330
TEC$^{smad4-/-}$	151	24.4	619
TEC$^{smad4-/-}$	120	38.6	311
TEC$^{smad4-/-}$	56	52.7	106
TEC$^{smad4-/-}$	453	42.9	1056

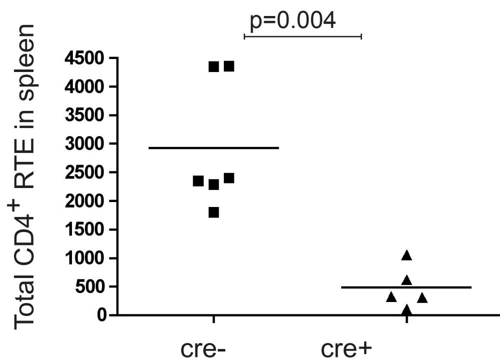


Figure 25B
Reduced thymic output in cre+ mice
FITC+ CD4+ T cells 17h after intrathymic FITC injection. Labelling efficiency of thymocytes was substantially different in cre- and cre+ mice as a consequnce of the size difference. Table III displays how the total number of CD4+ recent thymic emigrants (RTE) in spleen during 17h was calculated. The total thymic output is significantly reduced in cre+ mice compared to cre- littermates.

B.3.5.3.2 Hypothesis 2

Absence of signals inducing lymphopenia induced proliferation in TEC$^{smad4-/-}$ mice

B.3.5.3.2.1 TEC$^{smad4-/-}$ mice can provide homeostatic proliferation signals to adoptively transferred wildtype T cells

In order to test whether the signals for LIP were present in TEC$^{smad4-/-}$ mice or whether the lymphopenia could not be “sensed” by T cells, exogenously CFSE labeled wildtype T cells were adoptively transferred into TEC$^{smad4-/-}$ or control hosts. When a cell divides, the intensity of CFSE fluorescence decreases by about half and therefore provides an accurate count of the number of cell divisions achieved [160]. At day 6, CFSE^{+} T cells were analyzed in peripheral blood, allowing a discrimination into divided and undivided T cells. The relative number of undivided T cells was higher in control than in TEC$^{smad4-/-}$ hosts (data not shown). In contrast, CD4^{+} and CD8^{+} T cells showed a significantly higher percentage of proliferating T cells measured by CFSE dilution when injected into TEC$^{smad4-/-}$ hosts than injected into control hosts (Figure 26 a+b). At day 10, a more detailed analysis could be performed since mice were sacrificed. Again, both, CD4^{+} and CD8^{+} T cells had undergone higher proliferation when transferred into TEC$^{smad4-/-}$ hosts than in control hosts (Figure 27). Significantly more CD4^{+} T cells had divided at least once when transferred into TEC$^{smad4-/-}$ than into control mice (panels a-c) and significantly more CD8^{+} T cells had divided up to three times in TEC$^{smad4-/-}$ hosts than in control hosts (panels d-f).

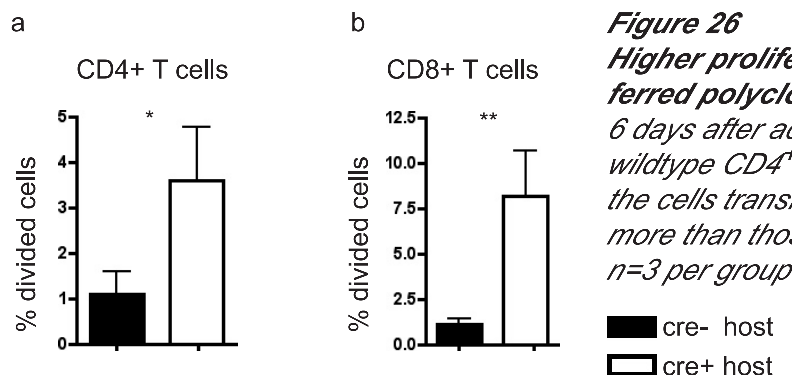


Figure 26
Higher proliferation rate of adoptively transferred polyclonal T cells in cre+ than cre- hosts
6 days after adoptive transfer of CFSE labelled wildtype CD4^{+} (panel a) and CD8^{+} (panel b) T cells the cells transferred into cre+ hosts had proliferated more than those transferred into cre- hosts.
n=3 per group

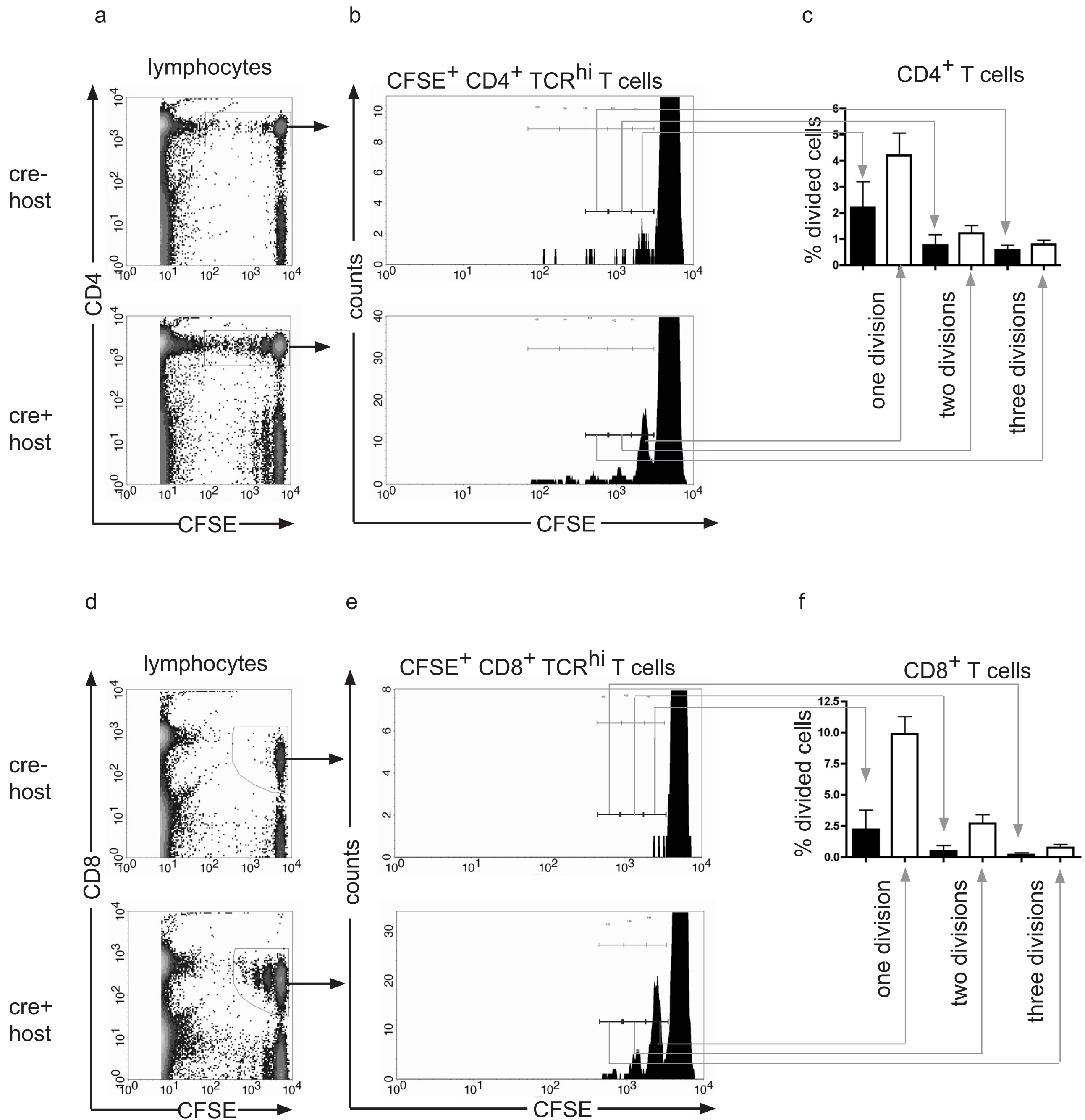


Figure 27

Adoptively transferred CFSE labelled polyclonal CD4+ and CD8+ T cells proliferate more in cre+ than in cre- hosts

10 days after transfer, proliferation of donor T cells was analyzed. CD4+ CFSE+ cells (panel a) or CD8+ CFSE+ cells (panel d) were analyzed for CFSE fluorescence intensity (panels b + e). Statistical analysis of 3 hosts per genotype revealed that both, CD4+ (panel c) and CD8+ (panel f) T cells proliferate more in cre+ than in cre- hosts.

Numbers indicate percentages of cells that underwent one, two or three divisions.

cre- host
cre+ host

B.3.5.3.2.2 TEC$^{smad4-/-}$ mice can provide homeostatic proliferation signals to adoptively transferred oligoclonal T cells

The same assay was performed with oligoclonal H-2^{b} class I restricted TCR transgenic CD8^{+} OT-I T cells (Figure 28). Rag2$^{-/-}$ hosts served as a positive control for proliferation. Cells were gated on Vα2^{+}Vβ5^{+} T cells that are chiefly OT-I TCR transgenic T cells (panel a). Six days after transfer, all OT-I T cells were proliferating in the Rag2$^{-/-}$ host (panel b). Substantially less T cells had proliferated in control and TEC$^{smad4-/-}$ hosts (panel b) indicating that the proliferation stimulus was weaker than in the Rag2$^{-/-}$ hosts. OT-I T cells transferred into TEC$^{smad4-/-}$ mice had, however, undergone significantly more divisions than OT-I T cells transferred into control hosts (Figure 28).

Taken together, the adoptive transfer experiments with polyclonal and oligoclonal T cells clearly demonstrated that TEC$^{smad4-/-}$ can provide lymphopenia induced proliferation signals. Moreover, the signals inducing LIP showed a dose response curve. A decreasing prolifera-tion stimulus can be assumed in the completely empty Rag2$^{-/-}$, the lymphopenic TEC$^{smad4-/-}$ and the normopenic smad4$^{loxP/loxP}$ control host. Thus, hypothesis 2 can be rejected.

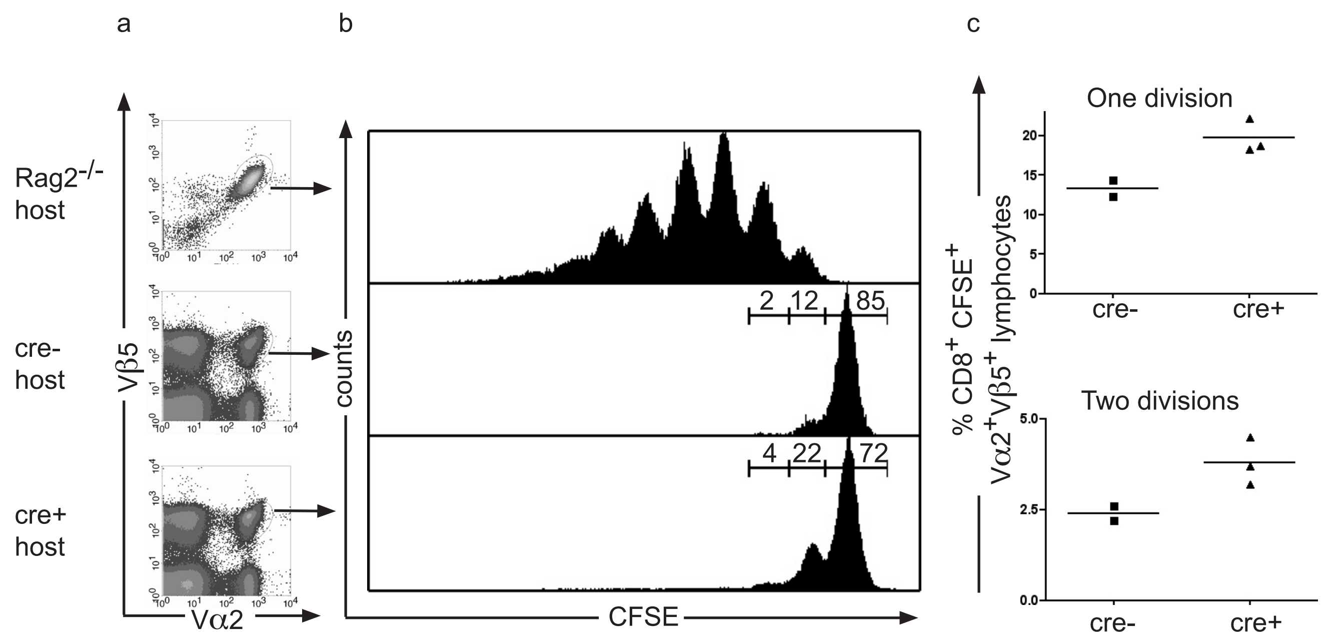


Figure 28
Adoptively transferred oligoclonal OT-I TCR transgenic CD8+ T cells proliferate more in cre+ than in cre- hosts
6 days after adoptive transfer, proliferation of OT-I T cells was analyzed. TCR Vα2^{+} Vβ5^{+} (panel a) T cells were analyzed for CFSE fluorescence intensity (panel b). The statistical distribution of the divided OT-I T cells is shown in panel c.

B.3.5.3.3 Hypothesis 3

T cells from TEC$^{smad4-/-}$ mice have a proliferation defect “imprinted” during development and selection in a Smad4-deficient thymic environment

B.3.5.3.3 Regular *in vitro* proliferation of purified T cells from TEC$^{smad4-/-}$ and control mice

To test the proliferation capacity of T cells that had matured in a Smad4-deficient thymic microenvironment, naïve (Figure 29, panel a) or memory (panel b) CD4^{+} T cells from control and TEC$^{smad4-/-}$ mice were purified and stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies. T cells from mice with both genotypes proliferated equally well showing a peak proliferation in both populations at 1 µg/ml anti-CD3 and subsequently decreased proliferation at higher concentrations, possibly due to activation induced cell death. Based on these results hypothesis 3 was rejected.

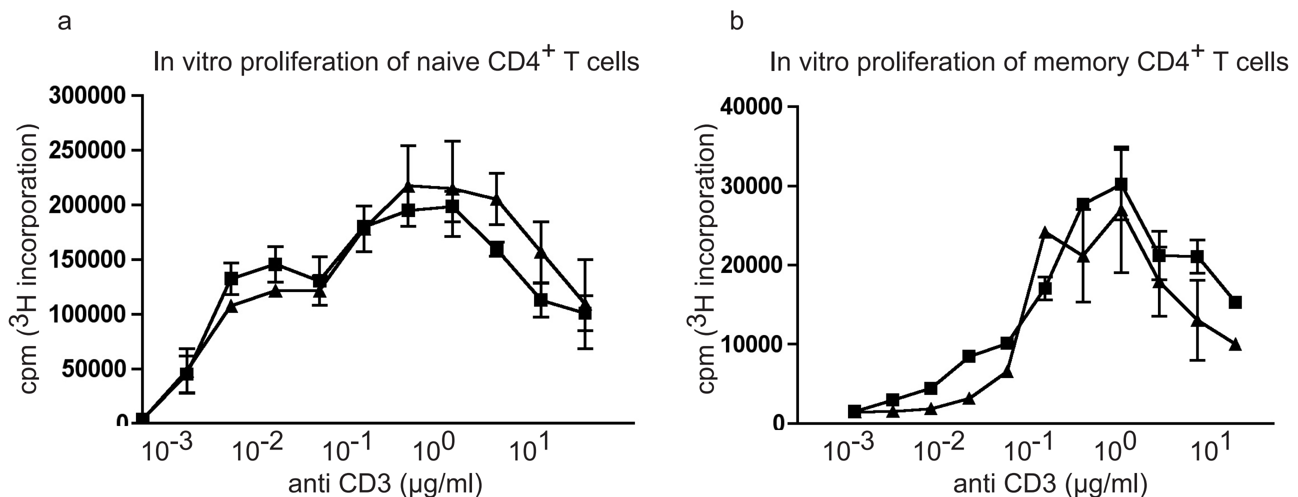


Figure 29
Purified CD4^{+} T cells from cre- and cre+ mice proliferate equally well in vitro
a) In vitro proliferation of purified naive CD45RB^{hi}CD4^{+} T cells (a) and purified memory CD45RB^{lo}CD4+ T cells (b) stimulated with anti-CD3 in vitro. T cells were cocultered with equal numbers of irradiated (3000 rad) splenocytes from a female Rag2$^{-/-}$ donor for costimulation.

cre-

cre+

B.3.5.3.4 Hypothesis 4

T$_{Reg}$ cells prevent lymphopenia induced proliferation

B.3.5.3.4.1 Increased frequency of CD4^{+}CD25^{+} T cells in TEC$^{smad4-/-}$ compared to control mice

To test whether increased numbers of T$_{Reg}$ cells prevented lymphopenia induced proliferation in TEC$^{smad4-/-}$ mice, the frequency of CD4^{+}CD25^{+} splenic T cells was determined. The relative number of CD4^{+}CD25^{+} T cells was significantly increased in TEC$^{smad4-/-}$ mice

(Figure 30 a). Despite the relative increase, the total number of $CD4^+CD25^+$ T cells was reduced in $TEC^{smad4-/-}$ mice in comparison to control mice (panel b). In parallel to the relative increase of T cells with an activated/memory phenotype in $TEC^{smad4-/-}$ mice, relative numbers of $CD4^+CD25^+$ T cells were increased in mice aged 3-19 weeks (panel c) when compared to control mice. Next, relative and absolute numbers of $CD4^+CD25^+$ T cells were determined in thymi from $TEC^{smad4-/-}$ and control mice to test whether the relative increase observed in the periphery might be a consequence of increased thymic production. Comparable relative and decreased total numbers of $CD4^+CD25^+$ T cells among the two groups argued against this possibility (panels d+e).

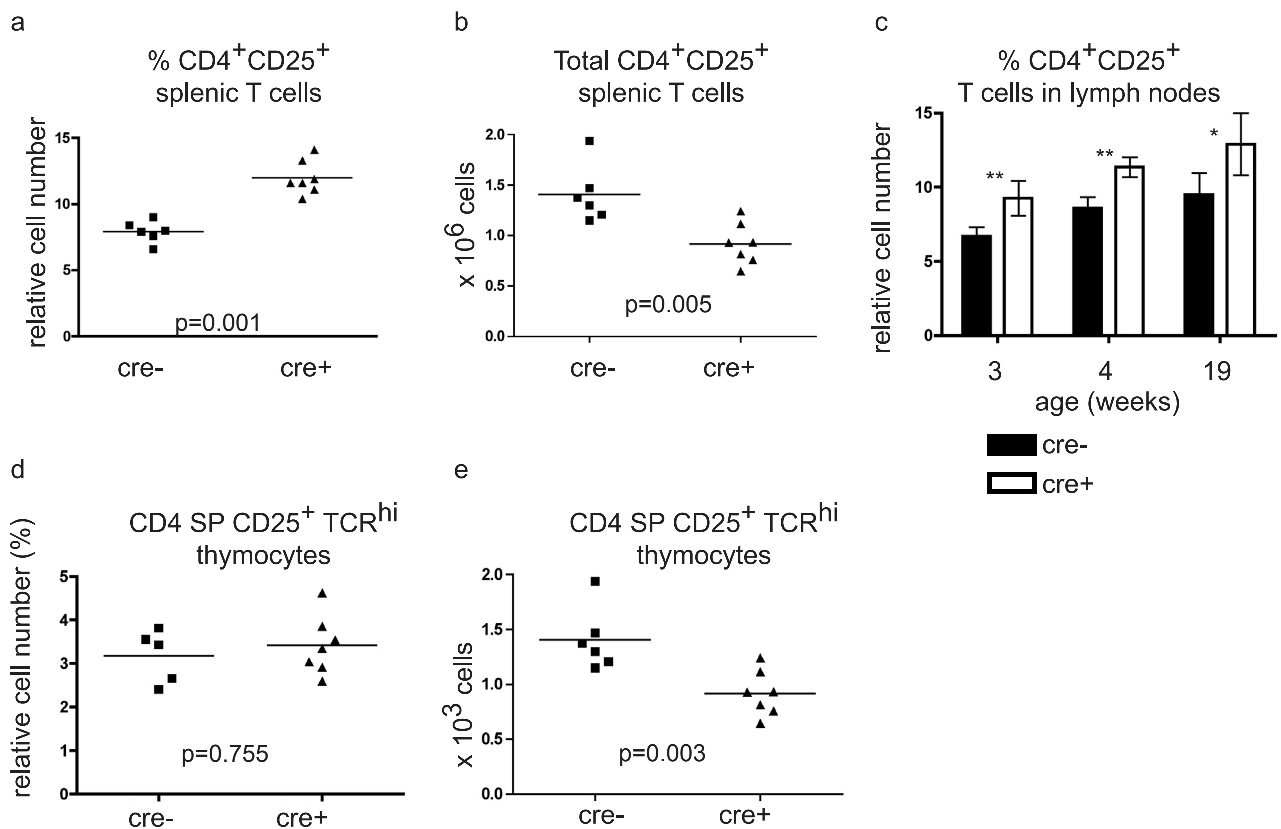


Figure 30
Cre^+ mice have increased relative but decreased absolute numbers of peripheral $CD4^+CD25^+$ T cells when compared to cre- mice while thymic production is reduced proportionally to the cellularity
Relative numbers of $CD4^+CD25^+$ T cells in spleen of cre+ mice are substantially increased (panel a) when compared to cre- mice. However, absolute numbers of $CD4^+CD25^+$ T cells are reduced in cre+ T lymphopenic mice compared to cre- mice (panel b). The relative increase of $CD4^+CD25^+$ T cells in the periphery is detectable from week 3 on and persists up to 19 weeks (not analyzed later).
Analysis of thymic $CD4^+CD25^+$ T cells revealed equal relative numbers in cre+ compared to cre- mice (panel d). Due to the overall reduced thymic cellularity, the absolute number is reduced substantially though (panel e).
** stands for $p<0.05$, ** stands for $p<0.01$; The Mann-Whitney test was used for all statistical analysis exept for panel c, 4 weeks, where an unpaired student t-test was used.*

B.3.5.3.4.2 Increased *in vitro* suppressive capacity of CD4⁺CD25⁺ T cells from $TEC^{smad4-/-}$ compared to control mice

$CD4^+CD25^+$ T cells contain T cells with regulatory function that can suppress LIP. However, CD25, the IL-2Rα chain, is a surface molecule that is not only expressed on T_{REG} cells but also on antigen-activated T cells. Furthermore, CD25 can be upregulated during LIP. Antigen-induced upregulation was unlikely since the mice were kept under spf conditions. Therefore, to discriminate between relatively increased numbers of $CD4^+CD25^+$ T cells as a consequence of homeostatic expansion (without regulatory activity) on one hand and increased numbers of T_{Reg} cells with suppressive capacity on the other hand, *in vitro* suppression assays were performed. Naïve ($CD45RB^{hi}$) $CD4^+$ T cells were purified by FACS omitting anti-TCR antibodies to avoid TCR stimulation. Naïve T cells were mixed with irradiated $Rag2^{-/-}$ splenocytes to activate the T cells. Titrations of purified $CD4^+CD25^+$ T cells were then added at ratios indicated in Figure 31a+b. $CD4^+CD25^+$ T cells purified from $TEC^{smad4-/-}$ exerted a higher suppressive activity than their counterparts isolated from control mice. At a ratio of one $CD4^+CD25^+$ T cells to 16 naïve T cells, T_{REG}s from $TEC^{smad4-/-}$ mice still suppressed proliferation by 25% while T_{REG}s from control mice did not contain any regulatory activity at this ratio any more. The increased suppressive capacity was independent of whether naïve T cells were from $TEC^{smad4-/-}$ or control mice.

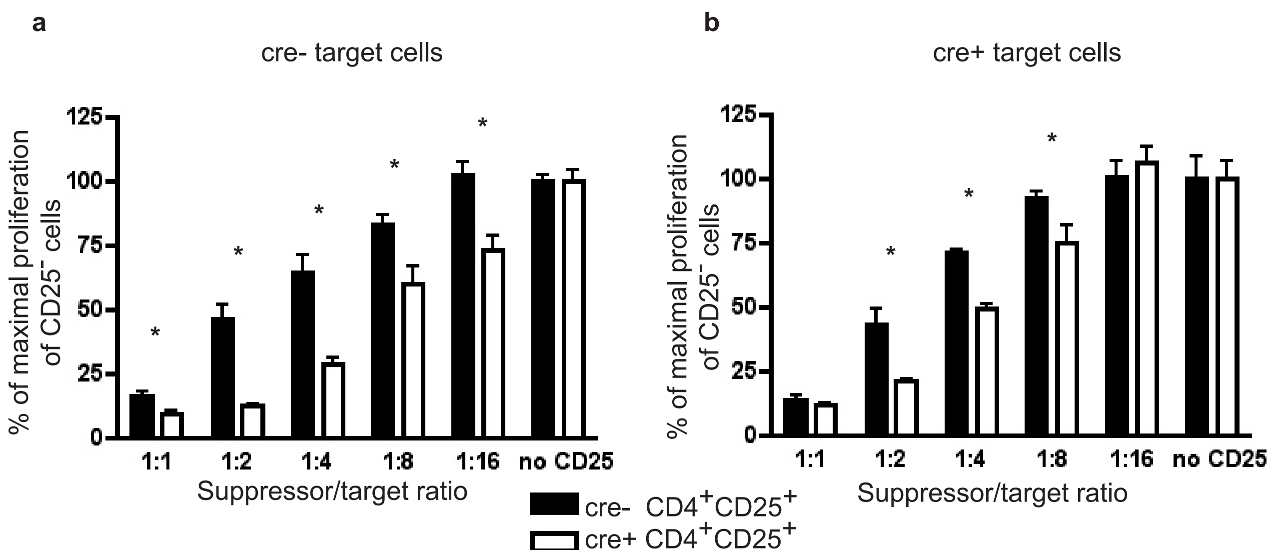


Figure 31
$CD4^+CD25^+$ T_{Reg} cells from cre+ mice exhibit increased suppression of naive T cells compared to cre- control cells
a+b) In vitro suppression of naive $CD4^+$ T cells from cre- (panel a) and cre+ (panel b) mice cocultured with sorted $CD4^+CD25^+$ T cells.
Cre- cells were purified from pooled lymph nodes from six 5-8 weeks old females. Cre+ cells were FACS purified cells from pooled lymph nodes from seven 5-9 weeks old females.
** stands for $p<0.05$*

B.3.5.3.4.3 Relative increase of $CD103^+$ cells among $CD4^+CD25^+TCR^{hi}$ T cells in $TEC^{smad4-/-}$ mice

The increased *in vitro* suppressive capacity of $CD4^+CD25^+$ T cells from $TEC^{smad4-/-}$ compared to $CD4^+CD25^+$ T cells from wildtype mice could be a consequence of a cell autonomously increased capacity to suppress, or, alternatively, could result from an increased frequency of T cells with suppressive potential within the heterogenous population of $CD4^+CD25^+$ T cells. To address this point, the relative number of $CD103^+$ cells among $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from $TEC^{smad4-/-}$ or control mice was examined. Both, $CD25^-$ and $CD25^+$ $CD4^+$ T cells contained elevated frequencies of $CD103^+$ T cells in $TEC^{smad4-/-}$ mice when compared to control littermates (Figure 32 a+b). The increase was strong enough that $TEC^{smad4-/-}$ mice contained equal numbers of $CD4^+CD25^-CD103^+$ T cells as wildtype mice despite the pronounced lymphopenia (panel c). The relative increase of $CD4^+CD25^+CD103^+$ T cells was even so marked that the absolute number of $CD4^+CD25^+CD103^+$ T cells in $TEC^{smad4-/-}$ mice was higher than in control littermates despite the lymphopenia (panel d). Thymic production of $CD4^+CD25^-CD103^+$ and $CD4^+CD25^+CD103^+$ needs to be addressed in future experiments, as preliminary data suggested that relative numbers of $CD4^+CD103^+$ T cells were at least equal in $TEC^{smad4-/-}$ and control mice (data not shown).

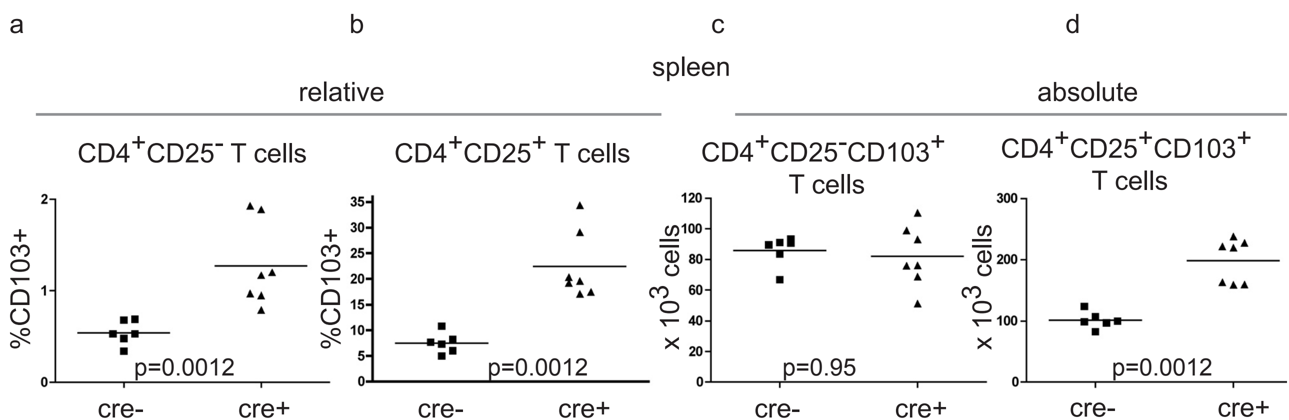


Figure 32
Increased total number of $CD4^+CD25^+CD103^+$ T cells in $TEC^{smad4-/-}$ mice
Cre+ animals contain a higher relative (panel a+b) and absolute (panel c+d) number of splenic $CD4^+CD25^+CD103^+$ T cells in spleen (a-d) and lymph nodes (data not shown).

B.3.5.3.4.4 Increased *in vitro* suppression of naïve CD4+ T cells by CD4+CD25+CD103+ T cells from TEC$^{smad4-/-}$ mice

In order to verify if the in vitro observed increased suppressive capacity of CD4+CD25+ T cells from TEC$^{smad4-/-}$ mice compared to CD4+CD25+ T cells from wildtype mice (see section B.3.5.3.4.2) was indeed a direct consequence of the relative increase of CD4+CD25+CD103+ T cells that possess a known inherently higher suppressive capacity than CD4+CD25+CD103- T cells [161], in vitro suppression assays were performed with purified CD4+CD25+CD103- and CD4+CD25+CD103+ T cells. Comparing equal numbers of suppressor cells from TEC$^{smad4-/-}$ and control donors, both, the CD4+CD25+CD103- (Figure 32 panel e) and CD4+CD25+CD103+ (panel f) purified from TEC$^{smad4-/-}$ mice were more potent to suppress proliferation of T cells in vitro.

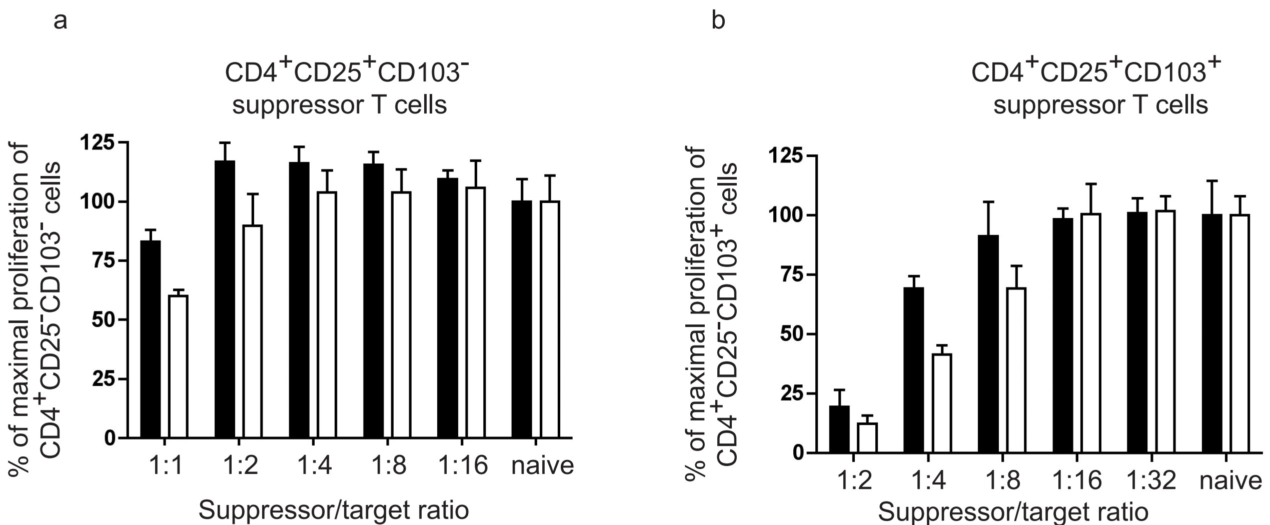


Figure 32B
Enhanced in vitro suppression capacity of CD4+CD25+CD103+ T cells in TEC$^{smad4-/-}$ mice
In vitro suppression of naive CD4 T cells cocultured with purified CD4+CD25+CD103- (panel a) or CD4+CD25+CD103+ (b) T cells.
Suppressor cells were purified from pooled lymph nodes from six (cre-) or seven (cre+) 5-9 weeks old females.
** stands for $p<0.05$; naive = naive T cells only*
The experiment was performed once in quadruplicates.

cre-
cre+

In summary, TEC$^{smad4-/-}$ mice contained a higher frequency of CD4+CD25+ T cells and these cells were as a whole population more suppressive than their counterparts from control mice. This can be explained by an increased frequency and absolute number of CD4+CD25+CD103+ T cells that had as a population again a higher suppressive potential than their counterparts from wildtype control mice. Therefore it is possible that LIP is counterbalanced by T_{Reg}s in TEC$^{smad4-/-}$ mice.

B.3.5.3.5 Hypothesis 5

Inability to respond to homeostatic signals

B.3.5.3.5 Purified T cells from TEC$^{smad4-/-}$ mice proliferate at least equally well as control T cells in lymphopenic hosts

The experiments performed thus far demonstrated clearly that the lymphopenia in TEC$^{smad4-/-}$ mice could be sensed by transferred T cells. Furthermore, T cells from

TEC$^{smad4-/-}$ mice could proliferate equally well as T cells from control mice when stimulated with anti-CD3 antibody *in vitro*. To test whether T cells from TEC$^{smad4-/-}$ mice could sense lymphopenia, T cells were purified from TEC$^{smad4-/-}$ and control mice, mixed at a 1:1 ratio, labeled with CFSE and then transferred into Rag2$^{-/-}$ hosts. Five days after transfer, both, CD4^{+} and CD8^{+} T cells from TEC$^{smad4-/-}$ mice had proliferated at least equally well as their counterparts from control mice (preliminary results, data not shown). Thus, in a competitive situation *in vivo*, T cells from TEC$^{smad4-/-}$ mice were able to respond to lymphopenia with quantitatively normal proliferation. Therefore, failure of T cells from TEC$^{smad4-/-}$ mice to sense and respond to lymphopenia cannot be accounted for the lymphopenia.

B.3.5.3.5 Hypothesis 6

Increased cell death of peripheral T cells in TEC$^{smad4-/-}$ mice

In order to answer point 6, naïve and regulatory T cells from control and TEC$^{smad4-/-}$ will be purified and subsequently be transferred into control, TEC$^{smad4-/-}$ or Rag2$^{-/-}$ hosts, respectively (see discussion).

B.3.5.4 Abnormal Vβ usage in peripheral T cells from TEC$^{smad4-/-}$ mice

Despite the statistically significant difference for Vβ6 usage in CD4 SP and Vβ12 in CD8 SP thymocytes, the biologic relevance remained questionable (see B.3.4.4). Clonal T cell expansion (CTE) is a phenomenon observed in aged mice [162]. It was hypothesized that the small change of Vβ usage by thymocytes could accumulate in aged mice. Therefore, the frequency of Vβ6 and Vβ12 usage in peripheral blood T lymphocytes of mice aged 9 months was measured. Aged TEC$^{smad4-/-}$ mice did indeed show an altered Vβ usage (Figure 33) when compared to control mice. While CD4^{+} T cells had equal frequencies of Vβ6 and Vβ12 in control and TEC$^{smad4-/-}$ animals (panel a), CD8^{+} T cells from TEC$^{smad4-/-}$ animals showed increased usage of Vβ6 and Vβ12 in comparison to control mice (panel b). Thus, the altered thymic output in TEC$^{smad4-/-}$ mice might indeed affect TCR Vβ usage or, alternatively, T cell

clones bearing TCRs with Vβ6 and Vβ12 could have a growth or survival advantage over T cells using other TCR Vβ in mice with chronic lymphopenia.

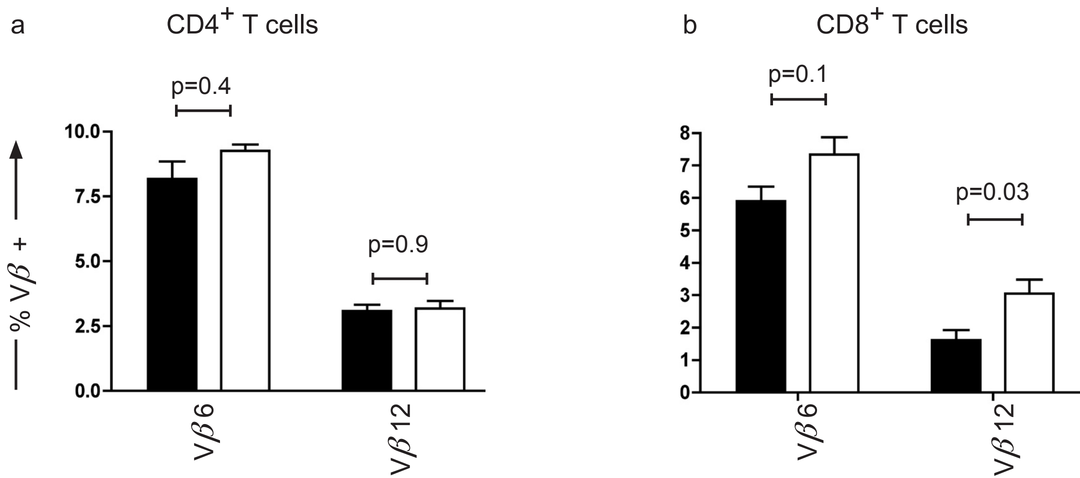


Figure 33
Aging cre+ mice contain CD8+ T cells with shifted TCR Vβ usage when compared to cre- littermates

Analysis of theTCR Vβ usage by peripheral CD4+ (a) and CD8+ (b) T cells from 9 months old cre- and cre+ mice.

n= 3 per genotype for Vβ6 and n=4 per genotype for Vβ12

■ cre-

□ cre+

B.3.5.5 Regular effector functions of T cells of TEC$^{smad4-/-}$ mice

B.3.5.5.1 Normal ability of T cells of TEC$^{smad4-/-}$ mice to provide B cell help

Immunization with Nitroiodophenol (NIP) conjugated to Ovalbumin (OVA) induces a strong T-dependent B cell response. To test B cell help in TEC$^{smad4-/-}$ animals, mice were immunized intraperitonally with Alum precipitated NIP-OVA and boosted on day 17. Serum was collected on days –1, +5, +12 and +23 after immunization. The increase of the NIP-specific IgG titer of TEC$^{smad4-/-}$ mice paralleled the response observed in control littermates implying that T cells from TEC$^{smad4-/-}$ mice can provide regular B cell help for immunoglobulin isotype switch (Figure 34a).

B.3.5.5.2 Colitis induction by transfer of naïve CD4^{+}CD45RB^{hi} T cells into a lymphopenic host

Adoptive transfer of purified naïve (CD45RB^{hi}) CD4^{+} T cells into lymphopenic hosts induces colitis [163]. In order to test effector functions of CD4^{+} T cells from TEC$^{smad4-/-}$ animals, purified naïve CD4^{+} T cells from control and TEC$^{smad4-/-}$ donors were injected i.v. into Rag2$^{-/-}$. Diarrhea incidence and weight loss of hosts injected with T cells from both donors were identical (Figure 34 b).

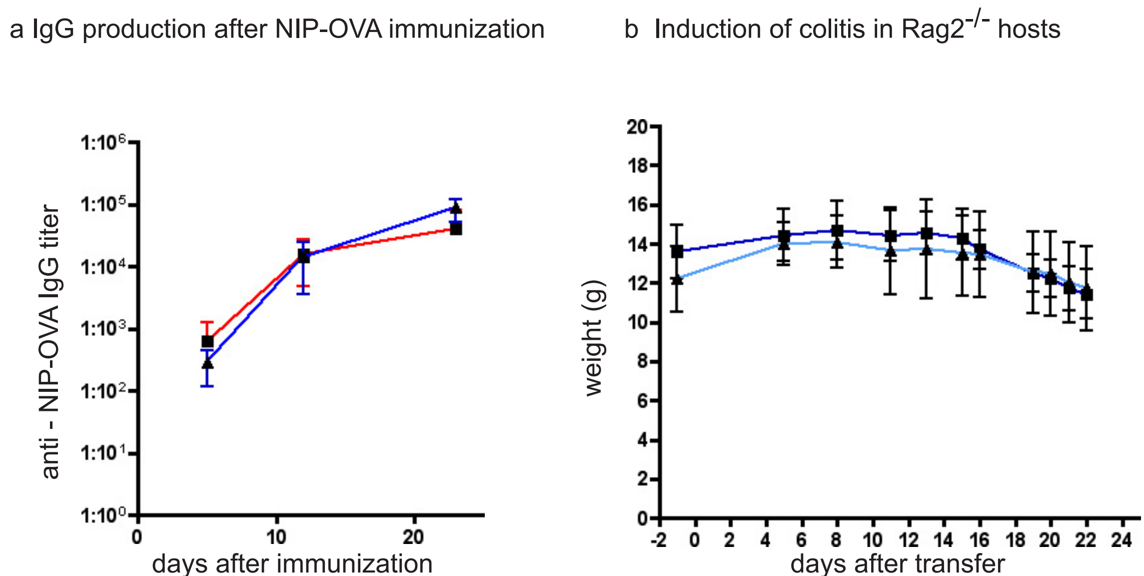


Figure 34
Normal effector functions of T cell from TEC$^{smad4-/-}$ mice
anti-NIP IgG in cre- and cre+ mice (a). Equal colitis induction induces comparable weight loss in cre- and cre+ mice.

cre-

cre+

B.3.5.5.3 MHC mismatched skin is equally rejected by T cells from control and TEC$^{smad4-/-}$ mice

Skin patches from female Balb/c (H-2^{d}) donors were transplanted onto control or TEC$^{smad4-/-}$ recipients. Graft rejection was similar for both groups (data not shown).

Taken together, T cells from TEC$^{smad4-/-}$ mice displayed normal effector functions when compared to T cells from control mice.

Discussion

Part A Aire

The autoimmune regulator (*aire*) gene is important for central tolerance induction by enabling thymic epithelial cells to display a wide array of self-antigens to developing thymocytes [76, 77, 79, 164-166]. This mechanism allows tolerance induction towards self-antigens with a restricted expression pattern. It is not clear, however, whether *aire* plays additional roles in antigen processing and/or presentation. In order to have a tool to genetically manipulate *in vivo* the rare population of mTECs that express Aire, a mouse, designated aire-cre, was generated that expresses the Cre recombinase under the transcriptional control of *aire* (Figure 6). This strategy should allow to activate or inactivate genes specifically in *aire* expressing cells. Crossing aire-cre mice to mice harboring a conditional transgene allows to specifically express the transgene in aire expressing cells. Thus, surrogate self-antigens could be expressed exclusively in aire expressing mTECs enabling to investigate the role of aire expressing cells in tolerance induction.

A.4.1 Generation of mice expressing the Cre recombinase under the transcriptional control of the aire promoter

To allow Cre expression in *aire* expressing cells, a gene targeting construct was cloned such that the transcriptional start codon ATG of the *cre* cDNA replaced the endogenous exon1 of the *aire* gene (see A.3.1.1). The final targeting construct yielded a very high targeting efficiency (see A.3.1.4). The reasons for this finding remained elusive. It could be speculated that the *aire* locus was well accessible since *aire* transcripts were expressed in ES cells (data not shown). Correctly targeted ES clones were used to generate aire-cre mice (see A.3.1.4 – A.3.1.7). The neomycine resistance gene cassette was removed *in vivo* since the latter can influence the gene expression of the introduced transgene [150, 167, 168]. Unwanted effects include additional, potentially toxic transcripts, aberrant alternative splicing, “gene knockdown” of neighboring genes and aberrant or premature expression of the transgene [167, 168].

A.4.2 Spontaneous infiltration of peripheral organs in aire-cre mice

Aire deficient mice develop spontaneous multiorgan autoimmune lymphocytic infiltration [78, 79, 169]. Since multipe *aire* splice variants have been described [136], it was unpredictable whether the targeting strategy to generate aire-cre mice would inactivate the *aire* gene or, alternatively lead to alternative splicing leaving some *aire* gene activity intact. To investigate whether aire-cre mice exhibit a similar phenotype as other aire-deficient mice, selected organs were examined histologically. Indeed, $aire^{CN/CN}$ and $aire^{CNDF/CNDF}$ mice developed spontaneous organ infiltration (Figure 10). Unlike other reports [78, 79], sections from some $aire^{CN/wt}$ heterozygous mice revealed lymphocytic infiltration as well. This finding might be explained by the different targeting strategy used. Reports in humans [170, 171] and mice [73] support the finding that heterozygous individuals can exhibit subclinical autoaggression.

Based on these results it was concluded that aire-cre mice can serve as a model to investigate the human autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome.

A.4.3 Characterization of Cre expression in aire-cre gene targeted mice

Aire-cre mice were generated not only to serve as a model to investigate Aire - deficiency but rather to have a genetic tool that allows to address more sophisticated questions with regards to the function of Aire. Protein domains suggested that Aire may act as a transcription factor [81, 172]. Furthermore, nuclear localization [142, 173], binding to the common transcriptional co-activator CREB [145] and transcriptional transactivator functions [145] supported the view that Aire may act as a transcription factor. It therefore did not come as a surprise that Aire regulates ectopic gene expression [79]. The regulation of ectopic gene expression, however, may not be the only role of Aire. This hypothesis is supported by a report that Aire acts as an E3 ubiquitin ligase [174] suggesting that Aire might be involved in antigen processing.

To test this hypothesis, a transgene acting as a surrogate self-antigen needs to be expressed in Aire expressing cells in the presence or absence of Aire. This could be achieved crossing hetero- or homozygous aire-cre mice to conditional transgenic mice. Thus, the transgene would be activated through Cre-mediated recombination in Aire expressing cells while Aire would only be present in heterozygous but not homozygous mice. To verify if aire-cre mice can be used to specifically regulate genes in mTECs, the Cre expression pattern in thymi of aire-cre mice was studied in relation to Aire (Figure 11). In adult mice, Aire was absent in $aire^{CNDF/CNDF}$ homozygous mice, indicating that the gene targeting indeed

abolished protein production. Cre was exclusively expressed in heterozygous aire$^{CNDF/wt}$ and homozygous aire$^{CNDF/CNDF}$ mice but not in wildtype mice, demonstrating specificity of the Cre signal. Cre expression was restricted to cells in the medulla, a pattern observed for Aire expression in wildtype mice [15, 16]. As expected, most Aire^{+} cells were also Cre^{+}. However, rare cells were either only positive for Aire, or, alternatively, Cre alone. Assuming that Aire and Cre have different half-lives, this result indicated that Aire was not constantly expressed. This hypothesis is supported by a number of findings: Aire expression is dependent on regular thymocyte maturation [16, 175] and it is known to be induced by triggering the lymphotoxin β receptor (LT-β R) on mTECs [18, 19]. The observation that only 1-5% of mTECs express Aire raises the question how thymocytes can be rendered tolerant to ectopically expressed genes by such a rare population of mTECs [74, 86]. The possibility that thymocytes meander randomly through the medulla and randomly encounter Aire expressing cells appears unlikely. A recent study described random migration of thymocytes in the thymic cortex but rapid, directed migration after positive selection toward the medulla [176]. This study is underscored by the finding that CCR7 is required for the migration from the cortex to the medulla [177-179]. It was proposed that the rare population of Aire expressing cells was sufficient to induce tolerance, as ectopic antigens are crosspresented by thymic professional APCs, suggesting that Aire^{+} mTECs act as a reservoir of ectopic antigens that spread to BM-derived APCs and thus amplify the distribution and presentation of ectopic genes [86]. Alternatively, it can be speculated that Aire expressing mTECs might attract immature SP thymocytes to expose them to ectopic antigens. This hypothesis is supported by the finding that rare mTECs express CCL22 in a pattern reminiscent of Aire expressing cells and that CCL22 attracts CD4^{+}CD8^{lo} thymocytes [180, 181]. Furthermore, several chemokines are regulated by Aire [182] and analysis of raw data from microarrays from Ref [79]. Collectively, it is possible that mTECs attract thymocytes that subsequently induce or perpetuate Aire expression. This would result in undulating Aire expression among mTECs which could explain that Aire or Cre were expressed in some mTECs alone.

A 4.3.2 LacZ reporter mice suggest Cre expression in aire-cre mice prior to the thymus formation

Knowing that Cre is specifically expressed in adult thymi of aire-cre mice, enzymatic Cre activity was studied by crossing aire-cre mice to ROSA26 lacZ indicator mice (see Materials & Methods). In contrast to the specific expression of Cre when assessed by direct staining of the protein, lacZ positive cells appeared in many more cells than what would be expected from Aire immunohistochemistry (Figure 12). Moreover, lacZ^{+} cells could be detected in the

thymic cortex, a site where Aire expression has not been reported with the exception of one survey [164]. Cre mediated recombination of loxP sites is an irreversible event [183]. Therefore, the increased number of $lacZ^+$ TECs when compared with direct Aire staining could be explained by the accumulation of recombination in mTECs if Aire was indeed induced in rare mTECs. Alternatively, this finding could be the consequence of recombination in a common precursor of cortical and medullary TECs secondary to physiologic Aire expression prior to thymus formation or aberrant *cre* transgene expression. Several investigators have reported aberrant expression of transgenes [150, 184, 185]. The potential influence of the neomycine resistance cassette on Cre expression was already discussed (A.4.1) but could be excluded as aire-cre mice without neomycine resistance cassette crossed to lacZ indicator mice displayed the same number of $lacZ^+$ cells in their thymi. A spontaneous mutation in ES cells leading to aberrant Cre expression [186] was unlikely as two independent aire-cre mouse lines yielded the same result when crossed to lacZ indicator mice. The genetic background has also been reported to influence Cre expression [187]. At present, influence of the genetic background on Cre expression cannot be excluded as the aire-cre mice used were backcrossed for less than four generations. To analyze whether β-galactosidase activity was restricted to thymic cells, non-lymphoid tissues were analyzed in [aire-cre x ROSA26lacZ] mice for lacZ. Extensive lacZ staining was found in several organs. As a whole, it was concluded that the most likely explanation for the unexpectedly high number of $lacZ^+$ cells in thymi from [aire-cre x ROSA26lacZ] mice was secondary to Cre activity in a precursor of TECs.

A.4.3.3 Widespread Aire expression in multiple lymphoid and non-lymphoid organs

The physiologic Aire expression pattern remains at present highly controversial. Aire was reported to be mainly expressed in lymphoid tissues [16], while other investigators found extensive *aire* mRNA expression in multiple non-lymphoid organs [136, 141]. One research paper reported that the Aire protein could not be found outside the thymus while by nested RT-PCR aire transcripts were found in several non-lymphoid organs [17]. In order to investigate the physiologic expression of *aire* outside the immune system and to dissect the contradicting results from the analysis of Cre expression using immunohistochemistry and lacZ reporter mice, RT-PCR was used to determine *aire* mRNA expression in multiple organs. Aire mRNA was found in almost all organs that were examined (Table I). It is possible that Aire is expressed in most cells of the body at low levels therefore escaping capture by techniques with low sensitivity. The highly sensitive RT-PCR could, however, explain the widespread expression of β-galactosidase activity found in [$aire^{CNDF/wt}$ x ROSA26 lacZ] mice. It remains

to be kept in mind though, that Aire is expressed in DCs that are present in many organs and could account for signals detected by RT-PCR.

A.4.3.4 Proof of Cre activity during mouse embryonic development

Two reasons remained that could have explained the unexpected lacZ findings. First, Aire and thus Cre expression prior to the thymus formation and second, low level ubiquitous Aire expression. Independent of the physiological expression pattern of Aire, the first scenario would exclude the use of aire-cre mice to induce transgene expression specifically and uniquely in Aire expressing mTECs since irreversible recombination in a precursor to thymic epithelial cells would result in ubiquitous recombination in all TECs. Therefore, [aire$^{CNDF/wt}$ x ROSA26 lacZ] mice were analyzed for lacZ expression during embryonic development. LacZ expression could clearly be demonstrated as early as E7.5 (Figure 13). Remarkably, E7.5 embryos were only partially blue, indicating that recombination had occurred in differentiated cells rather than omnipotent cells of the inner cell mass of the blastocyst. This finding implied that Aire plays a role during embryonic development. Indeed, APECED patients [82] and aire-deficient mice have a reduced fertility [78] which has been attributed to sterility secondary to autoimmune destruction of reproductive organs. Yet, an additional embryonic defect in Aire-deficient individuals contributing to reduced fertility cannot be formally excluded at present.

A.4.3.5 Alternative strategies to achieve transgene expression in Aire expressing mTECs

As transgene expression cannot be expressed specifically in Aire expressing mTECs when crossing Aire-cre mice to conditionally transgenic mice despite the highly specific Cre expression in adult mice, alternative strategies enabling the study of Aire expressing cells to induce tolerance were worked out. 1) Aire-cre targeted mice where Cre can be induced after physiological aire expression has been turned on in mTECs (i.e. approximately E 14.5) should allow to achieve the aim that was initially formulated. However, the obvious drawback would be another substantial effort of time to generate such mice. Moreover, inducible Cre expression can be inefficient, resulting in mosaic recombination [188]. 2) Alternatively, thymi from aire-cre mice could be isolated after E14 when a regular Cre expression pattern has been achieved in order to subsequently transduce the cells with a conditional transgene of interest, e.g. by the means of lentiviral transduction. Thymi could then be transplanted under the kidney capsule of host mice. The obvious drawback of this solution would be the extensive *in vitro* manipulation. On the other hand, such a system would be much more flexible

than crossing mice to conditional transgenic mice therefore allowing to vary the surrogate antigen considerably.

Part B Smad4

B.4 Effects of thymic epithelial-specific *smad4* deficiency on thymopoiesis and peripheral T lymphocytes

B.4.1 Deciphering thymic expression of mRNA transcripts of the TGF-β family of signaling transduction molecules

Fgf, Tgf-β, Wnt and Hh signaling are evolutionary conserved signal transduction pathways involved in many aspects of organ development (see Figure 1). The family of TGF-β signaling molecules (TGF-β, Bmp and Activins) are critical for the development of epithelial organs [45]. TGF-β signaling is required for thymocyte development [43], however, very little is known about the necessity of TGF-β signals for thymic epithelial cell development [189]. It was hypothesized that the biochemical intracellular signals triggered by TGF-β, Bmp and Activins contribute to thymic development and/or maintenance. To test this contention, the expression of several members of the TGF-β family in whole thymus preparations and thymic epithelial cell lines was analyzed. The abundance of mRNA transcripts for extracellular morphogens of the extended TGF-β family, their receptors and intracellular signal transduction molecules in unseparated thymus as well as TEC lines of cortical and medullary origin (table II) suggested a role for TGF-β signaling in TECs. Since Bmp2, Bmp4 and Noggin are secreted, these molecules might be produced by the stroma and act in a paracrine fashion on thymocytes. To discriminate between expression by stromal and lymphoid cells, the mRNA expression was tested in thymic epithelial cell lines (Table II, panel b) as well as sorted thymocyte subpopulations (Table II, panel c). Cortical and medullary TEC lines expressed transcripts for receptors and intracellular signal transduction molecules belonging to the canonical signal transduction pathway of the Tgf-β family of signaling molecules. This suggested that Tgf-β signaling was involved in the development and/or maintenance of TECs. The presence of *smad5* transcripts implied that Bmp signaling was involved while the presence of *smad3* hinted at a role for Tgf-β signaling as Smad5 belongs to Bmp-associated receptor-smads while Smad3 transmits signals induced by TGF-β [190]. Interest-

ingly, thymocyte subpopulations did neither express *bmp2* nor *bmp4*. Furthermore, none of the receptors analyzed could be found (Table II, panel c). Thus it appeared that Bmps were majorily synthesized by TECs acting in a para- and autocrine manner on TECs. In addition, since some receptors were expressed by thymocytes as well, Bmps secreted by TECs might act on distinct thymocyte developmental stages. Since end-point RT-PCR was used in these analyses, a quantitative assessment of the expression levels could not be drawn from these results. In particular, a comparative analysis and interpretation of this data was obviously limited by the differences in the number of thymic stromal cells relative to thymocytes as the latter increases substantially during development. Independent of this shortcoming, these results demonstrated that signals transmitted by Tgf-β family members are likely to be required for proper TEC development and/or function.

To study the function of Tgf-β signaling in thymic epithelial cell development in vivo, it was decided to block the canonical pathway triggered by Tgf-β or members of the extended Tgf-β family of signaling molecules.This is best achieved by removing the central co-Smad molecule, Smad4, which is needed for the activation of the canonical signaling pathway by Tgf-β, Bmp and Activin (see Figure 1). Since mice rendered deficient for Smad4 die at E 7.5 [191], i.e. before the development of a thymus anlage, it was decided to conditionally ablate *smad4* specifically in thymic epithelial cells using the cre/loxP system [183]. For this purpose, FoxN1-cre transgenic mice (Zuklys et al., unpublished) were crossed with mice harboring a conditional *smad4* allele [148]. Since FoxN1 is chiefly expressed in TECs and skin [192, 193], Cre mediated inactivation of *smad4* in double transgenic mice will be achieved preferentially in TECs and keratinocytes while in cells with silent FoxN1, including thymocytes and T cells, a functional *smad4* conditional allele remains active (Figure 4).

B.4.2.1 Normal development and fertility of $TEC^{smad4-/-}$ mice

In contrast to mice with a complete lack of *smad4*, viability is not affected in $TEC^{smad4-/-}$ or control mice (data not shown). Mice of both genotypes are born with a Mendelian frequency, show normal weight gain, have a regular life span (assayed up to 11 months) and are fertile (data not shown). These results indicate that FoxN1-cre mediated *smad4* ablation is suited to investigate in vivo the role of *smad4* in TEC development in the absence of unwanted systemic effects. As FoxN1 is expressed in keratinocytes, $TEC^{smad4-/-}$ mice have increased hair loss and malformations of the nails revealing that Smad4 is involved in normal hair and nail development and/or maintenance (data not shown).

B.4.2.2 Thymic hypoplasia in TEC$^{smad4-/-}$ mice

Thymic epithelial-specific *smad4* deficiency leads to substantial thymic hypoplasia (Figure 14). Comparative analysis of thymic cellularity at all ages examined revealed that thymocyte cellularity was dramatically reduced in TEC$^{smad4-/-}$ animals when compared to control mice. It appeared that *smad4* deficient TECs could support thymic growth to physiologic stimuli, but that the increase of cellularity was hampered by the absence of Smad4. This was reflected in the growth curve that paralleled the change in thymic cellularity of control mice (Figure 14, panel b). The extent of the increase in thymic cellularity was, however, strongly reduced in TEC$^{smad4-/-}$ mice. This could be due to a reduced proliferative capacity of TECs or a consequence of increased cell death of *smad4* deficient TECs. Alternatively, reduced thymic cellularity might be due to secondary effects on thymocyte differentiation and/or survival. As the data presented in this thesis do not allow to discriminate between these possibilities, further experiments are needed in the future.

B.4.3 Validity of the experimental system

B.4.3.1 Genomic deletion of the conditional *smad4* allele in thymic epithelial cells from TEC$^{smad4-/-}$ mice

Cre/loxP mediated gene deletion can be limited -among other factors- by a chromatin configuration inaccessible for recombination [194]. Therefore, monitoring the efficiency of deletion in any particular combination of a Cre transgene with a conditional allele is critical in order to draw sound conclusions from an observed phenotype. RT-PCR based estimation of genomic deletion efficiency was used on purified TECs from TEC$^{smad4-/-}$ and wildtype mice (Figure 14B). The wildtype band was detected in TECs from both, control and TEC$^{smad4-/-}$ mice indicating incomplete recombination (Figure 14B, panel c). The recombined allele could only be detected in TECs from TEC$^{smad4-/-}$ mice but not in TECs from control mice demonstrating the specificity of gene deletion. In comparison to the wildtype band, the deleted band in TECs from TEC$^{smad4-/-}$ mice appeared weak. A direct quantitative comparison between the intensities of both bands would need to be based on equal efficiencies of PCR amplifications of both products, an assumption that cannot be taken for granted. Furthermore, the pronounced difference in total thymic cellularity between TEC$^{smad4-/-}$ and control thymi needs to be taken into consideration. As discussed (B.4.2.2), it cannot be assumed that thymi from control and TEC$^{smad4-/-}$ mice contain the same number of TECs. If Smad4 is required for proliferation of TECs, TECs having escaped recombination might overgrow the TECs with deleted *smad4*. Thus, differential proliferation of Smad4-wildtype and Smad4-deficient TECs

within TEC$^{smad4-/-}$ thymi could account for the weak amplification signal of the recombined locus. Alternatively, Smad4 may be required for survival of TECs. In that case, TECs without Cre-mediated recombination would survive while successful recombination would immediately lead to cell death and elimination of the whole cell including the recombined genomic DNA fragment. This would lead to traces of recombined DNA that can be amplified by PCR. Furthermore, it is not established whether there are TECs that do not express FoxN1 and whether TECs with different FoxN1 expression levels exist. Thus, again an alternative explanation of the stronger PCR amplification signal from the unrecombined allele compared to the signal from the recombined allele would be that TECs expressing FoxN1 have a proliferation and/or survival disadvantage as a consequence of the Smad4 deficiency while their counterparts with a silent *foxN1* gene are unaffected by the *smad4* mutation resulting in a relative overrepresentation of unrecombined genomic DNA when assessed by end point PCR. The obvious discrepancy between the dramatic effect of *smad4* deletion on thymic cellularity in TEC$^{smad4-/-}$ mice and the surprisingly weak signal of the recombined *smad4* locus needs further investigation since functional conclusions depend on this knowledge. Smad4 transcripts will be quantitated in comparison to *cre* and *foxN1* transcripts to test the hypothesis that only TECs with a silent foxN1 gene survive in TEC$^{smad4-/-}$ mice.

B.4.3.2 Effect of the conditional *smad4* allele and the FoxN1-cre transgene alone on thymic and splenic cellularity, thymocyte maturation and T cells

Since a dramatic change of the thymic cellularity in mice lacking *smad4* in thymic epithelial cells was observed, a set of experiments to exclude changes unspecific to the Smad4 deficiency in TECs was performed, i.e. side effects caused by the genetic manipulations applied to the *smad4* locus were investigated. Furthermore, any unwanted effects caused by the FoxN1-cre transgene or its integration into the genome were excluded.

The thymic and splenic cellularities of animals of *smad4* wildtype mice either transgenic for FoxN1-cre or not were identical (Figure 15, panel a), excluding that the FoxN1-cre transgene itself accounts for the reduced thymic cellularity. To test whether the conditional *smad4* allele by itself had an effect on thymic or splenic cellularity, mice that were either wildtype for *smad4* or hetero- or homozygous for the *smad4* conditional allele were investigated (Figure 15, panel b). No differences could be observed comparing the thymic or splenic cellularity of these three genetically different mice. Particularly, in any of these experiments relative and absolute numbers of $CD4^+$ and $CD8^+$ T cells in spleen were normal. Thus, the genetic manipulations required to generate mice that allow TEC specific Smad4 ablation do on their own not influence thymic function.

B.4.3.3 Absence of a T cell intrinsic defect in TEC$^{smad4-/-}$ mice

To exclude an unphysiological, cell-autonomous effect on hematopoietic cells, bone marrow chimeras with donor bone marrow (BM) derived from TEC$^{smad4-/-}$ mice were generated. As expected from the prevalent view that *foxN1* expression is restricted to thymic epithelial cells and keratinocytes, the FoxN1-cre transgene did not influence the development of thymocytes or T cells intrinsically (Figures 16 + 17). However, a recent report described a role of FoxN1 in bone marrow [195]. The authors conclude that *foxn1* does not only play a role in the differentiation of TECs but is also important prior to thymic colonization. They suggest that *foxN1* deficient (nude) mice have a defect in bone marrow stromal cells that affects early prethymic progenitor development. The results of the control bone marrow chimeras performed showed that this defect was not influencing our system. Taken together, the control experiments demonstrated that any observed phenotype in TEC$^{smad4-/-}$ was a consequence of the absence of Smad4 in thymic epithelial cells.

B.4.4 Thymic epithelial cell-specific *smad4* deficiency leads to subtle thymocyte developmental defects

B.4.4.1 Normal thymic stromal architecture in TEC$^{smad4-/-}$ mice

The normal histology of the TEC$^{smad4-/-}$ thymus with a clear distinction into cortex and medulla indicated that the morphological differentiation of the two compartments was independent of *smad4* (Figure 18). This finding is further corroborated by the regular expression pattern of cytokeratin 18 as a marker for differentiated cTECs and cytokeratin 5 and MTS-10 as markers for differentiated mTECs. Since the formation of a medulla is a relatively late event and depends on the formations of $\alpha\beta$ TCR^{hi} SP thymocytes, it appears that the canonical Tgf-β signaling pathway is dispensable for the major differentiation steps of thymic epithelial cells.

B.4.4.2 Cellular composition of TEC$^{smad4-/-}$ thymi

To analyze whether the reduced thymic cellularity was due to a block in thymocyte development, embryonic and postnatal thymocyte maturation was examined in TEC$^{smad4-/-}$ and control littermates (Figure 19). Overall, the proportions of thymocytes were interchangeable between control and TEC$^{smad4-/-}$ mice. Thus, the reduced thymic cellularity is not due to a thymocyte developmental block.

B.4.4.3 Minor maturational defect of CD4 and CD8 single positive thymocytes in TEC$^{smad4-/-}$ mice

The relative number of CD8 SP thymocytes was normal in control and TEC$^{smad4-/-}$ mice. Further subdivision of CD8 SP thymocytes into CD8^{hi}CD4^{int} and CD8^{hi}CD4^{-} cells revealed, however, that TEC$^{smad4-/-}$ mice had reduced numbers of CD8 SP with a completely down-regulated CD4 coreceptor (Figure 20). Lineage commitment to the CD4 or CD8 T cell line-age is generally thought to occur at the DP stage and to result in silencing of the opposite coreceptor gene [196]. The "strength of signal" model proposes that strong/long TCR signals promote the CD4 lineage and weaker/shorter signals favor the CD8 T cell lineage [197]. A variation of this model is termed "kinetic signaling model". According to this model, all DP thymocytes sensing a TCR signal proceed to a CD4^{+}CD8^{lo} stage. At this stage, sustained TCR signals lead to CD4 T cell commitment, but CD8 T cell commitment results if TCR signals cease [197]. The relative reduction of mature CD8 SP thymocytes could have resulted from a partial lack of TEC-mediated signals necessary for the complete downregulation of CD4 on the surface of CD8 SP thymocytes. Alternatively, the dramatic reduction of thymic size in TEC$^{smad4-/-}$ mice might account for a reduced intrathymic maturation time leading to incomplete downregulation of the CD4 coreceptor molecule despite the presence of all signals required for complete thymocyte development. If this were the case, then thymocyte maturation in a wildtype fetal thymus lobe transplanted heterotopically under the kidney capsule of a syngenic host should result in reduced numbers of CD8 SP with a complete downregulation of the CD4 coreceptor.

The defect in thymocyte development in TEC$^{smad4-/-}$ mice was confirmed using two other cell surface markers used to characterize thymocyte development. CD24 (aka as HSA) is highly expressed on DN and DP thymocytes and subsequently downregulated during maturation [198-200]. The biological role of CD24 remains to be determined as CD24 deficient mice do not have any overt thymocyte maturational phenotype. CD69 is a member of the NK gene complex family of C-type lectin-like signaling receptors that is upregulated after positive selection (see Figure 3); as for CD24, the function of CD69 in thymocyte development is presently unknown although a recent report proposes a role for CD69 in thymocyte export [157].

Analysis of CD24 expression on SP thymocytes in TEC$^{smad4-/-}$ animals confirmed a late developmental defect in CD8 SP thymocytes and, in addition, revealed a reduction of mature CD4 SP thymocytes (Figure21, panels a+b). For CD8 SP thymocytes, this is in concert with the observation of a reduced frequency of CD8^{+}CD4^{-} SP mature thymocytes in TEC$^{smad4-/-}$

thymi. On the other hand, a comparable difference was not observed among CD4 SP thymocytes where a complete downregulation of CD8 occured during the transition to full maturity in TEC$^{smad4-/-}$ thymi. Surprisingly however, the reduction of mature CD8 SP thymocytes could not be found by the analysis of CD69 expression (data not shown).

Taken together, the deficiency of Smad4 in thymic epithelial cells leads to minor changes in the maturation of SP thymocytes. The functional relevance of the maturational delays or defects remain to be tested though.

B.4.4.4 Regular usage of most TCR Vβ chains by single positive thymocytes in TEC$^{smad4-/-}$ mice

The absolute reduction of mature SP thymocytes may be a consequence of a proportional reduction of all developing thymocytes (Figure 19). Alternatively, this finding might reflect a selective loss of thymocyte clones bearing specific Vβ TCR chains that are less frequently positively selected or preferentially deleted in a thymic microenvironment of *smad4* deficient TECs. This would ultimately lead to a shift in Vβ usage.

A selective increase of usage of a defined Vβ chain can substantially impair the overall immune response to particular antigens [201]. In aged mice, individual Vβ chains can be detected at increased frequencies, a phenomenon called age-related T cell clonal expansion (TCE) [202]. Chronic infections and homeostatic proliferation might contribute to TCE and thus disturb the balance of Vβ usage [108]. TCE occurs in the periphery and their onset can be accelerated by decreased thymic output and/or function [202]. Therefore, the Vβ usage in mice with *smad4* deficient TECs was analyzed in SP thymocytes.

The frequencies of usage of almost all Vβ chains studied was comparable among TEC$^{smad4-/-}$ and control animals (Figure 22). Although the usage of two Vβ chains had statistically significant differences, the small changes were unlikely of biological relevance. Vβ6 was underrepresented by 9% in CD4SP thymocytes of TEC$^{smad4-/-}$ mice compared to control littermates while Vβ12 was used 21% more frequently by CD8 SP of TEC$^{smad4-/-}$ mice when compared to CD8 SP of control mice. Yet, chronic thymic output of T cells bearing TCRs with a slightly shifted frequency of Vβ usage might result in the accumulation of certain Vβ specificities in the periphery over time and enhance TCE. Therefore the Vβ usage of peripheral T cells in aged mice was analyzed (see below).

B.4.4.4b Smad4 deficient thymic epithelial cells fail to respond to KGF

Thymic epithelial cells but not thymocytes express FGFR2IIIB, the receptor for FGF7 (aka KGF) [42]. Exposure of young and aged mice to KGF increases thymic size while main-

taining the regular structure and composition of the thymic microenvironment [203]. These beneficial features can be used in mouse models of experimental graft-versus-host disease to preseve thymic function [204]. Therefore, it is important to elucidate the molecular mechanism by which KGF acts on TECs in order to improve and fine-tune existing experimental treatments.

The expression of members of the TGF-β family of signaling molecules in TECs is regulated by KGF (Rossi et al., manuscript in preparation). In order to test whether Smad4 is involved in the observed effect of KGF, mice that were either deficient for the expression of Smad4 in TECs or that were wildtype were injected with KGF and HBSS, respectively. Control mice injected with KGF showed a 60% increase in thymic cellularity while thymi with an epithelial specific Smad4 deficiency did not significantly increase their cellularity. These results suggested that Smad4 is involved in the effect of KGF on thymic growth.

B.4.5 Thymic epithelial-specific *smad4* deficiency significantly alters the peripheral T cell pool

B.4.5.1 Splenic cellularity and composition of lymphocytes in secondary lymphoid organs of $TEC^{smad4-/-}$ control mice

As thymic epithelial-specific *smad4* deficiency dramatically reduced the thymic cellularity but had surprisingly little effects on thymocyte maturation, the question emerged whether $TEC^{smad4-/-}$ thymi had an impact on the function of peripheral T cells.

As expected, $TEC^{smad4-/-}$ mice had a normal splenic cellularity at any time point so far analyzed (Figure 23). However, the relative and thus, the absolute number of $CD4^+$ and $CD8^+$ T cells was strongly reduced when compared to control littermates. This T cell lymphopenia was most pronounced in young mice but remained present at least up to 19 weeks.

B.4.5.2 Increased frequency of T cells displaying an activated/memory phenotype in $TEC^{smad4-/-}$ mice compared to control mice

In light of the persistent lymphopenia in $TEC^{smad4-/-}$ mice in combination with a presumably reduced thymic output it could be assumed that peripheral T cells in $TEC^{smad4-/-}$ mice underwent lymphopenia induced proliferation and thus exhibited a memory-like phenotype. Indeed, T cells in $TEC^{smad4-/-}$ mice had a 1.5-2 fold relative increase in activated/memory(-like) T cells, both for the $CD4^+$ and $CD8^+$ subset (Figure 25). The most likely mechanism leading to this phenotype is the chronic lymphopenia by inducing LIP.

B.4.5.3 Possible mechanisms for the incomplete correction of T cell numbers in TEC$^{smad4-/-}$ mice

The chronic lymphopenia was unexpected as lymphopenia physiologically leads to lymphopenia induced proliferation (LIP) formerly termed homeostatic expansion [103, 159, 205, 206]. The term homeostatic proliferation for the description of the phenomenon of lymphopenia induced T cell proliferation is, however, somewhat uncorrect, as the naïve T cell pool is not really replenished but the T cell cellularity is corrected by the expansion of memory-like T cells [103, 117]. In TEC$^{smad4-/-}$ mice the extent of lymphopenia was gradually reduced over time (Figure 24 c+d), suggesting that some of the mechanisms to maintain normal levels of T cells were operational in mice deficient for Smad4 expression in TECs. The following mechanisms, mutually not all exclusive, might contribute to the chronic lymphopenia:

1 Reduced thymic output
2 Absence of homeostatic signals in the periphery
3 A proliferation defect of T cells “imprinted” during development and selection in a Smad4-deficient thymic environment
4 Increased anti-proliferative suppression by T_{REG} cells
5 Inability to respond to homeostatic signals
6 Increased cell death of peripheral T cells in TEC$^{smad4-/-}$ mice

The results of experiments to address these points are discussed below. As a reminder, for each experiment the corresponding hypothesis will be displayed in **grey**.

B.4.5.3.1 Hypothesis 1

TEC$^{smad4-/-}$ mice have a reduced thymic output

B.4.5.3.1 TEC$^{smad4-/-}$ mice have a reduced thymic output

It is conceivable that the reduced thymic size of TEC$^{smad4-/-}$ mice results in a decreased thymic T cell production. The size differences led to variable FITC labeling efficiencies between control and TEC$^{smad4-/-}$ thymi. Therefore, the number of measured RTE in spleen was corrected for the relative labeling efficiency (Figure 25B). Not surprisingly, the total number of $CD4^+$ recent thymic emigrants was significantly reduced in TEC$^{smad4-/-}$ mice when compared to the total number of $CD4^+$ RTEs in spleens of control mice. Hence, it was concluded that hypothesis 1 was correct and that reduced thymic output was likely to contribute to the lymphopenia in TEC$^{smad4-/-}$ mice.

B.4.5.3.2 Hypothesis 2

Absence of signals inducing lymphopenia induced proliferation in TEC$^{smad4-/-}$ mice

B.4.5.3.2 TEC$^{smad4-/-}$ mice can provide homeostatic proliferation signals to adoptively transferred T cells

A possible explanation for the persistence of lymphopenia in TEC$^{smad4-/-}$ mice could be an inadequacy in sensing a limited degree of lymphopenia. Threshold values necessary for LIP have not been determined and most studies focusing on LIP have been carried out by the transfer of purified T cells into mice genetically rendered devoid of T cells. In consequence, T cells enter niches that are completely empty. Alternatively, if the recipient mice were depleted of T cells, additional confounding difficulties to such experimental models have to be taken into consideration. For example, radiation induced cell damage alters tissues locally and leads to changes in the cytokine milieu [207-209] which do not represent the situation during steady state conditions. TEC$^{smad4-/-}$ mice are different for the reason that T cells are not in a completely empty environment, i.e. other lymphoid cells and cytokines are present and they are constantly exposed to this milieu. One may speculate that such a situation may lead to a phenomenon of adaptation. For example, the relative contribution to LIP by TCR and IL-7 mediated signals is not fixed and depends on the degree of lymphopenia [210], thus there must be as yet unknown feedback signals between the degree of lymphopenia and the two identified signals required for LIP. Since the observed lymphopenia in TEC$^{smad4-/-}$ mice is a result of a genetic defect restricted to thymic epithelial cells (and cells of the epidermis), it is justified to assume that peripheral tissue damage and its consequences do not play a role here. An alternative explanation for the persistent lymphopenia in TEC$^{smad4-/-}$ mice could be given by a mechanism whereby the periphery of these mice fails to provide the signals necessary for LIP. To test this contention directly in vivo, CFSE labeled wildtype CD4$^+$ or CD8$^+$ T cells that had matured in a wildtype thymus were transferred into either control or TEC$^{smad4-/-}$ mice hosts. In the event that wildtype T cells could sense the state of lymphopenia in TEC$^{smad4-/-}$ recipients, the transferred T cells should display a higher proliferation rate when compared to T cells transferred into control hosts. Indeed, T cells transferred into lymphopenic TEC$^{smad4-/-}$ hosts proliferated more than in control hosts (Figures 26+27). Interestingly, the relative difference of proliferating cells was higher for CD8$^+$ than for CD4$^+$ T cells. This might reflect the somewhat stronger thymocyte developmental defect of CD8 SP thymocytes leading to a stonger and more persistent lymphopenia for CD8$^+$ than CD4$^+$ T cells (see Figure 24).

These observations were extended with CD8$^+$ OT-I T cells which express a transgenic TCR that is MHC class I restricted. As for wildtype cells, OT-I T cells transferred into TEC$^{smad4-/-}$ recipients proliferated more vigorously than OT-I T cells transferred into control mice, confirming that the lymphopenia in TEC$^{smad4-/-}$ recipients can be sensed. Moreover, adoptive transfer of OT-I T cells into Rag$^{-/-}$ hosts provided an estimate of the extent of LIP in TEC$^{smad4-/-}$ in comparison to completely empty host (Figure 28).

It was concluded that the degree of lymphopenia present in TEC$^{smad4-/-}$ mice could be sensed by T cells that had developed in a regular thymic microenvironment. Thus, the signals for LIP were present in TEC$^{smad4-/-}$ mice. This finding disproves hypothesis B.3.5.3.2 but provides an explanation of the increased frequency of T cells with a memory-like phenotype in TEC$^{smad4-/-}$ mice. It is remarkable that a genetic defect affecting exclusively thymic epithelial cells has such a profound effect on T cell biology in the periphery. The observation that a TEC-specific defect beyond selection results in an altered T cell response is unique.

B.4.5.3.3 Hypothesis 3

T cells from TEC$^{smad4-/-}$ mice have a proliferation defect “imprinted” during development and selection in a Smad4-deficient thymic environment

B.4.5.3.3 Regular *in vitro* proliferation of purified T cells from TEC$^{smad4-/-}$ and control mice

The lymphopenia observed in TEC$^{smad4-/-}$ animals could have been secondary to a T cell intrinsic proliferation defect imprinted during the development in a *smad4*-deficient thymic microenvironment. Therefore, naïve (CD45RB^{hi}) or memory (CD45RB^{low}) T cells, respectively, were purified from TEC$^{smad4-/-}$ and control animals and analyzed for their proliferative capacity *in vitro*. Both, naïve and memory-like CD4$^+$ T cells from TEC$^{smad4-/-}$ mice were able to proliferate *in vitro* in a manner that is equal compared to that of control T cells. This result clearly refuted a gross defect in the capacity of TEC$^{smad4-/-}$ T cells to proliferate. However, more subtle deficiencies could not be excluded as the *in vitro* stimulation with anti-CD3 antibodies constitutes a very strong and unphysiologic stimulus.

B.4.5.3.4 Hypothesis 4

TReg cells prevent lymphopenia induced proliferation

B.4.5.3.4.1 Increased frequency of $CD4^+CD25^+$ T cells in $TEC^{smad4-/-}$ compared to control mice

CD25 is upregulated during activation and some reports describe CD25 upregulation during LIP [103] while others state that the lack of CD25 is characteristic of LIP [103, 114, 115]. Further controversy arises from the observation that $CD4^+CD25^-$ T cells gained CD25 expression while $CD4^+CD25^+$ T cells lost CD25 expression while undergoing LIP [211]. Therefore, the frequencies of $CD4^+CD25^+$ splenic T cells could not be predicted in $TEC^{smad4-/-}$. A relative but not absolute increase in $CD4^+CD25^+$ T cells was found in $TEC^{smad4-/-}$ mice that might have been accounted for by LIP (Figure 30). Alternatively, these cells may have corresponded to the naturally occurring $CD4^+CD25^+$ T_{Reg} cells [97]. The relative abundance of $CD4^+CD25^+$ T cells in $TEC^{smad4-/-}$ mice was observed at any age tested. Despite the relative increase, in absolute numbers, the $CD4^+CD25^+$ population was decreased compared to control littermates. It could have been that the lineage of naturally arising T_{Reg} cells and non-T_{Reg} $\alpha\beta$TCR T cell lineages have differential survival capacities leading to a higher proportion of $CD4^+CD25^+$ in $TEC^{smad4-/-}$ mice. Alternatively, thymic production of naturally occurring T_{Reg} cells could have been relatively increased in $TEC^{smad4-/-}$ mice. The frequency of $CD4^+CD25^+$ thymocytes was, however, normal in thymi from $TEC^{smad4-/-}$ mice, hence the absolute number was reduced, excluding the possibility of an increased thymic production of naturally occurring T_{REG} cells (Figure 30). A last explanation might be differential homeostatic regulation of the naïve $CD4^+$ and the naturally occurring T_{Reg} cell pools [159]. It appears that activated T cells contribute indirectly to their own regulation by providing IL-2 that $CD4^+CD25^+$ T cells require for survival [212] and function [159]. It was reported that maintenance of the ratio between naïve and T_{Reg} cell numbers is important to control T cell numbers and that this ratio remained constant independent of the absolute number of peripheral T cells [159]. The finding of an overrepresentation of T_{Reg} cell numbers in relation to naïve T cells illustrates, however, that this negative feedback loop is only unidirectionally operational, i.e. naïve T cell numbers are controlled by T_{Reg} cells but not vice versa.

B.4.5.3.4.2 Increased *in vitro* suppressive capacity of $CD4^{+}CD25^{+}$ T cells from $TEC^{smad4-/-}$ compared to control mice

To test whether the $CD4^{+}CD25^{+}$ T cells in $TEC^{smad4-/-}$ mice were naturally occurring suppressor cells or non-suppressive T cells displaying upregulated CD25, in vitro suppression assays were performed. Using this approach, purified $CD4^{+}CD25^{+}$ T cells from $TEC^{smad4-/-}$ mice displayed, on a population basis, a suppressive capacity that was even more pronounced than the one of T cells of the identical phenotype isolated from control mice (Figure 31). This finding could have been brought about by either a T cell intrinsic enhancement of the suppressive ability of $CD4^{+}CD25^{+}$ T cells from $TEC^{smad4-/-}$ mice or, alternatively, by an increased frequency of T cells with suppressor function within the population of $CD4^{+}CD25^{+}$ T cells. The first assumption is in concert with the finding that $CD4^{+}CD25^{+}$ T cells that underwent homeostatic proliferation have increased suppressive capacity [211].

B.4.5.3.4.3 Relative increase of $CD103^{+}$ cells among $CD4^{+}CD25^{+}TCR^{hi}$ T cells in $TEC^{smad4-/-}$ mice

It is the prevalent view that the population of $CD4^{+}CD25^{+}$ T cells contains cells that exert a suppressive function. However, the combination of these two markers or any other cell surface molecules is not exclusively specific for T_{REG} cells [97]. Independent of this lack of a diagnostic cell surface marker for T_{REG} cells, several studies have described T cell subpopulations with regulatory functions [161, 213-217]. Among the many candidate markers described, CD103 appeared promising to further subdivide $CD4^{+}CD25^{+}$ T cells with a suppressive function [161]. Analysis of CD103 expression on $CD4^{+}CD25^{+}$ peripheral T cells revealed a substantial relative and absolute increase of $CD103^{+}$ T cells among peripheral T cells of $TEC^{smad4-/-}$ mice when compared to control littermates (Figure 32). This result fitted well with the observation of increased *in vitro* suppression by the $CD4^{+}CD25^{+}$ T cells of $TEC^{smad4-/-}$ mice and would argue that on a per cell basis $CD4^{+}CD25^{+}CD103^{+}$ T cells from control and $TEC^{smad4-/-}$ mice exhibit the same suppressive activity.

The thymic production $CD4^{+}CD25^{+}CD103^{+}$ T cells needs to be determined as CD103 has been implicated in thymocyte development [218]. Moreover, peripheral production of $CD4^{+}CD25^{+}CD103^{+}$ T cells needs to be determined since T_{REG} cells can also be induced in the periphery [219-221]. Furthermore, to determine whether the relative increase of $CD4^{+}CD25^{+}CD103^{+}$ T cells in $TEC^{smad4-/-}$ mice is a consequence of T cell redistribution rather than increased production, the relative distribution of $CD4^{+}CD25^{+}CD103^{+}$ T cells in various LN, i.e. cervical, axillary, inguinal and mesenteric needs to be compared in control and $TEC^{smad4-/-}$ mice. Since $CD4^{+}CD25^{+}CD103^{+}$ are enriched in gut-associated lymphoid tissue

[222], it could be speculated that $CD4^+CD25^+CD103^+$ T cells are recruited to non-gut-associated tissues in $TEC^{smad4-/-}$ mice.

B.4.5.3.4.4 Increased *in vitro* suppression of naïve $CD4^+$ T cells by $CD4^+CD25^+CD103^+$ T cells from $TEC^{smad4-/-}$ mice

To test this conclusion formally, $CD4^+CD25^+CD103^+$ T cells were purified from $TEC^{smad4-/-}$ mice and control littermates and tested *in vitro* for their suppressive activity. Contrary to expectations, $CD4^+CD25^+CD103^+$ T cells from $TEC^{smad4-/-}$ mice displayed a stronger suppressive activity when compared to cells with an identical phenotype purified from control mice. While these results do not provide unequivocal evidence that the suppressive activity is increased in the population of $CD4^+CD25^+CD103^+$ T cells of $TEC^{smad4-/-}$ mice on a per cell basis, these findings fail to rule out such an explanation. Alternatively, these results could also be explained by an absolute increase of "real" T_{Reg} cells among the subpopulation of $CD4^+CD25^+CD103^+$ T cells. To assess unequivocally the frequency and absolute number of a specific subpopulation of suppressor T cells in $TEC^{smad4-/-}$ mice, the expression of FoxP3 among the subpopulations described will be necessary. FoxP3 is at present the only intracellular marker that defines suppressor T cells [96, 97]. Furthermore, the number of peripheral T cells seems to be influenced by the amount of the FoxP3 protein in peripheral T cells as transgenic FoxP3 overexpression resulted in lymphopenia [223].

Collectively, $TEC^{smad4-/-}$ mice contained elevated numbers of T cells with increased suppressive activity. The increased suppressive potential of $CD4^+CD25^+CD103^+$ T cells in $TEC^{smad4-/-}$ mice when compared to the T cells of the same phenotype from control mice can be estimated at about 8-fold as there were 4-fold more $CD4^+CD25^+CD103^+$ T cells in relation to non-regulatory T cells than normal and on a per cell basis this population exerted about 2-fold higher suppressive activity. As $CD4^+CD25^+$ regulate LIP [224-226], T_{Reg} cells could limit LIP in $TEC^{smad4-/-}$ mice preventing a correction of the lymphopenia. These findings support hypothesis 4.

B.4.5.3.5 Hypothesis 5

Inability to respond to homeostatic signals

B.4.5.3.5 Purified T cells from TEC$^{smad4-/-}$ mice proliferate at least equally well as control T cells in lymphopenic hosts

A puzzling question remained to be answered and that is why do naïve T cells upon adoptive transfer proliferate in both, control and TEC$^{smad4-/-}$ hosts but the endogenous T cells fail to do so? If the quantitative and qualitative differences of T_{Reg} cells in TEC$^{smad4-/-}$ were sufficient to explain the lymphopenia, it would be expected that adoptively transferred wildtype T cells would also be prevented from undergoing proliferation, which was obviously not the case (Figures 26-28). T cells in TEC$^{smad4-/-}$ mice fulfill the known molecular requirements for LIP, since they expressed normal levels (data not shown) of functional TCR (Figure 29) and normal cell surface concentrations of the IL-7 receptor α chain (CD127) (data not shown). It could be speculated, however, that T cells from TEC$^{smad4-/-}$ mice fail to sense signals for LIP, since these require low affinity peptide-MHC-TCR interactions which cannot be tested with anti-CD3 stimulation *in vitro*. For this reason, an *in vivo* proliferation assay was performed to compare the proliferative capacity of CD4^{+} and CD8^{+} T cells from control and TEC$^{smad4-/-}$ animals, respectively, by direct competition in a lymphopenic host. When transferred into Rag2$^{-/-}$ hosts, naïve T cells from TEC$^{smad4-/-}$ donors underwent LIP at least equally well as T cells from control donors (data not shown). This result clearly demonstrated, that T cells from TEC$^{smad4-/-}$ mice were able to sense lymphopenia and were able to proliferate *in vivo*. Thus, hypothesis 5 was refuted.

This finding left, however, the question unanswered, why T cells from TEC$^{smad4-/-}$ were able to proliferate in a lymphopenic host but failed to do so in TEC$^{smad4-/-}$ mice. It could be speculated that the discrepancy was a consequence of the applied experimental approach. In TEC$^{smad4-/-}$ mice, naïve and CD4^{+}CD25^{+}CD103^{+} T cells are in a steady-state. Possibly, naïve T cells in TEC$^{smad4-/-}$ mice are in physical contact with and thus controlled by T_{Reg} cells. Therefore, adoptively transferred T cells might escape this suppressive effect since they are injected intravenously where direct suppression by T_{Reg} cells is less likely to occur while other signals such as potentially increased IL-7 levels should be present, leading to the initiation of LIP. Such an assumption is supported by the finding that the maintenance of relative proportions of naïve to T_{Reg} cells is important to control peripheral T cell numbers [159]. To test this hypothesis, naïve T cells from TEC$^{smad4-/-}$ mice need to be isolated for subsequent adoptive transfer into control and back into TEC$^{smad4-/-}$ mice. If LIP is prevented by suppressive T cells in TEC$^{smad4-/-}$ mice in a steady-state situation, it can be expected that purified naïve T cells

from TEC$^{smad4-/-}$ transferred back into TEC$^{smad4-/-}$ hosts will proliferate.

B.4.5.3.6 Hypothesis 6

Increased cell death of peripheral T cells in TEC$^{smad4-/-}$ mice

Reduced survival of naïve T cells that have matured in a TEC$^{smad4-/-}$ thymic microenvironment could contribute to the state of lymphopenia. The relative increase of regulatory T cells would imply that CD4$^+$CD25$^+$ T cells are less and CD4$^+$CD25$^+$CD103$^+$ T cells are not dependent on survival signals imprinted by Smad4-deficient TECs. As initially discussed (see B.4.3.1), due to the discrepancy of the profoundly reduced thymic cellularity and the low ratio of recombined to unrecombined Smad4 in TEC$^{smad4-/-}$ thymi, it could be speculated that TEC$^{smad4-/-}$ thymi are enriched for TECs with a silent *foxn1* gene. This might explain the increased frequency of T_{Reg} to naïve T cells if FoxN1 expressing TECs imprint naïve but not T_{REG} T cells for survival before their thymic exit. To address this point, *in vivo* survival of naïve and regulatory T cells purified from control and TEC$^{smad4-/-}$ mice will need to be compared. These experiments will have to be carried out in control, TEC$^{smad4-/-}$ and Rag2$^{-/-}$ hosts to test whether the number of T cells influences the survival of the transferred T cells.

B.4.5.4 Abnormal Vβ usage in peripheral T cells from TEC$^{smad4-/-}$ mice

As discussed (see B.4.4.4) the small difference of Vβ6 usage by the TCRs of CD4 SP and Vβ12 usage by the TCRs of CD8 SP thymocytes of TEC$^{smad4-/-}$ mice could have accumulated in aged mice. Indeed, Vβ12 was overrepresented in TCRs of CD8$^+$ T cells of TEC$^{smad4-/-}$ mice aged nine months when compared to control mice (Figure 33). Low thymic output results in a shifted Vβ usage by peripheral T cells [227]. Furthermore, mild lymphopenia leads to the establishment of an oligoclonal T cell repertoire [228] since only about 10% of all TCR specificities proliferate during LIP [159]. It is likely, that the shift of Vβ12 usage by TCRs of CD8$^+$ T cells was a consequence of the shifted and low thymic output and the persistent lymphopenia. Interestingly, TCE in aged mice occurs only in CD8$^+$ but not in CD4$^+$ T cells [202]. Thus, if the usage of other Vβ chains is as well changed in aged TEC$^{smad4-/-}$ mice when compared to control mice, TEC$^{smad4-/-}$ mice could constitute a model for premature thymic aging.

B.4.5.5 Regular effector functions of T cells of TEC$^{smad4-/-}$ mice

Thymocyte maturation was grossly normal in TEC$^{smad4-/-}$ mice while the peripheral T cell pool was significantly affected. To further assess the peripheral T cells, the effector functions of T cells of TEC$^{smad4-/-}$ mice were characterized by testing 1) the ability to provide T cell help

to B cells, 2) by assaying the capacity to induce colitis in an adoptive transfer model and 3) by investigating the ability to reject H-2 mismatched skin transplants.

T cells from $TEC^{smad4-/-}$ were able to provide B cell help for immunoglobulin class switch as efficiently as T cells from control mice. Furthermore, disease in the colitis model was as efficiently induced by T cells from $TEC^{smad4-/-}$ as by T cells from control mice. This finding suggested that T cells from $TEC^{smad4-/-}$ were able to home to the gut and to efficiently exert effector functions in the colon. Finally, T cells from $TEC^{smad4-/-}$ were able to reject H-2 mismatched skin with the same efficiency as control T cells.

Collectively these data provide evidence, that T cells from $TEC^{smad4-/-}$ mice displayed normal effector functions.

Conclusions

- Homozygous aire-cre mice can serve as a model to investigate the human autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome.
- Aire is expressed during embryonic development before the formation of a thymus
- Aire is not constantly expressed by medullary thymic epithelial cells
- Aire-cre mice cannot be used to specifically activate transgenes *in vivo* in aire expressing cells by conventional mouse breeding. Alternative strategies need to be applied.
- Smad4 is required for normal thymic epithelial cell development. It appeared, that Smad4 was required in thymic epithelial cells for proliferation rather than differentiation.
- Thymic epithelial cell-specific Smad4 deficiency has profound effects on peripheral T cell biology.

Animals, materials & methods

If not indicated otherwise, general techniques were performed according to [229] [230] and [231].

5.1 Molecular Biology

5.1.1 Bacterial Transformation

In order to transform bacteria, competent cells were thawed on ice, plasmids were then incubated with competent cells for 30`on ice. Routinely, 100µl of DH5α E. coli bacteria were used if not indicated otherwise. For cloning with methylation sensitive restriction enzymes, dam- bacteria were used.
The suspension was then transferred into a waterbath at 42°C for 30`` for heat shock transformation. Samples were immediately put on ice for 2’. Then, 100µl prewarmed (37°C) SOC medium was added and the tubes were incubated for 1h at 37°C, shaking with 225 rpm in a bacteria shaker.
During the incubation time, plates containing 30ml LB were prewarmed and dried. If blue/ white screening was used plates were prepared with 35µl X-Gal (40mg/ml in DMSO from Promega) and 20µl IPTG.
100µl bacteria were streaked onto LB plates, containing the appropriate antibiotic (Ampicillin, Kanamycin, Sigma, Switzerland) the rest stored at 4°C.
Competent bacteria were either bought or made competent in-house according to general protocols.
DH5α E.coli were used for routine cloning, sure2 for larger plasmids and plasmids with multiple loxP sites, grown at 30°C overnight. For extra large plasmids XL10-Gold Ultracompetent Bacteria were used (Stratagene).

5.1.2 Growth conditions for bacteria and Plasmid extractions

Bacteria were grown in disposable 15ml polypropylene tubes (Falcon 2059) for low amounts of DNA and in 500ml glass Erlenmeyer flask. LB broth was supplemented with the appropriate antibiotic. Bacterial colonies from LB plates or glycerol stocks were used to inoculate the LB broth. Bacteria were grown for 12-16 h at 30°C or 37°C, respectively, depending on the plasmid and bacteria in an incubator shaking at 220rpm.
30° C was used for plasmids larger than 7kb
Ampicillin: 50mg/ml stock (use 1:1000)
Kanamycin: 25mg/ml stock (use 1:1000)

5.1.3 DNA extraction from bacterial colonies

DNA extraction was performed according to the manufacturer’s guidelines using plasmid extraction kits Miniprep (Macherey Nagel) or Maxiprep extraction kits (Qiagen or Macherey Nagel).

5.1.4 Agarose gel electrophoresis

Agarose gels were prepared with TAE and 1%o ethidium bromide.

5.1.5 Restriction endonuclease digestions

Restriction endonucleases were bought either from NEB (New England biolabs) or from MBI fermentas. Digestions were carried out as recommended by the manufacturer. In general, a total volume of 30μl was used and incubation time varied between 30' – 60' if not indicated otherwise.
Double digestions were carried out according to the manufacturer. If buffers for the two restriction enzymes could not be matched, subsequent digestions were performed with intermediate purification of the DNA fragments using MinElute PCR purifications kits (Qiagen).

5.1.6 Dephosphorylation of DNA

Heat labile shrimp alkaline phosphatase (New England Biolabs) was used according to the manufacturer`s recommendations to dephosphorylate cloning vectors to reduce self-ligation. After dephosphorylation the enzyme was heat inactivated (incubation at 65°C for 15`).

5.1.7 Dam- competent cells

For cloning with methylation sensitive enzymes, dam – bacteria were used.

5.1.8 Ligation reactions

Ligation reactions were carried out with 50-100ng total DNA in a final volume of 10-20μl at a molar ratio of 1:1 to 1:3 vector:insert. "Sticky ends" were ligated for 1h at RT with 1μl of T4 ligase (Sigma). 5μl of the reaction mix were transformed by the heat shock procedure. The rest was incubated at 16°C overnight. Blunt end ligations were carried out at 16°C overnight. For ligations of vector and insert cut with the same restriction enzyme, the vector backbone was dephosphorylated with alkaline shrimp phosphatase (New England Biolabs) to avoid self-ligation of the vector.

5.1.9 RNA isolation

RNA isolation was carried out in an RNase free environement wearing disposable gloves to avoid contamination of the samples with RNases. Frozen thymic tissue or frozen cell suspensions were suspended in 1 ml TRI-reagent (Molecular Research Center Inc.) and homogenized with a Polytron homogenizer (Kinematica PT 1200) for approximately 30 sec with increasing speed and all samples were incubated 10 min at room temperature. To extract the aquous phase, 100μl (1:10) bromochloropropanol (Molecular Research Center Inc.) was added. Samples were shaken for 10 sec and incubated for another 10 min at room temperature. The samples were centrifuged at 14000rpm in a tabletop microcentrifuge (Eppendorf) at 4°C for 10 min. The aquous phase was transferred to another Eppendorf tube and precipitated for 1 hour at room temperature with an equal volume of isopropanol (Sigma, Buchs). The samples were centrifuged again at 14000 rpm at 4°C for 30 min. The liquid was carefully aspirated and the pellet was resuspended

in 1 ml freshly made 75% ethanol, vortexed and centrifuged at top speed at 4°C for 10 min. The liquid was aspirated as much as possible and the pellet was air dried. The pellet was then dissolved in 30 μl highly pure H_2O and the total RNA concentration was measured with a Gene-Quant machine II (Pharmacia) (ratio 260/280 nm) and on a 1% agarose gel.

5.1.10 cDNA-synthesis from total RNA

To obtain cDNA, total RNA samples (4 μg) were mixed with 18 μl H_20 containing 500 μM dNTP, 10 mM DTT, 1 μl RNase free DNAse I and 1 μl of 1x1st strand buffer. This mixture was incubated for 30 min at 37°C. After the incubation, the dT_{20} and random hexamer primers were added at a final concentration of 500 nM and the mixture was heated for 5 min at 70°C. The samples were then quickly centrifuged at room temperature. Thereafter, 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was added and the samples were incubated at 42°C for 1 h. The reaction was quenched for 5 min at 95°C and then the samples were diluted in aliquots at a concentration of 20 ng/μl and stored at –20°C. All buffers are from Gibco BRL, Basel, CH.

5.1.11 PCR

5.1.11.1 PCR primer design

Oligonucleotides to be used as primers for PCR reactions or sequencing reactions and adaptor molecules for molecular cloning were designed in MacVector® and Primer Express® for qPCR. Where necessary, primers were hand chosen and verified using in silico PCR. Selected primers were blasted using NCBI Blast (see databases) to ascertain species specificity and to exclude that the primers bind to a repetitive sequence. For RT-PCR, if possible, primers were chosen to be exon spanning to exclude potential genomic contamination. Intron exon boundries were either searched at LocusLink or, alternatively, determined by blasting genomic DNA versus mRNA.
PCR primers including restriction sites were designed in MacVector for the sequences necessary for annealing. Restriction enzyme recognition sites were then added by hand. Additional oligonucleotides were randomly added as required for each restriction enzyme according to the manufacturer. Care was given not to introduce unwanted translational start or stop codons.

5.1.11.2 End point PCR

For end-point PCR analysis of mRNA expression, total RNA was isolated from whole thymic tissue, freshly isolated thymic epithelial cells, established thymic epithelial cell lines or thymocyte subpopulations (as indicated) were isolated. After reverse transcription, the cDNA was amplified for 30-36 cycles.

5.1.11.3 Polymerases used

Routine PCR: Taq DNA polymerase (Sigma). For cloning: Taq / Pfu (proof reading polymerase) mix, 15:1, with Sigma's long and accurate 10x PCR buffer (Sigma). For large amplicons KOD DNA polymerase (Novagen) was used.

5.1.11.4 Reaction mix

10ng DNA cDNA or 100ng genomic DNA
1 μl primer x at 10μM
1 μl primer y at 10μM
1 μl dNTPs at 10mM
5 μl buffer 10X, Sigma
0.2 –0.5 μl Polymerase
μl H_2O up to 50 μl (or 25μl final if oil was used)

5.1.11.5 Cycle conditions for routine PCR amplifications

94°C	3`
94°C	30’`
Tann	30`’
72°C	1`

repeat step 2-4 30-36 times
final elongation: 72°C 10`

5.1.11.6 Touch Down PCR for genotyping conditional *smad4* mice

Touchdown PCR is a modification of conventional PCR that may result in a reduction of nonspecific amplification. The principle is based on an annealing temperature that is higher than the target optimum in early PCR cycles. Subsequently, the annealing temperature is decreased by 1°C every cycle or every second cycle until a specified or ‘touchdown’ annealing temperature is reached. The touchdown temperature is then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product.

94°C	4’
94°C	30’’
65°C	30’’

decrease T_{ann} by 1°C at each cycle

72°C	1’

repeat step 2-5 nine times

94°C	30’’
55°C	30’’
72°C	1’

repeat step 6-8 24 times
72°C 10’

5.1.11.7 PCR conditions for cloning of long amplicons with high fidelity polymerases

10ng DNA cDNA or 100ng genomic DNA
1 μl primer x at 10μM
1 μl primer y at 10μM
5 μl dNTPs at 2mM
5 μl KOD buffer (Novagen)
2 μl $MgSO_4$ (25mM)
1.2 μl KOD Polymerase
μl H_2O up to 50 μl

HERC3 program:

92°C		2’
92°C		30’’
70°C		6’ (4x)
92°C		30’’
70°C	(-1)°C/cycle	30’’
70°C		6’ (4x)
92°C		30’’
55°C		30’’
68°C		6’ (+30’’/cycle) (32x)
68°C		20’

5.1.12 Purification of small DNA fragments (1-10kb)

Small DNA fragments such as PCR amplicons and digested plasmid fragments were purified using PCR purification kits (Macherey Nagel) and MinElute (Qiagen).

5.1.13 Generation of double-stranded DNA adaptor molecules

ssOligos were purchased from Thermo Hybaid, Germany, resuspended in T4 1x final ligase buffer (Sigma) at 200 μM.

Annealing:
10μl oligo x
10μl oligo y
4 μl T4 buffer (5x)
16μl H_2O

The final volume of 40μl was mixed in 0.2μl polypropylene tubes.

Annealing program:
95°C 4’
cool down to 20°C at 1°C/min.
freeze at –20°C

Concentrations were calculated based on the molecular weight. Annealed adaptors were diluted with milliQ H_2O to 1-10ng/μl in 4 ml total volume. The size of the annealed adaptors was checked on a 3% agarose gel.

5.1.14 DNA Sequencing

Sequencing reactions were initially carried out by Microsynth GmbH, Balgach, Switzerland. Trace files obtained from Microsynth were checked for quality and reliability by inspecting the obtained profiles of fluorescence peaks. Analysis was carried out using sequence alignments, Blast searches and using SeqMan and 4Peaks for reassembly of single sequencing reactions.
Alternatively, in-house sequencing was performed on an ABI sequencing machine according to the manufacturer's recommendations.
Trace files were analyzed using SeqMan and 4Peaks. (see software list)

5.1.15 Southern blotting

5.1.15.1 Gel and Transfer to Membrane

Genomic DNA was digested overnight. 30µg of digested DNA were separated slowly overnight on a 0.8% agarose gel without Ethidium Bromide. The next day, the migration was documented by PI staining. After washing in ddH2O the gel was depurinated in 0.25N HCl for 15' with subsequently rinsing the gel twice with ddH2O. DNA was then denatured in 0.5N NaOH for 30'. Then, the denatured, separated DNA was transfered in a BioRad Vacuum Blotter Model 785 onto a Hybond-N+ Membrane (Amersham) for 90 minutes at 5 inches Hg. Then the membrane was soaked twice in SSC for 5 minutes and air dried between two sheets of filter paper.

5.1.15.2 Prehybridization of the membrane

The membrane was transferred in a hybridization bottle and Church buffer was added at 100µl per cm^2. Pre-hybridization was performed for at least one hour at 65˚C in a rotating hybridizing oven.

5.1.15.3 Labeling of the Probe

30ng PCR product was boiled for 10' and then quickly cooled down on ice. 20µl ddH2O and 40µCi dCTP [$\alpha^{32}P$] were incubated with High Prime Roche (cat#1585592) at 37˚C for 15'. Then 2µl 0.2M EDTA and 80µl TE were added. The labeled probe was column purified using Micro Bio-Spin 6 columns (BioRad#732-6221) according to the manufacturer.
^{32}P Incorporation was measured by scintillation counting using IRGA-SAFE PLUS, (Packard #6013249).

100µl of labeled probe, 25 µl ddH2O, 5µl Cot-1 DNA 10µg/µl (Invitrogen #18440-016) (or alternatively salmon sperm), 50µl 20x SSC and 20µl 1% SDS were boiled for 5 min, then kept at 65˚C, 20min minimum.
The labeled probe was added to the prehybridized membrane.
Hybridization was carried out over night at 65˚C in a rotating hybridizing oven.

5.1.15.4 Washes

After incubation overnight, membranes were washed at 65˚C 2x 30' with wash solution 1, then 2x 15' with wash solution 2. Radioactivity was visualized using a Phosphoimager.

5.1.15.5 Stripping Probes from Nylon Membranes

For stripping probes from the membranes, 100-200ml 0.4M NaOH were added and the membrane was incubated for 30’ rotating at 42˚C. Then the membrane was washed subsequently in 0.1xSSC, 0.1%SDS, 0.2M tris-Cl (pH7.6) for 30’ at 42˚C.

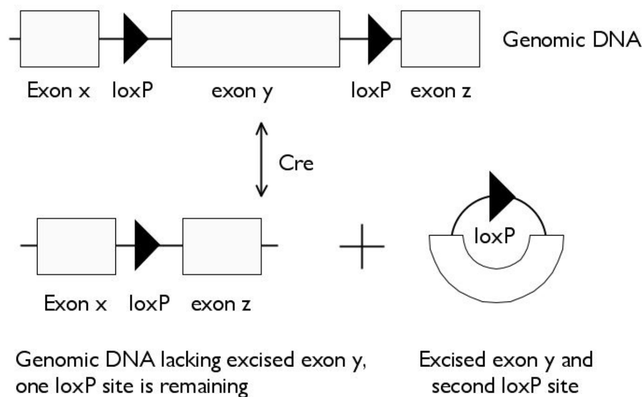
5.2 Generation of gene targeted aire-cre mice

5.2.1 Conditional gene targeting: the cre/loxP system

The development of genetic models for human diseases and the functional analysis of specific genes that are essential for embryonic development have necessitated the design of new genetic tools as simple gene targeting often leads to early embryonic lethality which in turn renders an analysis of the role of the targeted gene at later developmental stages impossible. The specific ablation of genes in the entire embryo or, alternatively, in only a restricted subpopulation of cells at later stages of development (even as late as adulthood) has now become possible due to the methodology of somatic cell mutagenesis and inducible gene ablation [183]. For this purpose, the Cre recombinase of bacteriophage P1, has proven to be an invaluable tool. This enzyme directs recombination of DNA between two specific nucleotide sequences designated as “locus of crossover”, or loxP. This recognition sequence comprises two 13 base pairs (bp) palindromes separated by an asymmetric 8 bp core. The Cre recombinase catalyses DNA strand exchange between two aligned loxP recombination sites and consequently results in the deletion of the intervening stretch of DNA. (Fig. 35) Thus, application of this technique in mice allows for the deletion of specific DNA sequences in a selected population of cells and at a desired point in time.

5.2.1.1 Loss of gene function

In order to delete specific sequences of genomic DNA by the use of Cre recombinase, it is first required to introduce the loxP recombination sites into those sequences of the genome where the deletion should eventually occur. Since this mutational change is silent, it does not affect the normal regulation and transcription of the modified gene. The loxP sites are introduced into the genome of embryonic stem (ES) cells using the methodology of homologous recombination. For the introduction of a genetic mutation into ES cells, a vector is first generated that contains two loxP recombination sites flanking the DNA sequences containing the locus to be deleted. This core construct is again flanked by DNA sequences homologous to the gene to be targeted. Next, the vector DNA is linearized and introduced by electroporation into ES cells, which bear the full potential to generate a viable and reproductively proficient animal. The ES



cells are then clonally expanded and tested by Southern blotting of genomic DNA for the correct occurrence of homologous recombination of the targeted locus. Finally, ES cells with proper targeting will be injected into blastocysts to generate genetically chimeric animals that are later bred to homozygosity for the introduced loxP recombination sites [149].

Figure 35: *The principal of the cre loxP system; shown is the excision of an exon from a genomic locus*

5.2.1.2 Gain of gene function

Tissue specific gene recombination cannot only be used to inactivate a specific gene by genomic deletion. The basic concepts can also be applied to induce site specific (and if desired temporally controlled) gene expression. To this end, recombinant DNA technology takes advantage of creating an artificial gene (designated transgene) which can only be transcribed if a genetic stop signal is removed 5' to its transcription initiation site. This stop cassette is flanked by loxP sites. The transgene is injected in the nucleus of a fertilized egg and will subsequently integrate into the genome of the cell. Validated transgenic mice will then be bred with animals expressing Cre under a tissue specific promoter. Upon Cre expression, the loxP sites are recombined and the stop cassette is removed which in turn leads to the transcription of the introduced gene [149].

For tissue restricted recombination of loxP sites the recombinase Cre has to be expressed in a manner limited to the cells of interest. This is best achieved using tissue specific promoters regulating Cre expression. Since only a few tissue specific promoters are known that allow for transgenic use so that an integration-independent but tissue-specific expression is maintained, many scientists have again taken advantage of the technology of homologous recombination to attain tissue-restricted Cre expression. To this end, the coding sequence for the translational start signal (most frequently the first exon) of a tissue specific gene is replaced by the recombinase Cre so that its expression is controlled identically to that of the replaced product. This method is referred to as “knock-in” and assures that all regulatory sequences which control the correct spatial and temporal expression of a given gene also apply in the instance that Cre has been “knocked-into” that locus.

5.2.2 Monitoring in vivo Cre activity using "reporter mice"

To verify the correct functioning of a Cre knock-in mice that express this recombinase can be crossed with animals that bear a transgene which is only transcribed and detectable as a protein if the "floxed" stop cassette has successfully been removed. Such reporter mice contain a ubiquitously expressed reporter transgene whose product can easily be detected. In the absence of Cre, a genetic element placed 5' of the reporter transgene prevents its transcription. This so called "stop cassette" is flanked by loxP sites and is removed from the genome as Cre becomes active. As reporter genes the cDNAs for green fluorescent protein (GFP) and ß-galactosidase (lacZ) are commonly used. This assemblage of genetic elements provides a functional in vivo assay for Cre activity [154, 232].

5.2.3 Cloning of the aire-cre targeting construct

First plasmid pIRES-hrGFP-2a (Invitrogen, Switzerland) (designated p19, Annex I) was modified for subsequent cloning steps. The Mlu I restriction site at nucleotide position 738 within the triple hemagglutinin (3xHA) tag was removed by replacing a XhoI / BsiWI fragment of the original vector by an adaptor molecule (#1395/#1396 see Annex III) containing a point mutation that destroys the MluI site while leaving the amino acid sequence of the 3xHA tag unchanged. The resulting plasmid was designated p36 (Annex I). Removal of the undesired MluI site was monitored using enzymatic restriction analysis and direct sequencing. Then, the complete open reading frame of the bacteriophage P1 recombinase cre was released as a 1.2kb fragment from plasmid NLS-cre (p25) using MluI [233]. The 1.2kb fragment carrying the nuclear localization signal of the SV40 large T-antigen and the coding sequence of cre including the translation initiation codon (ATG) and the termination codon (TAG) but without the poly A signal was gel purified, blunt-polished using T4 polymerase and then ligated into SmaI of p36 resulting in p67. Correct orientation and insertion of a single copy of the fragment was verified by asymmetric restriction enzyme digestion and then confirmed by direct DNA sequencing. Next, a 3.5kb 3' arm of homology of the murine aire locus was amplified by PCR from genomic DNA of Ola129 ES cells (a kind gift of Dr. R. Fässler) using KOD polymerase (Novagen) and the HERC 3 PCR program for cycling conditions. PCR primers contained mismatching overhangs to include NotI (#1635) and SacII (#1636) restriction sites. The resulting PCR amplicon was digested using NotI and SacII, gel purified and then ligated into plasmid loxPFrtNeoDuoNeoloxPFrt (p72) [188] digested with NotI and SgrAI. In addition to the 3.5kb arm of homology and the linearized p72 an adaptor molecule hybridized from oligonucleotides #1637 and #1638 (containing restriction sites for SacII-MluI-ScaI-SgrAI) was included in the ligation reaction resulting in p75 (Annex I). The adaptor molecule was used to prepare subsequent cloning steps and to include a ScaI site for southern blotting. The nucleotide sequence of the adaptor molecule was designed such that the desired restriction sites could be introduced, containing where necessary random spacer nucleotides while avoiding the generation of translational start or stop codons. This cloning step removed one loxP site of p72 while the second loxP site remained in place, flanked by PacI and AscI restriction sites. Next, p75 was digested with SacII and MluI and a NLS-CRE-3xHA-IRES-hrGFP-SV40pA fragment released from p67 using SacII and MluI was ligated to create p77. Finally, a 4.4kb arm of homology of the murine aire locus was amplified by PCR from genomic DNA from Ola129 ES cells including mismatching

overhangs for AscI and PacI using primers #1633 and #1634. The PCR amplicon was digested using AscI and PacI, gel purified and ligated into p77 resulting in p79. The final targeting construct was linearized using AscI for electroporation into ES cells.

5.2.4 Gene targeting

Gene targeting was performed in Munich at the Max Planck Institute for Biochemistry in the laboratory of Dr. R. Faessler. Electroporation of ES cells, selection and expansion of targeted ES cells and production of chimeric mice was performed according to [234]. Briefly, 5×10^7 ES cells were electroporated with 100µg of AscI linearized targeting construct and then plated onto irradiated feeder cells. 24h after electroporation positive selection was initiated using 500µg/ml G418. Resistant ES clones were manually picked using a stereomicroscope in a laminar flow hood after 6-8 days. 360 picked colonies were shortly trypsinized and then cultured on freshly plated feeder cells with ES medium and G418. 3-5 days after picking the expanded clones were washed, shortly trypsinized, resuspended in ice-cold freezing medium and then split for freezing at –80°C and the other half was used for further culture. After expansion of cell numbers, DNA was extracted and analyzed by southern blotting. All clones were screened using the 5' external probe after ScaI digestion of genomic DNA (see Figure 6). Confirmation was achieved using the 3' external probe after Hind III digestion. Two internal probes were used to avoid clones with integration of several copies of the targeting construct.

5.2.5 Production of chimeric mice and germline transmission

Five correctly targeted clones (#1, #103, #253, #292 and #336) were thawed and expanded for blastocyst injections. Blastocysts were from 3.5day pregnant C57bl/6J mice. Based on morphology after expansion, ES clones #103, #292 and #336 were injected into blastocysts at about 12 ES cells/blastocyst. Microinjected blastocysts were then transferred into the uterus of pseudopregnant BDF1 females. 28 chimeras were obtained representing animals from all 3 injected ES clones. Chimeras were shipped from Munich to Basel and bred to C57bl/6 mice for germline transmission (data not shown).

5.3 Histology

5.3.1 Tissue embedding

Freshly dissected organs were embedded in OCT compound (Medite, Switzerland) in Tissue-Tek Cryomolds (Miles Inc., Elkhart, USA). Tissues were frozen in methyl-butane cooled with dry ice and then stored at –70°C.

5.3.2 HE staining

OCT (Medite, Switzerland) embedded tissues were cut at 5-12 µm thickness using a cryostat. Tissues were air dried for at least 4 h at RT, then either frozen at –70°C or processed directly. Sections were fixed in Delaunay's fixation solution for 1', then rehydrated in a series of ethanol dilutions for 1' each: 100% ethanol, 96% ethanol, 70% ethanol, 50% ethanol and finally H_2O. Tissues were then stained with Meyer's Hämalaun for 2'. Afterwards they were washed with warm H_2O 3 times 1' before staining with 1% Erythrosin. After washing with H_2O for 1', sections were dehydrated in a series of ethanol solutions: 50% ethanol, 70% ethanol, 96% ethanol for 1' each, then ethanol 100% for 2'.

Slides were then air dried before mounting cover slips with Pertex.

5.3.3 Immunohistochemistry

For immunohistochemical detection of indicated proteins, thymi were isolated and embedded in OCT (Tissue-Tek, Sakura Finetec, Netherlands). Frozen samples were cut at 5-12 μm thickness, fixed with 4% paraformaldehyde/PBS and blocked with Biotin and Avidin. Endogenous peroxidase activity was blocked with H_2O_2. Sections were then incubated with primary antibodies for one hour at RT or at 4°C overnight. After washing 3x 5’, sections were either incubated with secondary antibody for 30’ at RT or with streptavidin-conjugated horseradish peroxidase. Sections were then incubated with AEC and counterstained with hemalaun.
Panels of antibodies and lectins have previously been used to characterize different thymic epithelial cell subsets. In brief, thymic epithelial cell subsets were identified using combinations of anti-cytokeratin 18 moAb and UEA-1 lectin, polyclonal anti-cytokeratin 5 antibody and the epithelial cell-specific MTS-10 antibody. The particular staining protocol was adapted from Klug and coworkers. Two- and three-color immunofluorescent sections were analyzed using a confocal microscope (Carl-Zeiss AG, Feldbach. Switzerland).

5.3.4 β-galactosidase (LacZ) staining; protocol modified from [235]

5.3.4.1 Isolation of tissue/embryos

For embryos E10.5 whole mount.
For embryos E10.5 - E12.5 cut into thirds.
Greater than E12.5 dissect individual organs.
For adult and 18.5 PO tissues, cut organs to size of » E 10.5
Wash in PBS

5.3.4.2 Fixation of tissues

Tissue sections were fixed in LacZ fix according to the recommended time in [235]. For whole mount lacZ stainings E12.5 embryos were fixed for 2.5 h gently shaking at 4°C.

Sections or embryos were then washed three times in PBS for 5’ each time.

5.3.4.3 X-gal Tissue Staining

Whole Mount Tissues

1. Incubate in LacZ stain at 30°C overnight
2. Wash in PBS 5min X 3
3. Post fix in 0.2% glutaraldehyde/PBS overnight at 4°C
4. Store in NaAzide/PBS or process for cryosectioning or paraffin sectioning

All other Tissues:
Tissue was dehydrated overnight in 20% sucrose/PBS at 4°C. When the tissue was equilibrated it was embedded in OCT compound (see above). Cryosections were done using a cryostat at 8-16μm. Sections were then air dried overnight and either processed the following day or frozen at –70°C wrapped in aluminum foil.

Tissues were fixed in LacZFix for 10min at room temperature before washing in LacZ Wash Buffer for 3x 5’. LacZ staining was performed overnight at 30°C and then washed 3x 5’ in PBS. Sections were then post-fixed in 0.2% glutaraldehyde/PBS at room temperature for 10’ and then washed in PBS for 3x 5’. Tissue was rinsed in water for 5’ and then counterstained in nuclear fast red (NFR, 0.1% NFR in 5% $Al_2(SO_4)_3$ sol.) for 5’ at RT and washed in H_2O for 5’. Sections were mounted in permount.

5.4 Cell Biology

5.4.1 Cell culture of TEC, EL-4, HEK 293, phoenix and NIH3T3 cells

The medullary thymic epithelial cell line mThymic epithelial cell 2-3 was a gift from Dr. M. Kasai (Tokyo, Japan). TEC were cultured in TEC medium in the absence of antibiotics at 37°C 5%CO_2.

5.4.2 Transfection of cells

Cells were transfected at an approximate density of 50-70% confluency using FuGene6 (Roche diagnostics) transfection formulation. The procedure was performed as recommended by the manufacturer. Briefly, DNA was incubated for 15 `at RT with FuGene6. The DNA/transfection reagent mixture was then evenly distributed on the cells by dropwise adding them to the cell culture dishes and gently swirling.

5.5 Mice

5.5.1 Genetic background of cells and mice used for the generation of Aire-cre gene targeted mice

ES cells: 129Sv
Blastocysts: C57bl/6J
Foster mothers: BDF1
Vasectomized males: FVB
Germline: Chimeras were crossed to C57bl/6 mice to obtain germline transmission.

Initial experiments with Aire-cre and *Smad4* conditional knock-out mice were performed on a mixed background 129Sv x C57bl/6. Aire-cre mice were backcrossed onto C57bl/6 and Balb/c backgrounds. If backcrossed animals were used, the number of backcross generations is indicated.

ROSA26 lacZ indicator

B6;129-Gt(ROSA)26Sor<tm1Sho> were purchased from Jackson, stock number 003504

Flp Deleter mice

129S4/SvJaeSor-Gt(ROSA)26Sor$^{tm1(FLP1)Dym/J}$ were purchased from Jackson, stock number 003946 [147]

Cre deleter mice

N6-Del, a kind gift of Dr. M. van den Broek, Zürich [236].

Rag2$^{-/-}$ mice

C57bl/6 Rag2$^{-/-}$

OT-I transgenic Rag2$^{-/-}$ mice

OT-I Rag2$^{-/-}$, a kind gift of Dr. E. Palmer, Basel [237].

Smarta1 mice

BM from smarta1 mice [238] was a kind gift from R.M. Zinkernagel.

C57bl/6 CD45.1 (Ly5.1)

B6.SJL-*Ptprc*^{a}*Pep3*^{b}*/*BoyJ congenic mice were purchased from Jackson

C57bl/6 mice

C57bl/6 CD45.2 (Ly5.2) congenic mice were purchased from Jackson

Balb/c mice

Balb/c mice were purchased from IFFA Credo, France

Smad4 conditional knock-out mice

Smad4 conditional ko mice, a kind gift of Dr. X. Deng, NIH, USA [148] are of a mixed genetic background (C57bl/6 x SvEv129 x FVB); chimeras were crossed to Black Swiss mice for germline transmission; The breeding stock of conditional *smad4* mice was a mixture of CD45.1 and CD45.2. For bone marrow chimeras and transfer experiments, a pure *smad4* lox/lox CD45.1/CD45.1 x smad4 lox/lox FoxN1-cre CD45.1/CD45.1 breeding as well as a pure *smad4* lox/lox CD45.2/CD45.2 x smad4 lox/lox FoxN1-cre CD45.2/CD45.2 breeding was established.

FoxN1-cre mice

FoxN1-cre mice were produced as PAC transgenic animals. (Zuklys S. & Holländer G. unpublished). Briefly, the cds for iCre (for eukaryotes codon usage optimized version of the Bacteriophage p1 cre recombinase, [239] a kind gift of Dr. E. Casanova was inserted into PAC clone RPCI21-436p24. Successfully modified BACs were purified and injected into C57bl/6 x BDF1 pronuclei by the Transgenic Mouse Core Facility (TCMF) of the Biozentrum at the University of Basel. Offspring was screened for transgene integration by PCR using primers #1621/#1622. Positive founder mice were confirmed by southern blot analysis. FoxN1-cre mice were bred to homozygous smad4 conditional mice resulting

in a mixed genetic background of 129Sv x C57bl/6.

5.5.2 Housing and breeding

Mice were kept under pathogen free conditions according to the Swiss law and regulations of animal welfare. Sex and age of mice are indicated in each experiment. If not indicated otherwise, mice were between 4-10 weeks.

5.5.2.1 Aire-cre mice

Gene targeted mice were designated aire$^{CN/wt}$ (heterozygous for **C**re **N**eo(=CN)) or aire $^{CN/CN}$ (homozygous). After crossing to Flp-Deleter mice (see above) mice were designated aire $^{CNDF/wt}$ (for **C**re **N**eo **D**eleted **F**lipase) and aire $^{CNDF/CNDF}$ respectively. Deletion of the neo cassette was monitored using PCR and southern blotting. After further backcrossing –when the Flipase transgene was lost- mice were called aire $^{CND/wt}$ and aire $^{CND/CND}$. Mice were backcrossed onto C57bl/6 (H2-^{b}) and Balb/c (H-2^{d}) haplotypes for several generations. Mice used in these experiments were F1-F4. Two mouse lines derived from two independent ES clones were used and compared (Clone 292 and 336).
Reporter mice were kept as homozygous breeding stocks. Aire-cre mice were crossed to homozygous reporter mice (B6;129-Gt(ROSA)26Sor^{tm1Sho} and B6;129-Gt(ROSA)26Sor^{tm2Sho}) for analysis of cre expression and analyzed as heterozygous reporters either cre^{+} or cre^{-} littermates.

5.5.2.2 *Smad4* conditional mice

Smad4 lox/lox mice were bred to FoxN1-cre mice. *Smad4* lox/wt FoxN1-cre offspring was then crossed to *Smad4* lox/lox mice to get *Smad4* lox/lox FoxN1-cre mice. Mice were analyzed from breedings of *Smad4* lox/lox x Smad4 lox/lox FoxN1-cre, resulting in 50% cre$^{–}$ control littermates and 50% cre^{+} TEC specific smad4 knock-out mice.
Smad4 lox/lox mice were also bred to N6-Del mice to get *Smad4*$^{-/wt}$. These were bred to *Smad4* lox/lox to get *Smad4* -/lox which were then bred to *Smad4* lox/lox FoxN1-cre mice. As offspring from this breeding did not show any differences compared to breedings *smad4* lox/lox x *Smad4* lox/lox FoxN1-cre, this breeding was discontinued.

5.6 Mouse manipulations and immunological procedures

5.6.1 Time mating

For timed pregnancies one male and two females were separated in the same cage for 2 days using a grid to prevent uncontrolled mating. After 48h the grid was removed at 17h00. After 15h males were separated and females were checked for vaginal plugs. Plug positive females were assumed to be at gestational age 0.5 days.

5.6.2 Collection of mouse blood

For small amounts of blood (e.g. for repetitive blood collection), 2mm of tail tip was cut in anesthesia and blood collected. For larger amounts, tails were cut with a razor blade and blood collected. For large quantities, mice were anesthesized and blood was collected by external heart puncture.

5.6.3 Genomic DNA extraction from mouse tails

Mouse tails were cut between 1-3 weeks of age. 5 mm of tail tissue were digested in 500-600µl tail lysis buffer at 55°C for 12h in a 1.5ml tube, shaking at 1000 rpm in a Eppendorf Thermomixer Comfort shaker. Undigested tissue and bones were pelleted in a tabletop mini centrifuge (Eppendorf Centrifuge 5417 C/R) for 5’ at 13000 rpm. Supernatant was poured into an equal volume Isopropanol. Tubes were then inverted several times until DNA precipitated. DNA was fished and transferred into 300µl 70% Ethanol. After washing, DNA was pelleted at 13000 rpm for 5’. DNA was air dried and resuspended in an appropriate volume of TE. (100-300µl to yield approximately 100ng/µl). To ease resuspension, samples were heated to 55°C. DNA was stored at –20°C.

5.6.4 Genotyping

Genotyping was performed using PCR as described above. The following primer pairs and PCR conditions were used:

Aire-cre: Primers #2323/#2324/#2368:
touch down PCR
upper band = wt/wt
2 bands = cre/wt
lower band = cre/cre

Smad4 conditional allele
Primer pair #2332/#2626
Upper band 450bp (conditional allele)
Two bands (heterozygous)
Lower band 390bp (wildtype allele)
Touch down PCR, Sigma’s long and accurate PCR buffer.

FoxN1-cre (iCre): Primers #1621/#1622
standard PCR program

Smad4$^{loxP/loxP}$ mice were genotyped using oligonucleotides #2332 and #2626 for several generations. The same was done for Smad4$^{loxP/loxP}$ FoxN1-cre mice. Primers used were slightly different from the published ones. For experiments where the mouse was sacrificed, the size of the thymus was checked and compared to the genotype observed by PCR. Where the genotype did not match the thymic phenotype, DNA was reextracted and regenotyped. For experiments where cells from several individuals were pooled, the fur phenotype was checked the day before the analysis and compared to the PCR genotype and on the day of analysis, the thymic size was checked. Only animals matching all 3 criteria were included in the analysis to avoid cross-contamination of cre- with false negative cre+ and vice versa.

5.6.5 Cell separation

Freshly isolated thymocytes, thymic epithelial cells or peripheral lymphocytes were stained with the appropriate moAbs and then sorted into subpopulations with the use of

a FACSvantage cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) or a MoFlo Cell sorter (Dako Cytomation).

For thymic epithelial cell isolation, thymic lobes were separated and small cuts were made. Thymic lobes were then stirred twice in fresh IMDM-2 (IMDM + 2% FCS) for 30 min on ice. The thymic lobes were transferred in new tubes containing Collagenase IV 1mg/ml + DNAse 10µg/ml in IMDM-2 and digested at 37°C for 20 min; every 10 min the suspension was mechanically disrupted using pipette tips with decreasing diameter. After mechanical disruption of the lobes done with a Pasteur pipette with a cut-off tip, the supernatant containing thymocytes was either used for thymocyte stainings or, alternatively, discarded and fresh digestion medium was added. After three rounds of digestion all supernatant was carefully discarded and fresh trypsin was used for the last digestion round. The cell suspension was carefully washed in FACS buffer with 5 mM EDTA and filtered trough a 40 nm mesh before starting the staining procedure. Adherent cells were stained with a combination of anti-IA^b (MHC class II) and CD45 moAbs. $IA^{b+}CD45^-$ cells were sorted on a FACSvantage (Becton Dickinson, Franklin Lakes, NJ, USA).

5.6.6 Early thymic emigrants detection

Anesthetized mice were injected in one thymic lobe with 10 µl of 125 µg/ml FITC (Sigma, Buchs, CH) diluted in PBS (Stock solution 1mg/ml). 16 hours later the mice were sacrificed; thymus, lymph nodes and spleen were dissected and sinlge cell suspensions were analyzed for the presence/absence of $FITC^+$ lymphocytes with corresponding surface markers ($CD3^+$, $CD4^+$ or $CD8^+$).

5.6.7 KGF treatment

KGF treatment was performed as described previously (Rossi et al.)

Briefly, mice were injected intraperitoneally for a period of 3 subsequent days with HBSS (Hank's balanced salt solution) or recombinant human KGF (rhKGF; solubilized in HBSS) at a dose of 5 mg/kg per day. rhKGF was produced in E.coli and had an ED50 of 40.02 ng/ml (kindly provided by Amgen, Thousand Oaks, CA, USA). Analysis was performed 14 days after the last injection.

5.6.8 NIP-OVA Immunizations

Mice were immunized intraperitoneally with 100µg of Alum precipitated NIP-OVA using 1ml syringes (Primo) and 25G needles. 20µg NIP-BSA was injected subcutaneously on day 17 in the base of the tail. 100-200 µl blood was collected from tail veins at days –1, +5, +12 and +23 (FACS analysis of lymph nodes) in BD Microtainer tubes. Samples were incubated at room temperature for at least 30' and then centrifuged at 14000rpm (Eppendorf centrifuge 5417C) for 5' to separate serum from cells. Sera were frozen at –20°C for short term (weeks) storage or at –70°C for long-term (months) storage.

Mice were boosted with 20µg of Alum precipitated NIP-OVA in the base of the tail at day +18.

Analysis: FACS analysis of inguinal and axillary lymph nodes. Anti-CD4, anti-CD8, and OVA specific tetramers (a kind gift from Dr. M. Daniels, Basel).

5.6.9 Bone marrow chimeras

a) Recipients

Recipient mice were starved for 3h before irradiation to reduce small bowel peristaltic. They were then lethally irradiated with 2x 550 rad with an interval of 3h to reduce toxicity with a γ-ray ^{137}Cs source.

b) Donors

Bone marrow was either flushed from femur and tibia or -for large numbers of donor mice (>5)- bones were crushed in IMDM 10%FCS. The supernatant was saved and replaced with fresh medium. The procedure was repeated with new bones, supernatant pooled and this procedure was repeated until the medium was remaining clear during manipulation. Cells were washed and filtered through a nylon mesh. T cell depletion was achieved using Low-Tox®-M rabbit complement lysis (Cedarlane, Bioreba AG, Switzerland) using mAbs anti-CD4 (clone RL172), anti CD8 (clone 31M) and anti- Thy1.2 (clone H01349) according to the manufacturer. 1 – 5 x 10^6 T cell depleted donor bone marrow cells were injected i.v. in a volume of 200-500µl HBSS. Transplanted mice were housed in scantainers or single ventilated cages.

5.6.10 Detection of donor/host chimerism

3-4 weeks posttransplantation 100-200µl blood was collected and anticoagulated with 1 drop of heparin (5000U/ml). Leucocytes were separated by Ficoll-Paque TM PLUS (Amersham Biosciences) gradient centrifugation for 25' at 15°C spinning with 805 rcf (G). Relative chimerism was determined using 2-color FACS analysis with anti-CD45.1-Fluos and anti-CD45.2-Cy5 or, alternatively, for transgenic donor BM the appropriate combinations of anti-Vα and anti-Vβ TCR chains were used if CD45 isoforms could not be used. Thymocytes and/or splenocytes were isolated from transplanted and appropriate control mice.

5.6.11 B cell depletion

When unsorted T cells were prepared for i.v. transfers, LN were isolated, single cell suspension made as described and B cells were depleted using anti B 220 mAb and anti-rat magnetic beads (Dynal Biotechnology). Depletion efficiency was monitored by FACS (data not shown).

5.6.12 In vitro proliferation assay

Antibodies were diluted in 50µl FCS free PBS/well in 96 well plates. Coating was performed in a humid chamber at 4°C overnight or a few hours at room temperature. Unused wells were filled with PBS. Peripheral wells were not used to improve accuracy. Wells were coated with purified anti CD3 at various concentrations as indicated and 4µg of anti CD28. The next day wells were washed once with 100µl medium (IMDM 10% FCS, β-mercaptoethanol, kanamycine). 5x10^4 cells/well were seeded in 200µl total volume.
After 2 days 100µl supernatant was removed, transferred to a sterile flat bottom 96 well plate (for in vivo assays with IL-2 dependent CTL cell line) and frozen. In the evening 3H thymidine was added at 1µCi/well (bottle = 5ml with 5 mCi, i.e. 1µl=1µCi). Cells were incubated overnight. The next day cells were harvested and counted in a β-scintillation counter or frozen for later analysis.

Alternatively 5 x10^4 sorted CD45RB^{hi} CD4 T cells were stimulated with 0.5 µg/ml soluble anti-CD3 mAb and 5 x10^4 irradiated Rag$^{-/-}$ splenocytes as antigen presenting cells in the absence or presence of indicated numbers of sorted CD25$^+$CD4 T cells. Proliferation was measured by ^{3}H thymidine incorporation for 16h. Cells were harvested using a harvester and incorporated ^{3}H was measured using a beta counter.

% inhibition was calculated as

$$\% \text{ inhibition} = \frac{100 - (\text{cpm naïve CD4 T cells} + \text{CD4}^+\text{CD25}^+ \text{ T cells}) \times 100}{\text{cpm naïve T cells alone}}$$

5.6.13 CFSE labeling

CFSE stock solution (carboxyfluorescein diacetat succiminidyl ester), Molecular Probes, Cat. No: C-1157) diluted in DMSO at 10mM [ours: 5mM].

Cells were washed twice in PBS (NO FCS!) and resuspended at 2 x 10^6/ml in PBS (serum-free) at RT. Having no serum in the PBS will make the dead cells clump and enclose some live cells within that clump. The stock CFSE solution was diluted 1:2000 in PBS, left at RT. Cells were then mixed at a 1:1 ratio of cells and diluted CFSE. The two components were initially well mixed, then turned slowly in the dark for 8 min at RT [final concentration of CFSE: 2.5µM]. The labeling reaction was quenched by adding an equal volume of FCS and immediately mixing well. Cells were then incubated for 1' before spinning and then washed twice more with medium containing 10% FCS. The CFSE labeled cells were counted and resuspended in the appropriate volume and medium (HBSS for i.v. injections).

5.6.14 In vivo proliferation assay

a) Oligoclonal in vivo proliferation assay: OT-I Rag2$^{-/-}$ i.v. injections after CFSE labeling

Pooled female OT-I Rag2$^{-/-}$ splenocytes and lymph node cells were labeled with CFSE (2.5µM final concentration) after red blood cell lysis. 5 x 10^6 cells resuspended in 400µl HBSS were injected i.v. into female mice. Recipients were Smad4$^{loxP/loxP}$ and TEC$^{Smad4-/-}$ mice. Rag2$^{-/-}$ mice served as positive controls for proliferation. 6 days after injection, spleen and lymph nodes were collected and the CFSE labeling intensity of Vα2$^+$ Vβ5$^+$ CD8$^+$ cells (OT-I cells) was analyzed by FACS.

b) Polyclonal in vivo proliferation assay (Purified CD45.1 congenic T cell i.v. injection after CFSE labeling)

Single cell suspension was made from pooled lymph nodes of 7 female B6.SJL-*Ptprc^{a}Pep3^b/*BoyJ congenic mice. B cells labelled with a purified anti B220 antibody (clone RA36B2) 1.4mg/ml diluted 1:500. B 220 expressing cells were then eliminated using magnetic Dynabeads (Dynal Biotech, Oslo, Norway) coated with sheep anti-rat IgG. B cell depletion efficiency was monitored by FACS. Purified cells were labeled with CFSE (2.5µM final concentration) and resuspended at 12x10^6 cells/ml in HBSS. 420µl of cell suspension were injected i.v. into Smad4$^{loxP/loxP}$ and TEC$^{Smad4-/-}$ females. A Rag2$^{-/-}$

mouse served as a positive control for proliferation.
7 days after injection the recipients were bled and FACS analysis was performed. 5 days post transfer mice were sacrificed and analyzed in detail.

5.6.15 In vivo proliferation competition assay

Smad 4 lox/lox mice were bred onto a CD45.1 background. LN single cell suspensions were B cell depleted. $CD4^+$ $CD45RB^{lo}$ cells were sorted from CD45.2 and CD45.1 $TEC^{Smad4-/-}$ mice. Equal numbers of purified cells were injected i.v. into the tail vein of $Rag2^{-/-}$ recipients. Analysis was performed 4.5 days after injection.

5.6.16 Colitis induction by adoptive transfer of naïve $CD4^+$ T cells

$Rag2^{-/-}$ females were injected i.v. with various populations of sorted cells as indicated.
4 x 10^5 FACS sorted cells were injected i.v. into 6-10 week old C57bl/6 $Rag2^{-/-}$ females. Recipient mice were monitored every other day for clinical signs of disease (behavior, diarrhea, fur, hunched back and kachexia) and weight was measured. Mice were either bled or sacrificed as indicated. Mice with 20% weight loss were sacrificed even before the desired time points according to the Swiss Law and regulations.

5.6.17 ELISA

ELISA was performed in 96 well flat bottom plates. Plates were coated with 10µg NIP-BSA/ml in PBS in a humid chamber at 4°C overnight or for 1h at RT. Plates were washed 3x with PBS. Uncoated plastic was then blocked with PBS 2% BSA at RT for 3h and was then washed 3x with PBS. Sera were added at the indicated dilutions (diluted in blocking buffer) and incubated for 90'. After washing 3x with PBS, biotinylated goat anti-mouse total IgG was added at 1:1000 diluted in blocking buffer and incubated for 60'. After washing 3x with PBS, Streptavidin-alkaline phosphatase (RPN1234, Amersham) was added, diluted 1: 1000 in blocking buffer. After incubation of 20' and washing 3x with PBS, 50µl of substrate was added. 50µl of stop solution (1M NaOH) was added after 13' to terminate the enzymatic reaction. Optical densities were measured at 405nm in a ELISA reader. SoftMax was used to analyze the data.

5.7 Statistical analysis

The overall statistical significance level was set to 5% if not indicated otherwise. Data are represented as mean +/- SD. Numbers of individuals per experiment are indicated where necessary (bar diagrams) i.e. if not evident from scatter plots. Significance levels of <0.05 are indicated by * and <0.01 by ** if not indicated otherwise.

Analysis of Numeric data

Comparing means of 2 groups

The decision whether to take a parametric or nonparametric test to analyze data was based on the following reasoning:
a) Parametric tests can only be used when the examined population is distributed in a Gaussian distribution

b) Nonparametric tests are less powerful than parametric ones but are independent of a Gaussian distribution.
c) Nonparametric tests are more robust to outliers.
d) Normality tests (i.e. tests that check if the examined population has a Gaussian distribution) are only useful when large samples (>100) can be compared.
e) Practical considerations: Breeding space availability, likelihood of getting the desired genotypes according to Mendel's rules
f) Small statistically significant differences are not necessarily biologically relevant.

For small numbers of samples scatter plots allow to estimate the shape of the distribution as well as the sample size. Since most experiments were performed with small numbers of mice, scatter plots were used most often.
Practicability only rarely allowed to have groups with n>10. This makes testing for normality of the distribution unreliable. As an estimation, the distribution of a scatter plot was considered and mean and median were compared. However, since Gaussian distributions could not be assumed for most of the data, the nonparametric Mann-Whitney rank test was preferably used. The use of this test reduces the likelihood to get a p value of <0.05. Since the threshold value of p=0.05 is arbitrary, statistically non significant values can still be biologically important and vice-versa statistically significant differences may not be biologically relevant. Therefore, the p value is indicated as calculated and only indicated where appropriate.

For comparing mean values of groups of normally distributed samples but with significantly different variances either Welch's correction was performed on a student t-test or a Mann-Whitney test was used.

Statistical analysis and plotting of graphs was done with GraphPad Prism 4.0 for Mac OS X.

5.8 Software and databases used

5.8.1 Software

Operating system
Mac OS 9.2.2 and Mac OS X 10.1 through to 10.3.8

4Peaks 1.5 for OS X, freely available at http://www.mekentosj.com/4peaks/
Ambi Code, freely available from http://perswww.kuleuven.ac.be/~u0002316/RealBasic/Ambicode.html
Blast, Blast 2 sequences (NCBI) http://www.ncbi.nlm.nih.gov/BLAST/
Cell Quest Pro 4.0.2 for Mac OS9, Becton Dickinson
DNA Star 5.0 for Mac OS9
Edit Seq for Mac OS9
Endnote 6.0.1 and later 8.0, ISI Researchsoft
Enzyme X 1.1 (later 2.1), freely available at http://www.mekentosj.com/enzymex/
Mozilla Firefox 1.0
Graphpad Prism 4.0 for OS X (Graphs, statistics)

Illustrator 10 for OS X, Adobe (Aire-cre targeting strategy cartoon)
ImageQuant (southern)
InDesign 3.0, Adobe (Design of figures and final thesis layout, generation of index)
iSpinX
Mac Vector (Oligonucleotide design, restriction enzyme search, generation of plasmid maps)
MS office X for mac, Microsoft (Text editing)
Omnigraffle 2.1.1, Omnigroup (design of the signaling molecule overview)
Photoshop 7.0 for OS X, Adobe
Safari 1.0 through to 1.2.4 (Internet browser for literature search, database access, blasting etc.)
SoftMax 2.3.2 (ELISA reader)
VueScan

5.8.2 Databases

-PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
-UniGene	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene
-Genbank	
-OMIM	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
-NCBI	http://www.ncbi.nlm.nih.gov
-Locuslink	http://www.ncbi.nlm.nih.gov/LocusLink/
-Gene expression omnibus	http://www.ncbi.nlm.nih.gov/geo
-Mouse knockout and mutation database	http://research.bmn.com/mkmd
-Protocol database	http://www.protocol-online.org/

5.9 Lithuanian sausage recipe

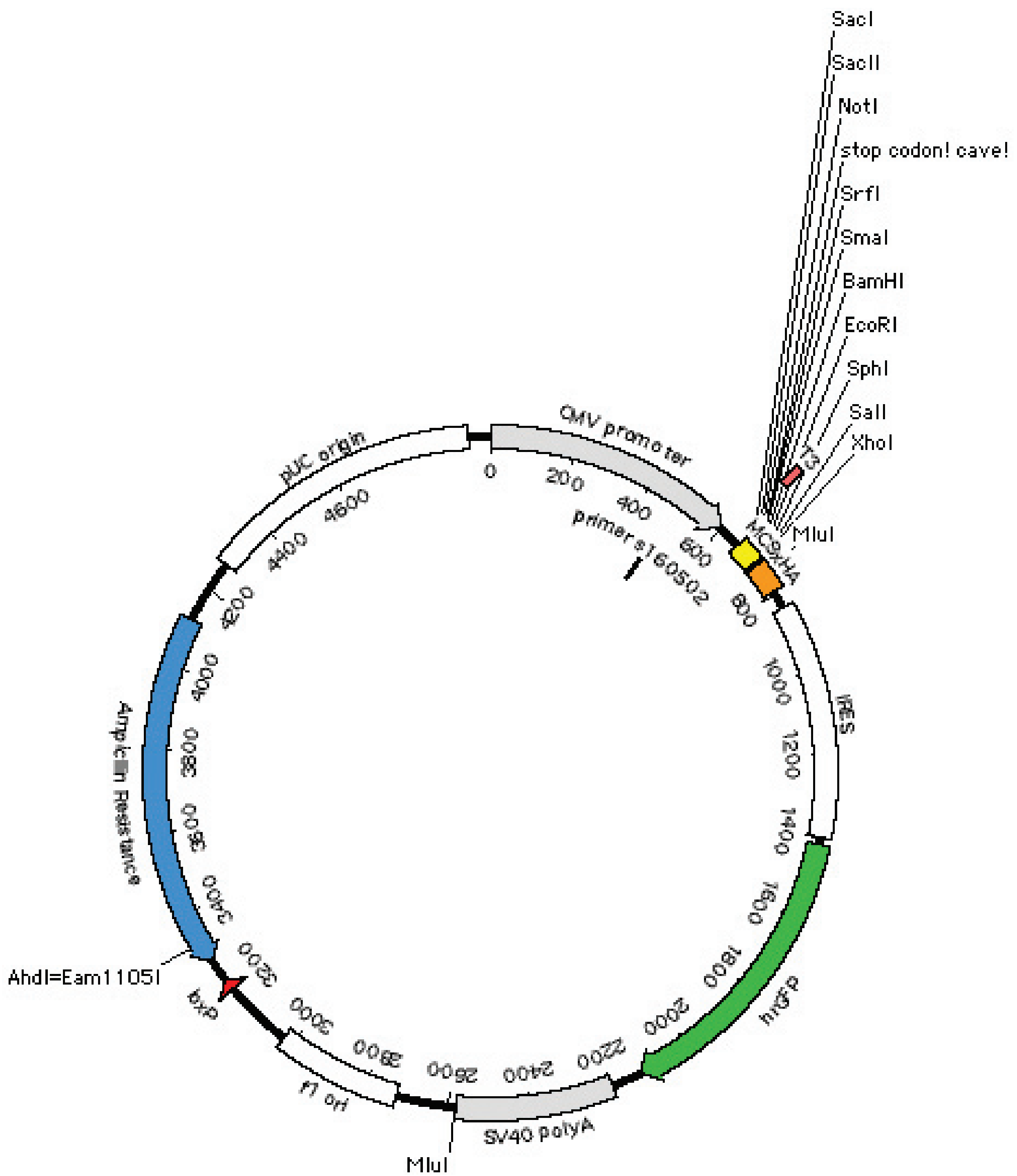
Lithuanian sausages were placed in a fire-safe bucket filled with 70% Ethanol. Matches or a lighter were used to light the ethanol. Once the ethanol was burnt and fat was flowing out, the sausages were served.

Annexes

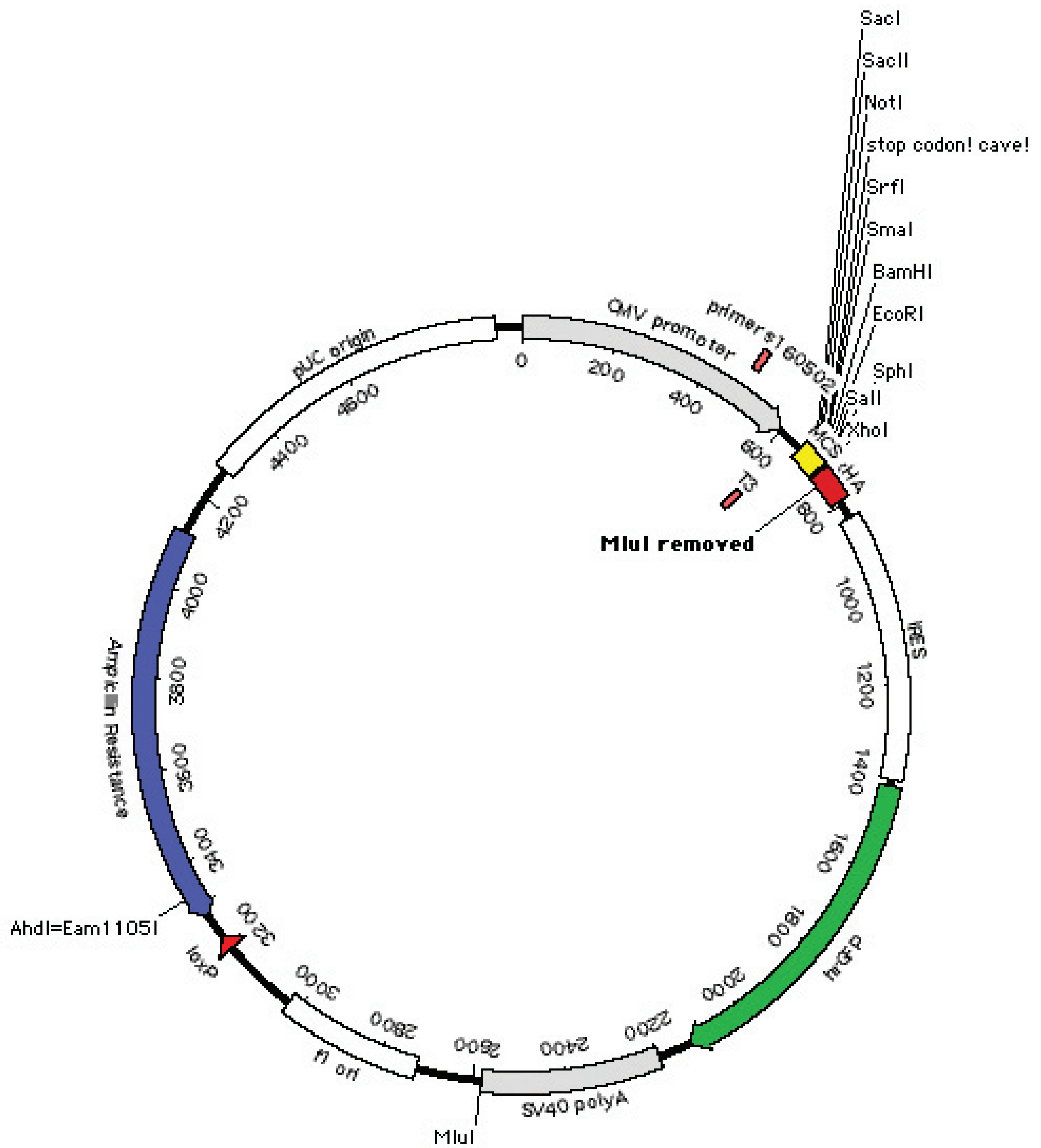
Annex I Plasmids
Annex II Antibodies
Annex III Oligonucleotides
Annex IV Buffers, solutions

Annex I: plasmids

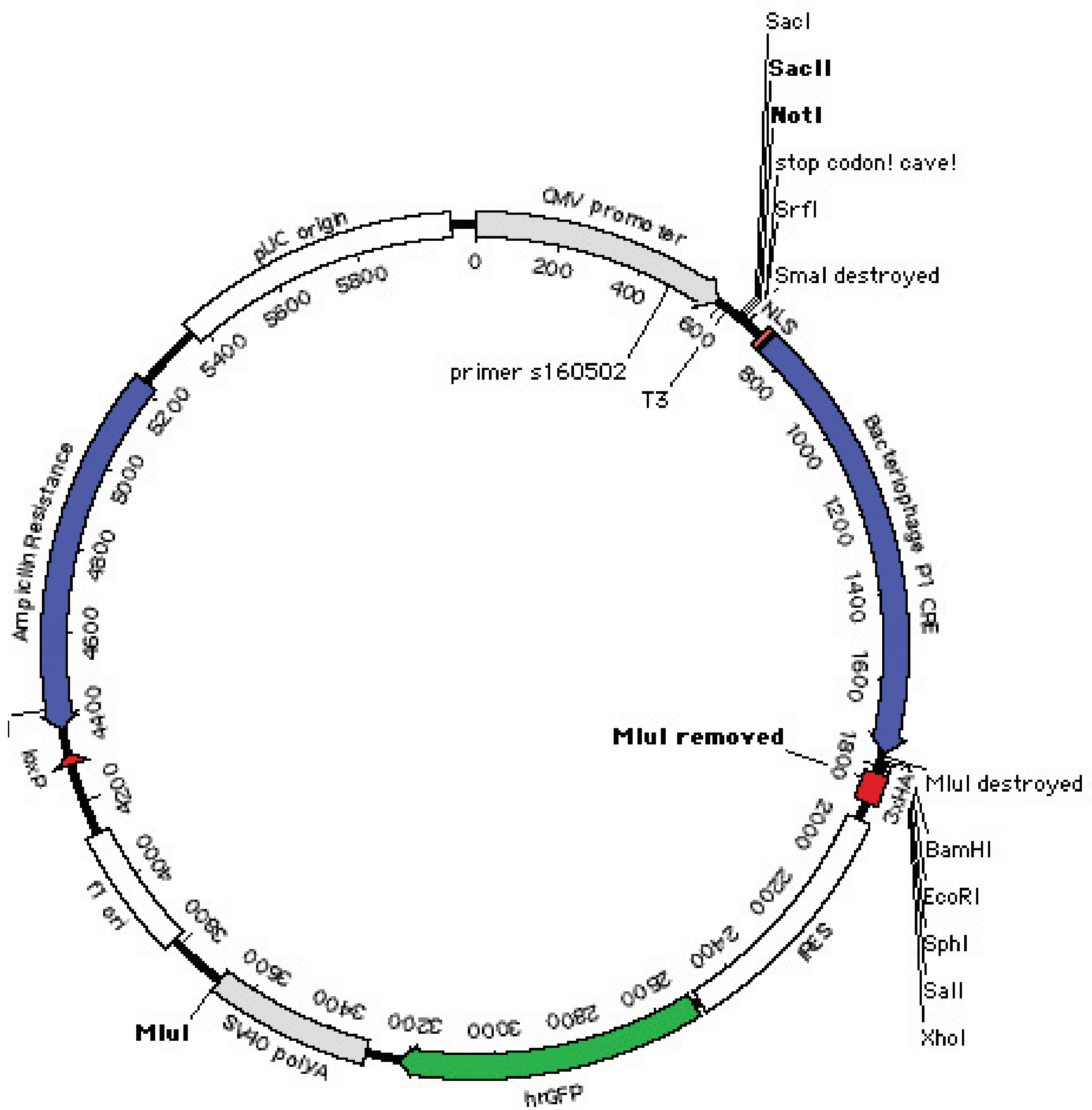
p19



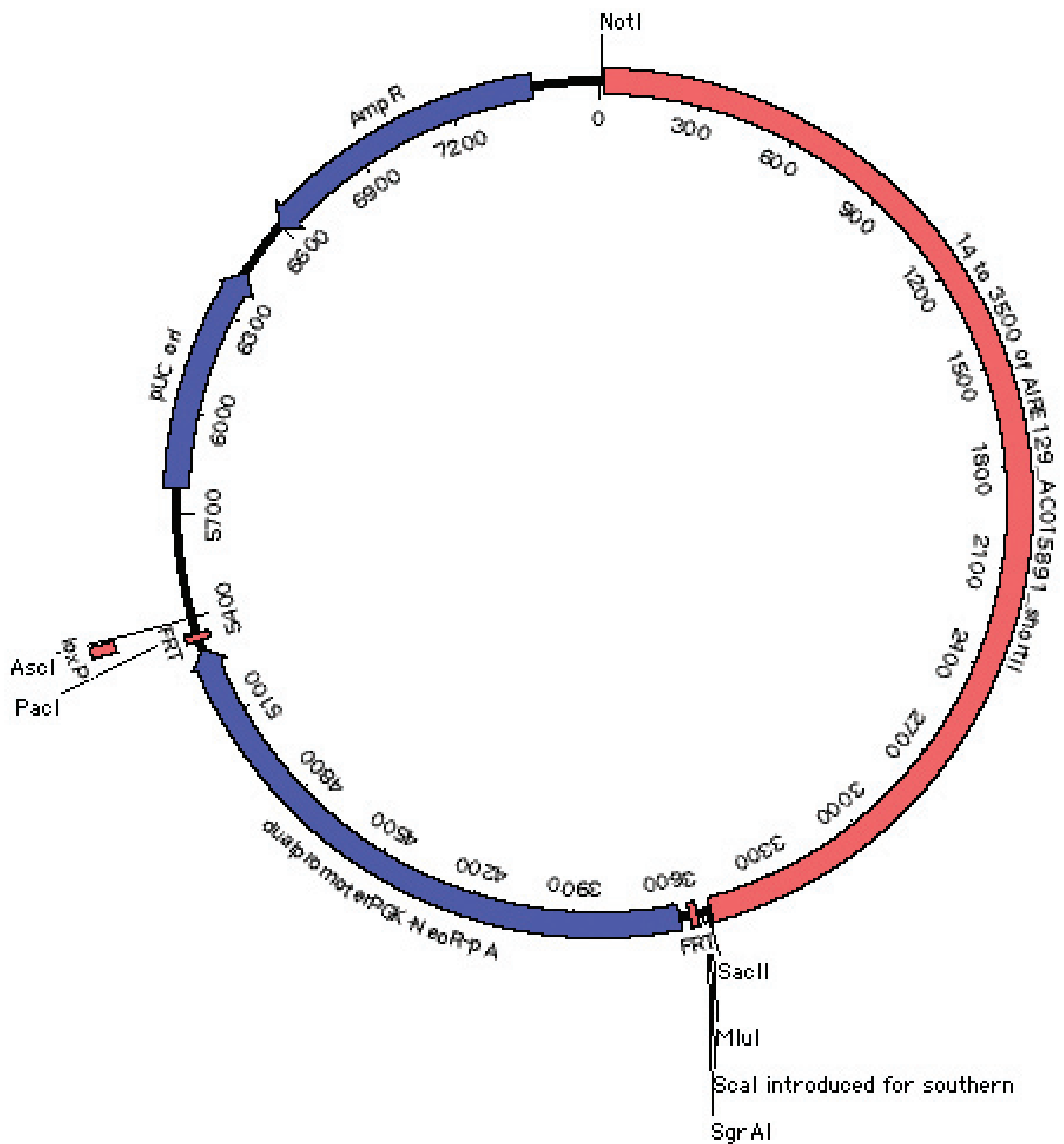
p36



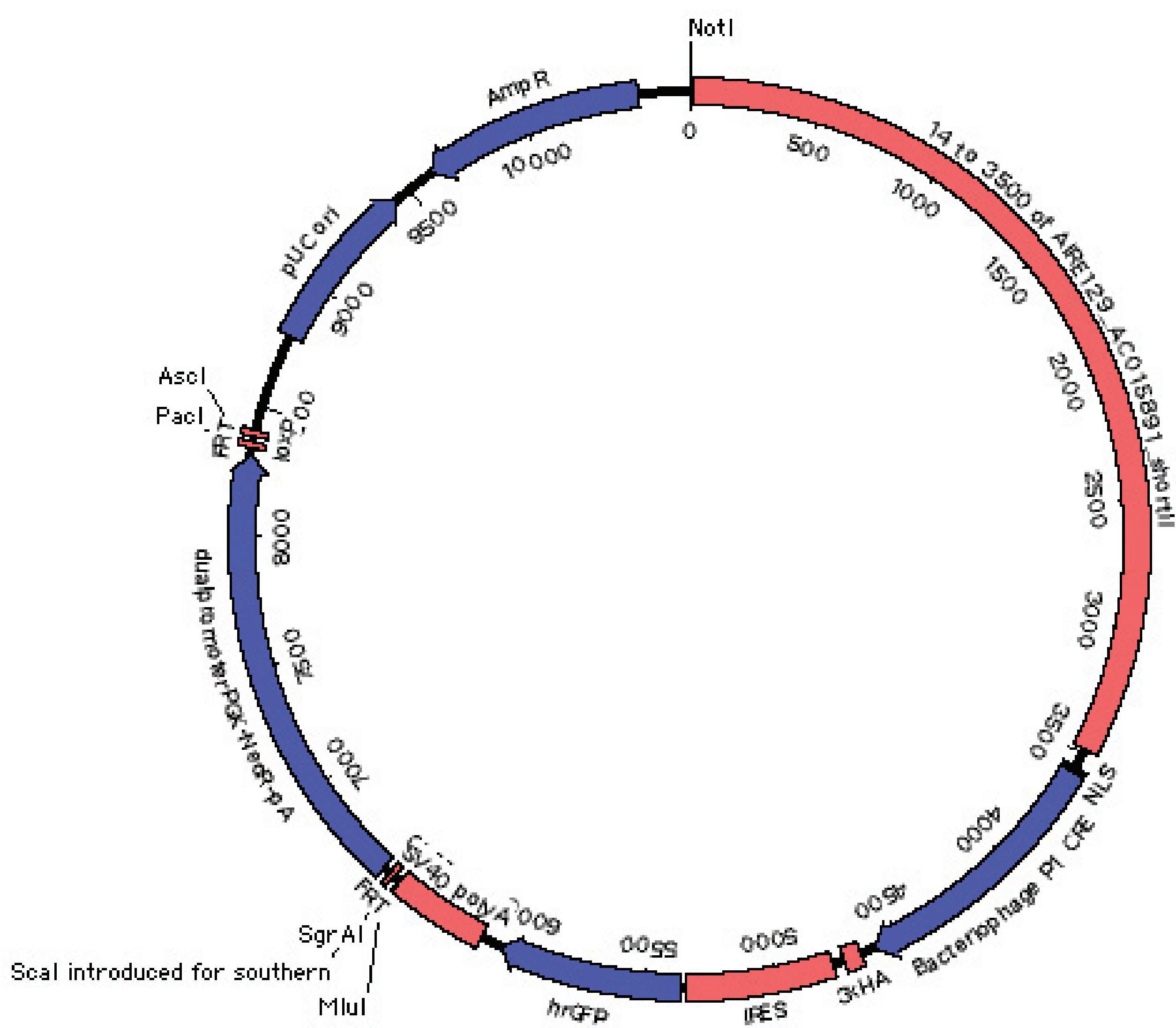
p67



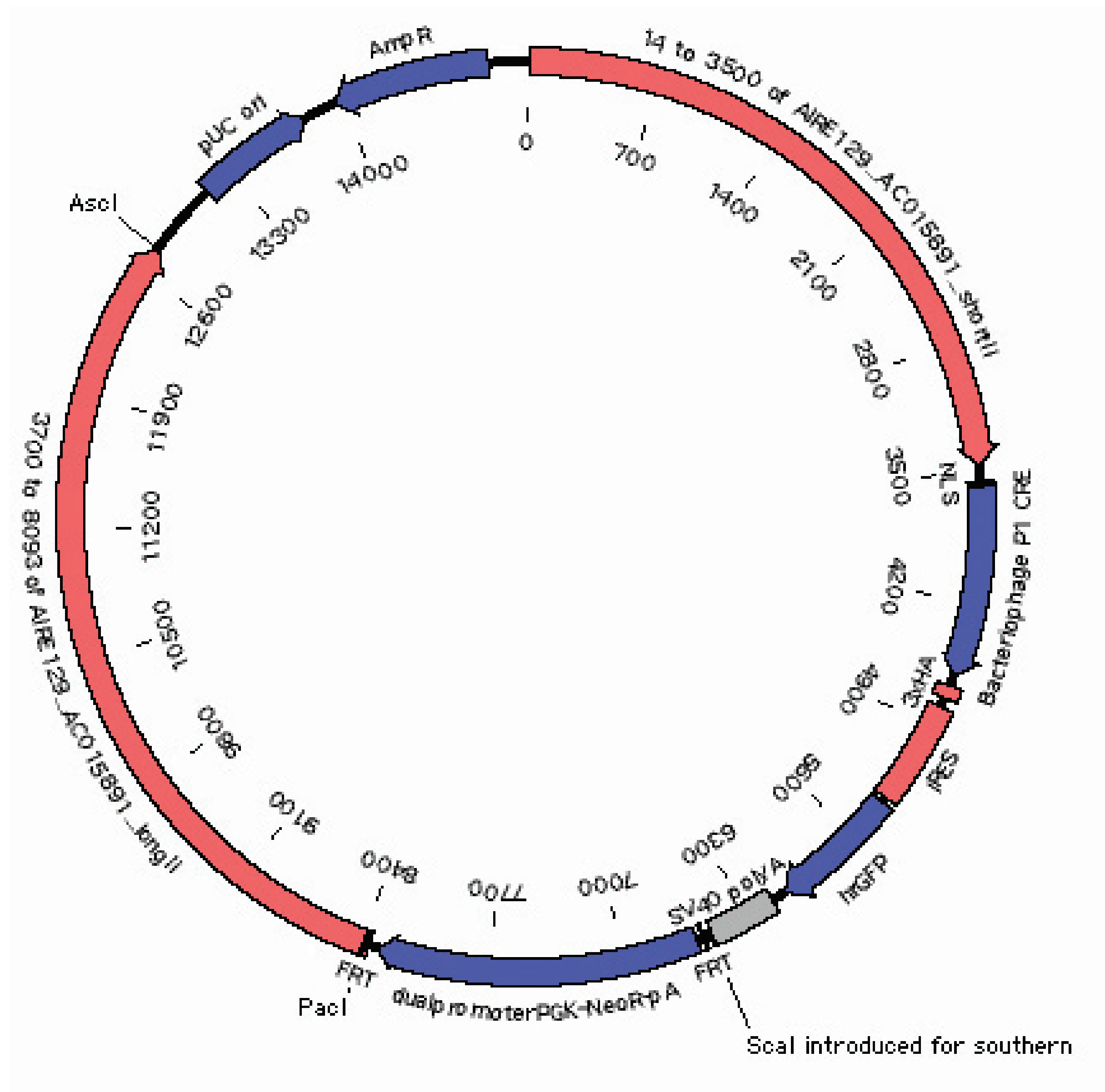
p75



p77



p79



Annex II: Antibodies

antibody and conjugate	clone	manufacturer
B220	RA36B2	
B7.1 Bio	GL1	
B7.2 Bio		
CD3 PerCP	145-2C11	
CD3 Cy5	145-2C11	
CD3 Bio	145-2C11	
CD4FITC	RM4-5	Becton Dickinson, Pharmingen
CD4 PE	RM4-5	Becton Dickinson, Pharmingen
CD4 PerCP	RM4-5	Becton Dickinson, Pharmingen
CD4 CyChrome	RM4-5	Becton Dickinson, Pharmingen
CD4 Bio	RM4-5	Becton Dickinson, Pharmingen
CD5 Bio	53-7.3	Becton Dickinson, Pharmingen
CD8 alpha FITC	53-6.7	Becton Dickinson, Pharmingen
CD8 alpha PE	53-6.7	Becton Dickinson, Pharmingen
CD8 alpha PerCP	53-6.7	Becton Dickinson, Pharmingen
CD8 alpha Cychrome	53-6.7	Becton Dickinson, Pharmingen
CD8 alpha Cy5	53-6.7	Katrin Hafen
CD8 alpha Bio	53-6.7	Becton Dickinson, Pharmingen
CD8 beta PE		Becton Dickinson, Pharmingen
CD11bPE(MacI)		
CD19	ID3	Katrin Hafen
CD24 FITC	M1/69	Becton Dickinson, Pharmingen
CD25 FITC	PC61	Becton Dickinson, Pharmingen
CD25 PE	PC61	Becton Dickinson, Pharmingen
CD25Bio	PC61	Becton Dickinson, Pharmingen
CD44 PE	IM7	Becton Dickinson, Pharmingen
CD44 Bio	IM7	Becton Dickinson, Pharmingen
CD45PE	30-F11	Becton Dickinson, Pharmingen
CD45RB FITC		eBioscience, USA
CD45.1 Fluos	A20	
CD45.1Bio	A20	
CD45.2 Cy5	104	
CD45.2 Bio	104	
CD62L Bio	MEL-14	

CD69 PE	H1.2F3	
CD103 Bio		Becton Dickinson, Pharmingen
CD127PE	A7R34	eBioscience, USA
Epcam Bio		
Fas PE		Becton Dickinson, Pharmingen
FasLBio	MFL3	
Gr-1 (Ly6G)	RB6-8C5	
H2-Kb Bio	AF6-88.5	
H2-KdPE	SF1-1.1	
IAb FITC	AF6-120.1	
NK1.1 Bio		
TCR FITC	H57-592	
TCR PE	H57-592	
TCR Cychrome	H57-592	
TCR Bio	H57-592	
TCR gamma delta PE	GL3	
Valpha 2.3PE		
V beta 8.3Bio		
V alpha 2		
V beta 5		
Vbeta2PE		Becton Dickinson, Pharmingen
Vbeta3Bio		Becton Dickinson, Pharmingen
Vbeta4PE		Becton Dickinson, Pharmingen
Vbeta5.1/5.2FITC		Becton Dickinson, Pharmingen
Vbeta6PE		Becton Dickinson, Pharmingen
Vbeta7PE		Becton Dickinson, Pharmingen
Vbeta8.1/8.2FITC		Becton Dickinson, Pharmingen
Vbeta9Bio		Becton Dickinson, Pharmingen
Vbeta10PE		Becton Dickinson, Pharmingen
Vbeta11PE		Becton Dickinson, Pharmingen
Vbeta12Bio		Becton Dickinson, Pharmingen
Vbeta13PE		Becton Dickinson, Pharmingen
Vbeta14Bio		Becton Dickinson, Pharmingen
Vbeta17aBio(KJ23)		Becton Dickinson, Pharmingen
Fc blocking		
CD16/CD32	2.4G2	Becton Dickinson, Pharmingen
Streptavidin		
SA APC		

SA Cy5		Zymed Laboratories, San Francisco, CA
SA PE		Southern Biotechnology, Birmingham, USA
SA PE Cy7		Becton Dickinson, Pharmingen
In vitro Stimulation		
purified anti CD3	145C11	
purified anti CD28		
ELISA		
Purified rat anti-mouse IgM mab		Becton Dickinson, Pharmingen
Depletion antibodies:		
BM chimeras: T depletion		
CD4	RL 172	
CD8	31M	
Thy1.2	H01349	
B220 depletion	RA 36B2	
Dynabeads M-450, sheep anti-rat IgG		Dynal Biotech, Norway
In vivo depletion		
CD25 depletion	PC61	Katrin Hafen
IHC antibodies		
Cytokeratin5		Progene GmbH, Heidelberg, Germany
biotinylated UEA-1 lectin		Vector Laboratories, Lausanne, Switzerland
MTS10		Pharmingen, San Diego, CA
rat anti-MTS24		supernatant, generous gift of R. Boyd, Melbourne, Australia
cytokeratin 18		Daco
anti-aire		generous gift of Dr. H. Scott, Australia

Annex III: Oligonucleotides

All oligonucleotides are indicated as 5' ➔ 3' sequences

Number	sequence	purpose
		Analysis of expression of signaling pathway molecules
1210	CCAGAGATGAGTGGGAAAACGG	mBMP2
1211	GTTAGTGGAGTTCAGGTGGTCAGC	mBMP2
1212	GGTAACCGAATGCTGATGGTCG	mBMP4
1213	TTCTGCTGGGGGCTTCATAAC	mBMP4
1216	CGAACATCCAGACCCTATCTTTGAC	mnoggin
1217	GCAGGAACACTTACACTCGGAAATG	mnoggin
1298	TGGGAAATGGCTCGTCGTTG	mBMPRecIa
1299	ATGTAGAGGAAGGAGTCTGGAAACC	mBMPRecIa
1302	TAAATGCCACCACCACTGTCCG	mBMPRecIb
1303	ATGTATGTCTCGTCCTGCTCCAGC	mBMPRecIb
1300	GGAGGGAACGGCCATTAGA	mBMPRecII
1301	GAATTGGGCCTCTGTGCTCTT	mBMPRecII
1306	ACTTGACCTGTTGCCTGTGTGAC	mTGF-bRecII
1307	TGGTAGTGTTCAGCGAGCCATC	mTGF-bRecII
1304	GCCACCCTATTACAACATTCTGCTG	mActivinRecIIA
1305	TGATTAGCCACAGGTCCACATCC	mActivinRecIIA
1370	CTGCATGACGTCCTCTCTCCT	mBambi
1371	TGGGAACCGCTATCACAGCT	mBambi
2109	CACTATTGAAAACACCAGGCGAC	mSmad1
2110	TTATCGTGGCTCCTTCGTCAGG	mSmad1
1308	TACAAGGCGACACATTGGGAGAGG	mSmad3
1309	TCTAAGACACACTGGAACAGCGG	mSmad3
2111	CGATTCAAACCATCCAACACCC	mSmad4
2112	ATCCATTCTGCTGCTGTCCTGG	mSmad4
1310	AGTTCACTGTGCCTTGTCCTTAGG	mSmad5
1311	GCAAAGCCACCCAATGGTTG	mSmad5

		Aire expression
490	TGTGCCACGACGGAGGTGAG	AIRE-8528-F
491	GGTTCTGTTGGACTCTGCCCTG	AIRE-9759-R
2144	CCAGTGAGCCCCAGGTTAAC	Aire real timef
2145	GACAGCCGTCACAACAGATGA	Aire real timer
2107	TCTGAAGGAGAAGGAAGGCTGC	mAire_3959f
2108	TCTCTTGGTGGGGGGTCTG	mAire_4440r
		Cloning of the aire-cre targeting vector
1635	TACAGCGGCCGCTGGCTTTCAAAACACACACTTGGCT	NotI_aire_14f
1636	TCATCCGCGGCTTCCTCACAGGGGCTGCGCCTCG	aire_3500r_SacII
1633	ATCGTTAATTAATTCCCTCCCAACCTCAGCCAAAAC	PacI_aire3700f
1634	TCAGGCGCGCCTTCCCCATCCCCTACCTTATCAGG	aire_8093r_AscI
		Adaptor molecules
1395	TCGAGTACCCATATGACGTTCCAGACTATGCGTATCC	MluIremovalUpper
1396	GTACGGATACGCATAGTCTGGAACGTCATATGGGTAC	MluIremovalLower
1637	GGTCGTTGACGCGTAGTACTCA	adSacII/MluI/ScaI/SgrAIupper
1638	CCGGTGAGTACTACGCGTCAACGACCGC	adSacII/MluI/ScaI/SgrAIlower
		Southern blot
1925	CAAGATGTGGAGAGACCCAGGTAG	5’ probe2
1926	CAGGGAGAGGGGAAACTCAAATC	5’ probe2
2127	TTTGGCTTCCCACCTATGAATG	3’ probe 3
2128	GAGAAGGAAAGGCTGAGTGTG	3’ probe 3
2053	TGGCTCGCACACATTCCACATC	internal_1
2054	TTCCCGCTTCAGTGACAACG	internal_1
2055	GCCCAAGAAGAAGAGGAAGGTGTC	internal_2
2056	AGACGGAAATCCATCGCTCG	internal_2

		PCR screening aire-cre targeted ES cells
1939	TTCCCTGCCACAGTCTGA	3’
1937	TACCATCCGTGCGACTGAAGAC	3’
		Genotyping
2332	GGGCAGCGTAGCATATAAGA	smad4
2626	GACCCAAACGTCACCTTCAC	Smad4
2323	TAAACGCTTACAGGTGCCTCTCCG	Aire-cre
2324	CCAGGTATGCTCAGAAAACGCC	Aire-cre
2368	CATAGGGTGTGAGGTTAGGGAACTC	Aire-cre
1621	CTCTCCTCCGAGTATCCAATCTG	FoxN1-cre
1622	CCCTCACATCCTCAGGTTCAG	FoxN1-cre
		smad4 deletion efficiency
3060	TCCCACATTCCTCTTAGTTTTGA	
3061	CCAGCTTCTCTGTCCAGGTAGTA	

Annex II: Buffer composition and solutions

FACS buffer

PBS, 2% FCS

FACS buffer for thymic epithelial cells

PBS, 2% FCS, 5mM EDTA to avoid clumping

RBC lysis buffer

- 8.84 g NH_4Cl
- 0.037 g EDTA
- 1 g $NaHCO_3$

in 1 liter milli-Q-H_2O, sterile filtered.

PI-solution for apoptosis detection with FACScan

- 200 μg/ml Propidium iodide
- 100 μl/ml 10% Triton-X100
- 154 μl/ml 1M NaCl
- 10 μg/ml 10 mg/ml RNAse

fill up to 1 ml with PBS
stored at 4°C in the dark.

Southern blotting

Church Buffer

BSA	10 g
0.5M EDTA	2 ml
1M $NaHPO_4$, pH7.2	500 ml
20%SDS	350 ml
H_2O	ad 1 liter

1M $NaHPO_4$

Na_2HPO_4-7H2O	134 g
85% H_3PO_4	4 ml

pH should be at 7.2

Wash Solution 1

BSA	10 g
0.5M EDTA	2 ml
1M $NaHPO_4$, pH7.2	80 ml
20% SDS	500 ml
H_2O	ad 2 liter

Wash Solution 2

0.5M EDTA	4 ml
1M $NaHPO_4$, pH7.2	80 ml
20% SDS	100 ml
H_2O	ad 2 liter

LacZ staining solutions

0.5M EGTA (250mls)

47.5g
pH to 7.5 with NaOH
adj vol to 250mls H_20
Note: EGTA will not go into solution until pH is neutral
Autoclave

lacZ Fix (50ml)

0.4ml 25% glutaraldehyde (Sigma)
0.5ml 0.5M EGTA (pH 7.3)
5.0ml 1M $MgCl_2$
44.1ml PBS (w/o $MgCl_2$, CaCl)(BioWhittaker)

lacZ Wash Buffer (500ml)

1.0ml 1M $MgCl_2$
5.0ml 1% NaDOC (final 0.01%)
5.0ml 2% Nonidet-P40 (final 0.02%) (Roche)
489ml PBS (w/o $MgCl_2$, CaCl)(BioWhittaker)

lacZ Stain (100ml)

96ml lacZ wash buffer
4.0ml 25mg/ml X-gal (dissolved in DMSO)
0.21g K-ferrOcyanide
0.16g K-ferrIcyanaide

Immunohistochemistry solutions

Fixation

1-4% PFA in PBS, 30 min RT (apply 50µl directly)
Or Aceton, 7min 4°C

Permeabilization and blocking

0.1% Tween20 (Sigma)+ 1% FCS in PBS, pH 7.3, 30 min, RT

Washing

0.1% Tween 20(Sigma)+ PBS, pH 7.3

Blocking Biotin and Avidin

Vector labs-Kit
Blocking endogenous peroxidase:
0.3% NaN_3 + 0.1% H_2O_2 in PBS

AEC-buffer

37ml 0.2N acetic acid, 88ml 0.2 Na-acetate, 375ml H_2O, pH5.0

Tail lysis buffer

		final conc:
10ml	1M Tris pH 8.5	100mM
1ml	0.5M Na-EDTA	5mM
1ml	20% SDS	0.2%
4ml	5M NaCl	200mM
ad 100ml	H_2O	
	proteinase K	100µg/ml (keep at –20°C, add freshly)

Buffers for Gene targeting

20x SSC

175.3 g of NaCl, 88.2 g of Na-citrate, adjust the pH to 7.0, autoclave
(can be stored at RT)

50x TAE-buffer

242 g of Tris-base, 57.1 mL of glacial acetic acid, 100 mL 0.5 *M*
EDTA at pH 8.0, fill to 1 L with deionized water (can be stored at RT).

5x TBE buffer

54 g of Tris-base, 27.5 g of boric acid, 20 mL 0.5 *M* EDTA at

pH 8.0, adjust to 1 L with deionized water (can be stored at RT).

10x phosphate-buffered saline (10x PBS)

80.06 g of NaCl, 2.01 g of KCl, 14.42 g
of Na_2HPO_4 °— 2 H_2O, 2.04 g of KH_2PO_4. Fill to 1 L with deionized water and autoclave (store at RT).

Trypsin/EDTA solution

10°— stock (Gibco, Paisley, Scotland) (store at –20°C)
made 1°— with 1°— PBS (aliquot and store at –20°C, store the presently used aliqout at 4°C).

EF medium

DMEM high glucose + Na-pyruvate (Gibco) supplemented with
10% fetal bovine serum (FBS) (Gibco) and 2 m*M* L-glutamine (Gibco).

ES medium

DMEM high glucose + Na-pyruvate (Gibco) supplemented with
15–20% FBS (FBS needs to be tested for ES cell use, or can be bought from Gibco already tested), 2 m*M* L-glutamine (Gibco), 0.1 m*M* 2-mercaptoethanol, 1°— nonessential amino acids of 100°— stock solution (Gibco), and 1000 U/mL leukemia inhibitory factor (LIF) (ESGRO from Gibco).

3 *M* Na-acetate at pH 5.2, adjust pH with glacial acetic acid (can be stored at RT).

Freeze medium

70% DMEM, 20% FBS, and 10% DMSO.
G418 (geneticin; Gibco or Sigma).

Molecular cloning

LB

10 g of bactotryptone (Difco, Detroit, MI), 5 g of bacto-yeast extract (Difco),
10 g of NaCl. Fill to 1 L with deionized water, adjust the pH to 7.0, and autoclave.

LB agar

Add 15 g of bacto agar (Difco) to LB, autoclave, and allow medium to
cool to 50°C before adding antibiotics and pouring. Plates can be stored for 1 mo at 4°C. When plates are fresh they will exude moisture when incubated at 37°C. This increases the risk of cross contamination and is avoided by drying the plates in a laminar flow hood for 20 min before use.

ELISA

Denaturation solution

1.5 M NaCl, 0.5 M NaOH (can be stored at RT)

Neutralization solution

1.5 M NaCl, 0.5 MTris-HCl at pH 7.2 (can be stored at RT)

ELISA blocking

PBS 2% BSA

Addendum

This paragraph goes to the brave readers who are still following me. The perception of the initially asked question might divide the attentive readers into scientists and gourmets. I do not intend to answer the question as I may be biased by my déformation professionelle. Rather I'd like to thank the reader who fought his way through the jungle of abbreviations.

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Languages

German	Mother tongue
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French	Fluent, written and orally
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Education

1982 - 1986	Primary School, Buchs (AG) and Basel
1986 - 1994	Realgymnasium Basel (Matura Type B)
1994 - 2000	Medical studies at the University of Basel
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1997- 1998	Medical studies in Paris, Université René Descartes, Faculté Necker – Enfants malades
1998- 1998	Visiting Scholar in the laboratory of Prof. N.R. Rose, Dept. of Molecular Microbiology & Immunology, Johns Hopkins University, Baltimore, Maryland, USA
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January 2001-June 2001	Assistenzarzt, Internal Medicine, Davos Hospital
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Functional Genomics	Prof. Dr. P. Philippsen
Transcriptional Regulation	PD Dr. R.G. Clerc
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Molecular & Cellular Basis of disorders of the Immune System	Prof. Dr. A. Rolink
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Bioinformatics I	Prof. Dr. M. Primig
Cancer Research	Proff. Dr. N. Hynes/M. Burger
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2001	ACLS (Advanced Cardiac Life Support), Diploma of the American Heart Association
2003	Laboratory Animal certificate, LTK 1

Scholarships

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Publication

Lukas T. Jeker, Mehrdad Hejazi, C. Lynne Burek, Noel R. Rose and Patrizio Caturegli: \`Mouse primary thyrocyte culture\`, (Biochemical Biophysical Research Communications, 1999, April 13;257(2):511-515)

Book chapter

Lukas T. Jeker and Georg A. Holländer, ‘Monogene Autoimmunkrankheiten’, in ‘Pädiatrische Allergologie und Immunologie’, 4th Edition, Urban & Fischer Verlag (in preparation)