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DISSERTATION

Titel der Dissertation

Direct antigen presentation by medullary thymic epithelial cells is essential for CD4 T cell tolerance

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SUMMARY

Medullary thymic epithelial cells (mTEC) play an essential role in the establishment of central tolerance as they express rare, otherwise tissue-restricted antigens. The overall contribution of direct antigen presentation by mTEC as opposed to mere provision of antigens, however, remains to be established.

Here, we generated a transgenic mouse (*C2TAkd*) expressing a “designer miRNA” against C2TA, the master regulator of MHCII, specifically in mTEC. Tissue-specific and efficient knock-down of C2TA was observed in mTEC, but not in other thymic stromal cell types. In *C2TAkd* mice the polyclonal $CD4^+$ single positive compartment was enlarged and $CD4^+$ thymocytes were self-reactive *in vitro*, presumably due to impaired negative selection. In several TCR transgenic models of tolerance to mTEC-derived self-antigens diminished negative selection was observed when interfering with antigen presentation by mTEC, irrespective of whether potentially cross-presenting dendritic cells (DC) were present or not. In addition we found that reduced antigen presentation by mTEC shifted the mode of tolerance from negative selection towards regulatory T cell (T_{reg}) development which is in support of an avidity model of T_{reg} induction.

Taken together we propose a dual function for mTEC in $CD4^+$ T cell tolerance as antigen reservoir and as antigen presenting cells.

ZUSAMMENFASSUNG

Medulläre thymische Epithelzellen (mTEC) spielen eine wichtige Rolle für die Entstehung zentraler Toleranz, da sie gewebsspezifische Antigene exprimieren. Die Relevanz direkter Antigenpräsentation, im Gegensatz zur ausschließlichen Produktion von Antigenen durch mTEC, ist undurchsichtig.

Diese Arbeit beschreibt die Generierung einer Transgenen Maus (*C2TAkd*), die eine „Designer miRNA“ gegen C2TA, den Hauptregulator von MHCII, spezifisch in mTEC exprimiert. Der gewebsspezifische und effiziente Knockdown von C2TA konnte in mTEC, nicht jedoch in anderen thymischen Stromazellen detektiert werden. In *C2TAkd* Tieren fanden wir eine vergrößerte $CD4^+$ einfach positive T-Zell Population, die $CD4^+$ Zellen mit autoreaktivem Potential enthielt, was wahrscheinlich auf einen Defekt in der negativen Selektion zurückzuführen ist. Des weiteren führte die Inhibierung von MHCII in mTEC in mehreren Mausmodellen, in denen ein von mTEC produziertes Antigen zusammen mit einem T-Zell Rezeptor spezifisch für das Antigen exprimiert wurde, zu einer Verminderung der negativen Selektion. Diese Beobachtung konnte in An- und Abwesenheit von Dendritischen Zellen (DC) gemacht werden. Darüber hinaus konnten wir in den T-Zell Rezeptor transgenen Modellen mit verminderter Antigenpräsentation durch mTEC eine Verschiebung von negativer Selektion Richtung Induktion von regulatorischen T-Zellen (T_{reg}) feststellen. Dies ist ein starker Hinweis darauf, dass die Entwicklung von T_{reg} auf einem Aviditätsmodell beruht.

Wir vertreten die These, dass mTEC eine zweifache Funktion in der Toleranzinduktion von $CD4^+$ T-Zellen innehaben, als Antigenreservoir und als Antigen präsentierende Zellen.

1. INTRODUCTION

The immune system is a remarkably old “institution” as even the most primitive multicellular organisms have some kind of defense mechanism against potentially dangerous pathogens. Innate immunity is a hallmark of all metazoan species and depends, amongst others, on the recognition of highly conserved pathogen associated molecular patterns (PAMPS) by germ line–encoded pattern recognition receptors. Microorganisms on the other hand steadily develop new strategies to evade these host defense tactics, thereby constantly putting immense selective pressure on the host. Thus, there is a permanent need to respond to new and improved invaders, which most likely led to the evolution of a new, highly sophisticated defense mechanism, the so called adaptive immune system (1, 2).

The adaptive immune system first can be found in jawed vertebrates, and in a rudimentary form also in jawless vertebrates. Unlike the innate immune system it is characterized by the remarkable ability to specifically respond to virtually any given pathogen. The enormous diversity and flexibility of the adaptive immune system is ensured by different lymphocyte species and their ability to perform somatic diversification of antigen receptor genes, thereby generating a vast repertoire of cells, each of which expressing a different antigen receptor. Antigen recognition by specific lymphocyte clones initiates a sequence of inter-depending processes including proliferation, differentiation and production of specific antibodies.

Representing the major lymphocyte populations, T cells are primarily responsible for cell-mediated immunity while B cells are mediating antibody responses or humoral immunity, whereby their cross-talk is indispensible for an effective immune response. Another hallmark of the adaptive immune system is its ability to memorize previously encountered pathogens, enabling the host to erase future infections more quickly and efficiently.

The generation of a nearly infinite antigen receptor repertoire is made possible by the perpetual development of new lymphocytes and the somatic rearrangement of their V-D-J immunoglobulin gene segments. As this process is entirely random it is an obvious consequence that also potentially harmful receptors recognizing self constituents are generated. Mechanisms of tolerance induction have therefore arisen, eliminating auto-reactive lymphocytes and leading to the ability to distinguish

harmful/foreign from self, which is a fundamental feature of the adaptive immune system.

1.1 The Thymus

Concomitant with the emergence of the adaptive immune system and its T cell arm 500 million years ago, a co-evolution of a specialized organ for T cell development took place, i.e. the thymus (2). T cell development is a non-autonomous process in which the thymus as a primary lymphoid organ provides the essential niches and constant signals for maturing T cells. The thymic stroma plays a key role at multiple stages of T cell development, on the one hand it ensures T cell lineage specification of common lymphoid precursors and on the other hand it is absolutely essential for MHC-self restriction via positive selection and the elimination of auto-reactive T cells via negative selection (3). Constituting the functional unit of the thymus, the thymic stroma consists of hematopoietic as well as non-hematopoietic components which are heterogeneous in their developmental origin, whereby dendritic cells (DC) and macrophages belong to the first group while epithelial cells and mesenchymal cells compose the latter. The postnatal thymus is compartmentalized into the inner morphologically lighter zone, the medulla, containing medullary thymic epithelial cells (mTEC), DC and macrophages and the outer morphologically darker zone, the cortex, mainly comprising cortical thymic epithelial cells (cTEC). While the cortex is operative in positive selection, the medulla has been shown to crucially contribute to tolerance induction by expressing otherwise tissue-restricted antigens (TRA), thereby mirroring the peripheral self.

1.1.1 Ontogenesis

Thymus ontogenesis can be divided into pre- and post-thymus committed stages, whereby thymus specification is strictly linked to the expression of the transcription factor Forkhead box N1 (Foxn1) (4, 5). To date Foxn1 is the only marker that identifies early thymic epithelial cell commitment and its expression can be first detected around embryonic day 11.5 prior to vascularization and colonization by hematopoietic cells, indicating that early thymus development is a cell autonomous process or at least independent of hematopoietic cell immigrants (4, 6). Fate

mapping studies using Foxn1-Cre mice crossed to the *Rosa26R*-eYFP reporter system revealed that more or less all adult TEC have expressed Foxn1 at some point during development (7). The crucial role of Foxn1, not only as a marker but also functionally, has been unambiguously shown in Foxn1-deficient nude mice that exhibited a total block in early TEC development and consequently did not harbor any differentiation into cTEC and mTEC. Moreover, nude mice did not support any T cell development demonstrating the importance of a fully differentiated thymic epithelium for this process. A detailed analysis showed that the Foxn1-deficient thymus anlage did not express CCL25 and CXCL12 as well as Notch ligands, all absolutely required for T cell development (8, 9). Importantly, mixed chimera experiments established a cell autonomous role of Foxn1 in thymus development (10).

TEC derive exclusively from the endodermal lining of the third pharyngeal pouch which was confirmed by grafting endoderm-only third pouch of day 9 mouse embryos into athymic mice and showing that this was sufficient to support T cell development (11, 12). In addition it was found that the neural crest-derived mesenchyme is crucial for early thymus organogenesis and thymus function (13). Factors that have been implied in thymus development prior to thymus specification are rare; Tbx1, a gene that has also been linked to the DiGeorge syndrome, was found to be essential for the formation of pharyngeal pouch-derived organs such as the thymus (14, 15). Of note, several other genes, like Hoxa3, Pax1 and Pax9, have also been shown to have functional roles in early thymus generation (16-19).

The typical medullary-cortical structures first become apparent around E12, interestingly coinciding with the first hematopoietic cell immigration. It is well established that proper TEC development into medulla and cortex is dependent, at least during some stages of maturation, on “thymic cross talk” which describes the requirement of immature TEC to interact with T cells in order to differentiate into cTEC and mTEC (20). This is based on the observation that T cell-deficient mice did not show the typical thymic architecture and exhibited a disrupted medulla-cortex organization (21-23). This cross talk involves various crucial cell surface molecules and pathways all of which will be discussed later in this section.

1.1.2 Common TEC progenitor

It had been a long standing issue whether cTEC and mTEC originate from a common progenitor. This idea first came up with the observation that rare TEC in the adult thymus simultaneously express cTEC and mTEC markers; keratin 8 (K8) is largely expressed by cTEC while keratin 5 (K5) is specific to mTEC, however a rare population of TECs in the adult thymus appears to be positive for both, thus possibly representing a bi-potential progenitor. This hypothesis was even bolstered by the finding that large clusters of $K8^+K5^+$ TEC accumulated in thymi of mice that showed a block in T cell development (24). An ensuing study proposed $MTS24^+$ cells, a subset of $K8^+K5^+$ TEC, to fulfill the criteria for a common progenitor, however this has been disproven later (25-27).

Today it is largely accepted that cTEC and mTEC both arise from the same progenitor which was convincingly proven by two independent studies. First, Foxn1-reactivation in single TEC of a Foxn1-deficient adult thymus led to the generation of a fully competent thymus capable of supporting T cell development (7). Second, a single yellow fluorescent protein expressing $keratin^+$ cell isolated from embryonic day 12.5 murine thymi gave rise to both cTEC and mTEC clusters when placed into an embryonic thymus (28). While these studies clearly demonstrate the existence of a bi-potent TEC precursor, yet no specific marker for the identification of a common progenitor has been described.

1.1.3 cTEC progenitor

Whereas the last few years brought some insight into the mechanisms of TEC development, especially mTEC development, still very little is known about cTEC differentiation. Bleul et al. could prove the existence of a cTEC-committed progenitor by fate mapping experiments using a YFP reporter, however, no specific marker of early cTEC has been defined in this study (7). cTEC in contrast to mTEC appear to be a largely homogenous population and are generally recognized by the surface expression of EpCAM-1 and Ly51. Very recently a potential cTEC precursor was identified in the embryo which was characterized by the expression of EpCAM-1 and CD205 while being negative or low for MHCII and CD40 expression (29). Moreover this presumable cTEC progenitor was highly proliferative and independent of

crosstalk with thymocytes while later stages of cTEC differentiation were found to require the presence of T cells.

1.1.4 mTEC progenitor

The earliest mTEC-specific progenitor that has been identified to date is a subset of Claudin-3 and -4 expressing TEC, isolated from E13.5 embryonic thymi (30). These cells were found to exclusively give rise to mTEC and not to cTEC when mixed into RTOC and grafted into nude mice, whereas Claudin3/4 negative TEC showed both, cTEC and mTEC potential. This data is indicative of a very early mTEC specification during ontogeny.

Based on the expression of CD80, MHCII and AIRE, mTEC can be subdivided into three major subsets: $CD80^{-}MHCII^{-}AIRE^{-}$ mTEC, $CD80^{+}MHCII^{+}AIRE^{-}$ mTEC and $CD80^{+}MHCII^{+}AIRE^{+}$ mTEC, whereby promiscuous gene expression (pGE), i.e. the ectopic expression of otherwise tissue restricted genes, increases in the same order, being highest in $AIRE^{+}$ mTEC. pGE, a phenomenon that has been shown to be essential for the establishment of central tolerance, is largely controlled by AIRE (31, 32). Initially it was found that the human Autoimmune Polyendocrine Syndrome Type I (APS-1) was linked to a mutation in the AIRE gene. Soon after, AIRE-deficient mice were generated, shedding more light on how AIRE enables tolerance induction to TRA. The function and molecular mechanism of AIRE is described below.

In the context of mTEC development there has been a long lasting debate whether AIRE-expressing mTEC represent the most mature or immature cell subset. Two models have been proposed: The progressive restriction model assumes that AIRE-expressing mTEC are progenitor cells that still have features of multipotent stem cells and constantly minimize pGE as they mature (33). The second competing hypothesis is the terminal differentiation model which implies that progressive differentiation leads to increased pGE. A prerequisite for this of course is, that $CD80^{-}MHCII^{-}AIRE^{-}$ mTEC develop into $CD80^{+}MHCII^{+}AIRE^{-}$ and finally into $CD80^{+}MHCII^{-}AIRE^{-}$ mTEC. In the last few years there was evidence accumulating, strongly arguing for the terminal differentiation model. For example $AIRE^{+}$ mTEC were found to be postmitotic and have an unexpectedly high turnover rate of 1 week which has been attributed to the pro-apoptotic function of AIRE (34, 35). The final prove was brought by reaggregate thymic organ culture (RTOC) experiments directly showing the progeny-precursor

relationship of $CD80^{-}MHCII^{-}AIRE^{-}$, $CD80^{+}MHCII^{+}AIRE^{-}$ and $CD80^{+}MHCII^{+}AIRE^{+}$ mTEC (Figure 1).

While early TEC development (before thymus specification) is independent of hematopoietic immigrants, proper cTEC and mTEC development requires interaction with T cells. Several surface molecules and signaling pathways have been implicated in this process: The NF-κB pathway plays a fundamental role in mTEC development. Interfering with components of the NF-κB pathway by inactivation of the TNF receptor-associated factor TRAF6 or the NF-κB complex component RelB and NF-κB inducing kinase (NIK) severely disrupted mTEC development and medulla formation (36). The lack of a functional medulla led to an uncontrolled release of auto-reactive T cells to the periphery and consequently to the development of multiorgan autoimmunity. In the same line it was shown that receptors activating the NF-κB pathway, namely the receptor activator of nuclear factor-kappa B (RANK) and CD40, critically contribute to normal mTEC differentiation. RANK-ligand/RANK and CD40L-ligand/CD40 interactions with T cells synergistically induce mTEC maturation in the adult thymus (37-39), whereas RANK signaling provided by $CD3^{+}CD4^{-}$ lymphoid tissue inducer cells was essential and sufficient to support the maturation of mature $AIRE^{+}$ mTEC in the fetal thymus (37).

The lymphotoxin-β receptor (LTβ-R) has also been implicated to be important for mTEC development, however its role has been controversial. While the LTβ-R was initially described to directly regulate AIRE and TRA transcription, this observation was soon challenged by the report of Boehm et al. showing that LTβ-R, instead of regulating AIRE itself, contribute to mTEC development (40-42). This controversy notwithstanding, there seems to be a role for LTβ-R signaling in mTEC development, albeit not a very fundamental one. Finally there was a report proposing that cognate interactions between mTEC and auto-reactive $CD4^{+}$ SP T cells crucially contribute to mTEC maintenance (43).

Collectively these data imply that a common TEC progenitor, that is present throughout life, differentiates into a cTEC or mTEC-commited progenitor in order to finally form mature cTEC and mTEC lineages respectively. The specification of these two lineages seems to undergo T cell-dependent as well as –independent stages (*Figure 1*).

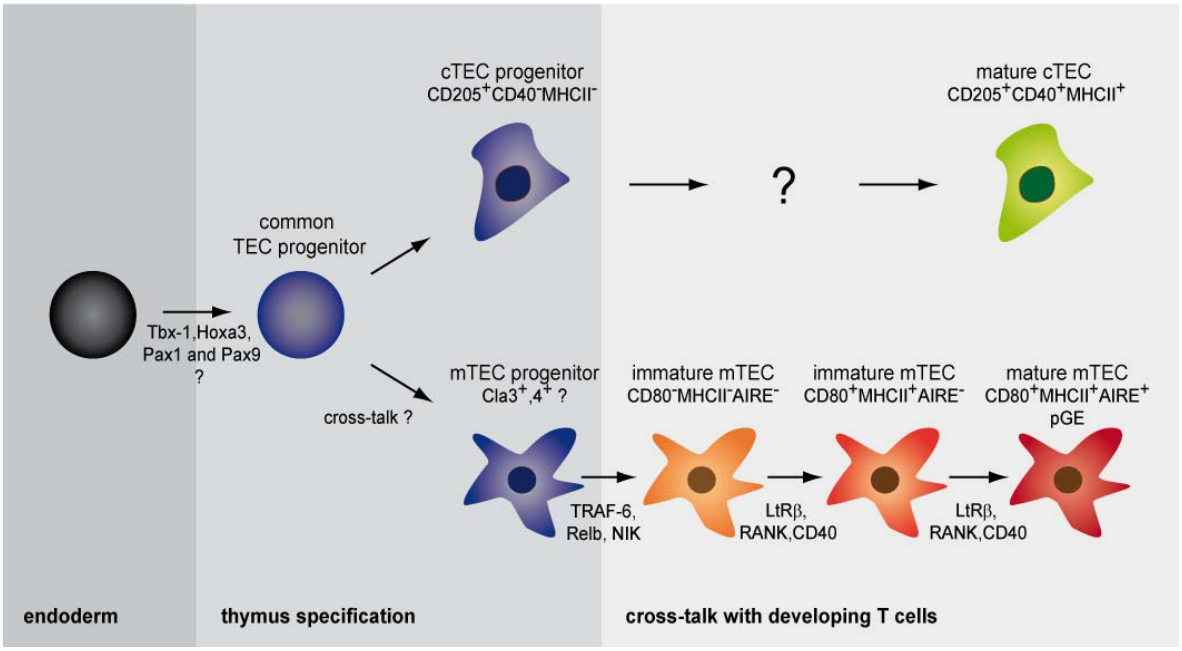


Figure 1 – TEC development. TEC development proceeds along several stages that are partially dependent on interactions with T cells. The thymus originates from the endodermal lining of the third pharyngeal pouch and gives rise to a common, bi-potent TEC progenitor which branches into the cTEC and mTEC lineage respectively. The receptors and signaling molecules involved in various developmental stages as well as the surface markers identifying the state of maturity are indicated.

1.1.5 The Autoimmune Regulator (AIRE)

The discovery of AIRE and its important role in tolerance induction is based on the genetic analysis of the autoimmune syndrome APS-1. Compared to other autoimmune diseases, the underlying genetics of APS-1 is rather simple with almost all identified mutations located exclusively in the AIRE gene (44). AIRE expression is restricted to very few cell types, being almost exclusively expressed in mature mTEC and to much lesser degree in DC in the thymus (45). With the generation of AIRE-deficient mice it finally became clear that AIRE is directly linked to central tolerance. Loss of AIRE expression led to the development of multiorgan autoimmunity with inflammatory infiltrates and autoantibody production, thus by and large recapitulating the disease pattern of APS-1 (46). These observations prompted the speculation that TRA expression might be controlled by AIRE and indeed detailed analysis of AIRE-deficient mTEC revealed that hundreds if not thousands of promiscuously expressed

transcripts are regulated by AIRE, e.g. insulin, salivary protein 1 and fatty acid binding protein. Of note not all TRA are under the control of AIRE, examples of which are C-reactive protein or GAD67, implying a potential role for additional, yet unknown factors (32).

The precise molecular mechanism of AIRE is still largely obscure. At first glance AIRE appears to be a classical transcription factor, however several findings hint at a broader function of AIRE. First, AIRE regulates the transcription of several thousand genes which is by far more than any other known transcription factor (32). Second, there appears to be an extremely high variability in the transcriptional footprint of AIRE between individual mTEC (47, 48). Third, the pattern of TRA transcription in mTEC differs from that in the respective tissue and beyond that it appears to be independent of the tissue-specific transcriptional regulators (49). Interestingly it was shown that transcriptional start sites can also differ in AIRE-dependent transcripts (49). Fourth, rather than qualitatively activating gene expression, AIRE quantitatively increases the level of transcription, thereby accentuating “transcriptional noise” (47, 50). Fifth, AIRE-regulated genes appears to be clustered in the genome (32).

Structural analysis of AIRE shed some light on how AIRE might exert its function. First, AIRE contains a SAND domain (Sp100 AIRE-1 NucP41/75 DEAF-1) that was suggested to be involved in DNA binding. But instead of a strong and specific binding to DNA, AIRE seemed to interact with DNA in a rather non-specific manner and no conserved DNA binding element has be identified (51). Second, the PHD finger (plant homeo domain), a typical protein-protein interaction site, was speculated to enable AIRE to interact with nucleosomal histones (52). In particular it was found that AIRE specifically binds to unmethylated lysin 4 histon 3 (K4m0H3), interestingly a marker for inactive regions. This might be operative in concentrating AIRE at transcriptionally inactive sites in order to activate transcription. A third important domain, the CARD domain (caspase recruitment domain), was shown to be a crucial protein interaction domain, which could for example serve as a site for interactions with other transcriptional regulators (53). Therefore the identification of potential interaction partners of AIRE has been of great interest to in order to understand its mode of action (*Figure 2*).

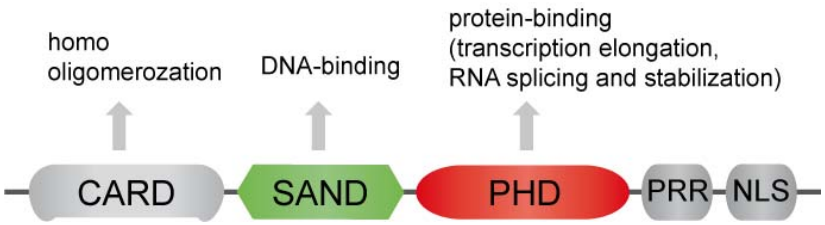


Figure 2 - Structural and functional domains of AIRE. AIRE contains three major domains, the caspase recruitment domain (CARD), Sp100 AIRE-1 NucP41/75 DEAF-1 (SAND) and plant homeo domain (PHD). The proposed functions of the different domains are depicted.

The first protein that was found to bind to AIRE was the ubiquitous transcriptional activator CREB binding protein (CBP), whereby CBP is translocated to the nucleus in an AIRE-dependent fashion (54). Another binding partner of AIRE is the DNA-dependent protein kinase (DNA-PK) which was described to be activated by double-stranded DNA breaks and to phosphorylate proteins that are involved in transcription such as Polymerase II (Pol II) (55). P-TEFb was a very promising candidate as it is involved in transcriptional elongation by helping Pol II to be released from the initiation complex (56). Interestingly, proteins involved in splicing have also been found to interact with AIRE.

The question of how AIRE can activate such a large battery of transcripts is of central importance in tolerance. A model, albeit highly speculative, has been proposed in which AIRE first binds in a rather non-specific manner to CpG-rich promoter regions, thereby being recruited to a large number of loci. Furthermore, the predisposition of AIRE to interact with H3K4m0 would favor its recruitment to transcriptional inactive sites. At that point several, probably synergistic, activities might come into place. Being located to the DNA, AIRE might recruit several transcriptional activators like P-TEFb and thereby help Pol II to disengage from the transcription initiation complex. Of note, it was found that a large number of inactive genes have a preformed initiation complex at their promoter regions that is however unable to be elongated. Finally AIRE could also interact with splicing factors in order to facilitate processing and export of transcripts (57).

The role of AIRE with respect to tolerance induction has been extensively explored. It is believed that the main role of AIRE is to ensure the deletion of TRA-reactive T cells which was confirmed by the detection of eye and stomach-specific T cell clones and auto-antibodies in AIRE-deficient mice (58, 59). In the same line, negative selection was diminished in TCR/neo-self-antigen double transgenic mice in the absence of AIRE (35, 60, 61). Another potential mode of tolerance that might be influenced by AIRE is the induction of regulatory T cells (T_{reg}). It was shown that $AIRE^+$ mTEC are capable of directly inducing T_{reg} in a TCR/neo-self-antigen transgenic mouse model (62). However, transplantation of WT and AIRE-deficient thymi into athymic mice did not rescue from autoimmunity which speaks in favor of a T_{reg}-independent defect in tolerance (35). In a similar vein crossing AIRE-deficient mice onto a T_{reg}-deficient background did even deteriorate the clinical picture strongly arguing for a defect in negative selection. Finally AIRE was also proposed to play a role in the periphery where it was found to be expressed by very rare cells in the LN and spleen that also express TRA, although with limited diversity (63). To date the physiological relevance of this phenomenon is unclear.

1.2 T cell development – thymic microenvironments

Whereas most hematopoietic cell lineages undergo differentiation and maturation in the bone marrow, T cells develop in a specialized organ, the thymus. T cell development proceeds in a temporarily and spatially strictly controlled order which is dictated by the microenvironment provided by the thymic stroma. The journey through the thymus is a matter of surviving; developing T cells have to go through several checkpoints where they either die or pass on until they are released to join the peripheral T cell pool as mature T cells. Only those T cells bearing a functional TCR that is self-MHC restricted and able to interact with peptide-MHC (pMHC) complexes in a certain window of affinity and/or avidity will pass the first checkpoints, the so-called β-selection and positive selection which takes place in the thymic cortex. After the first selection process that is accompanied by a tremendous cell loss, T cells translocate to the medulla and undergo tolerance induction during which self-reactive T cells are deleted, leaving a fully tolerant T cell repertoire (3, 64, 65).

1.2.1 Early T cell development in the thymic cortex

Thymus-resident T cell progenitors have no or only very limited self-renewing potential; thus, in order to generate new T lymphocytes throughout life, a continuous influx of bone marrow-derived hematopoietic progenitor cells from the blood is required (66, 67). The entry of early thymic progenitor cells into the thymus, a process that is thought to be a gated phenomenon, was suggested to happen through high endothelial venules (HEV) that are located at the cortico-medullary junction (68, 69). The mechanism of thymus homing is not completely understood, however the involvement of different adhesion molecules (P-selectin, VCAM-1, MadCAM-1, ICAM-1, laminin and fibronectin) and chemokines such as CXCL12, CCL21 and CCL25 has been proposed (8, 70-76).

Classically, early T cell development is subdivided into four stages defined by the cell surface markers CD25 and CD44 and location of the cell within the cortex (77). The earliest T cell progenitors are found within the most immature subset of thymocyte precursors, the double negative (DN) 1 cells that lack CD4 and CD8 expression. Typically enriched in the perimedullary cortex, DN1 cells are characterized by the surface expression of CD44 and CD117 and the absence of CD24 and CD25 (78). Not yet being committed to the T cell lineage, Notch-1 was shown to provide a crucial signal for T lineage specification as deletion of Notch-1 led to a complete block in T cell development and ectopic differentiation of B cells in the thymus (79-82). The requirement for Notch-1 signaling will not be lost until TCR rearrangement is complete in later stages of development.

Lasting up to two weeks in the DN1 stage until progressing to the DN2 stage, DN1 cells have by far the longest single period of intrathymic residence during which they expand by about 1000 fold (83, 84). The proliferation and survival of developing T cells is absolutely dependent on interactions with thymic microenvironments. Even though the molecular mechanisms involved are not fully understood, the Wnt signaling pathway and Kit ligand (stem cell factor) seem to play a role in providing signals for proliferation (85-87).

After this period of proliferation, cells differentiate into the more T cell-restricted DN2 stage that is marked by the up-regulation of CD25 and accompanied by migration outwards into the inner cortex. Note worthy, it is here that recombination-activating protein (RAG) gene expression is up-regulated for the first time and the first

rearrangements in the TCRγ/δ gene loci appear, whereas TCRα/β loci genes are still not accessible at this stage leading to TCRγ/δ versus TCRα/β lineage divergence (88). IL-7 has been shown to be essential for TCRγ/δ locus accessibility. Furthermore, IL-7 is required for survival and expansion of DN2 cells, as has also been shown for Kit ligand provided by cTEC (89-92).

The signals involved in emigration from the perimedullary cortex are largely obscure. CXCR4 mutant lymphocytes have been found to be blocked at the DN1 stage as a consequence of being unable to migrate into the central cortex (75). A prerequisite for directional cell migration is not only a polarizing signal, but also a substrate for cell adhesion. Much in contrast to conventional migration along an extracellular matrix, DN2 cells "crawl" along the stromal matrix involving VCAM-1 and E-cadherin on epithelial cells (73). It has been speculated that the need for direct interaction with thymic stromal cells might represent an intrathymic feedback loop controlling the size of the various differentiation stages of T cell development. Like DN1, DN2 thymocytes still retain the potential to develop into non-T cell lineages, i.e. DC and NK cells; thus, Notch-1 signaling in DN2 cells triggered by its ligand Delta-like 4 on cortical epithelial cells is indispensible for further T lineage specification (93, 94).

After a rather short residence time of about two days, DN2 cells precede their maturation and migrate into the outer cortex, where they enter the again two days lasting DN3 stage which is concurrent with the down-regulation of CD44 and CD117 and a low rate of cell cycling (83). Key events at this stage are the opening of the TCRβ locus, presumably regulated by IL-7 signaling, and the recombination of the germ line encoded VDJ segments of the TCRβ locus (95, 96). First D – J rearrangements take place on both alleles and subsequently the recombined DJ segments rearrange with the V segment forming the TCRβ chain. This second step however strictly underlies the so-called "allelic exclusion", meaning only one allele will eventually complete TCRβ recombination (97, 98). Implicit in this process is the formation of the pre-TCR, a complex consisting of a TCRβ chain, a pre-TCRα chain, CD3δ, ε, γ and TCRζ, an event that is decisive for the fate of the individual cell (99, 100). Pre-TCR formation is a key process in T cell development and represents the first checkpoint, known as β-selection, which ensures that only those T cells that have successfully rearranged their TCRβ gene segments and hence express a fully functional pre-TCR on their surface are permitted to survive and to undergo further

differentiation (101, 102). This is marked by proliferation, the up-regulation of the co-receptors CD4 and CD8 and finally irreversible T lineage commitment (103-105) .
The transition from CD4/CD8 double negative to double positive immature T cells is referred to as DN4 or pre-DP stage and coincides with the continued migration to the outermost cortex, the subcapsular zone, which is critically dependent on CCL25 (106). It is here that rearrangement of the TCRα gene locus is initiated but only after a massive expansion of those cells carrying a functional pre-TCR took place (107). During the whole expansion phase, the Rag genes are turned off to prevent any premature rearrangements of the TCRα locus. The enlargement of the precursor pool makes sure that every TCRβ positive cell gives rise to numerous progeny cells, each of which able to independently rearrange their α-chain, thereby increasing the possibility to survive and more importantly the diversity of the α/β–T cell repertoire.

1.2.2 Positive selection

The second important checkpoint in T cell development, the so called positive selection, is reached when DN cells become DP and ensures the generation of a self-restricted TCR pool. Here for the first time the polarity of cell migration is changed and DP cells migrate inwards towards the medulla (108). This developmental stage is mainly characterized by the rearrangement of the TCRα-locus and, albeit low level of TCRα-locus rearrangement is readily detected in DN4 cells, full-scale α-locus rearrangement does not take place until the DP T cell stage. In contrast to β-selection that simply requires T cell intrinsic pre-TCR signaling, positive selection is strictly dependent on TCR-pMHC interaction between T cells and cTEC (109-111). Accordingly it is not sufficient to simply express a functional TCR consisting of a successfully rearranged β- and α-chain, but to generate a TCR that additionally recognizes self-peptide–MHC complexes (112). This phenomenon was termed “recognition of self” and was first described in F1 bone marrow chimeras revealing that T cells did only recognize antigen when presented by MHC of the same type as the bone marrow recipient (113). Direct proof followed with the generation of the first TCR transgenic mouse; T cells ectopically expressing a

rearranged TCR were only positively selected in a thymus of the same haplotype the TCR originated from in the first place.

Intuitively one would expect that the possibility of a T cell receptor to recognize MHC by chance is extremely low, therefore it was speculated that the TCR has developed an inherent propensity to bind MHC (114). In support of this notion it was shown that random combinations of TCR α and β chains or pre-positive selection TCR react with a surprisingly high frequency with MHC (115-117). In the same line it was found by studying the crystal structure of the TCR that the CDR1 and CDR2 loops encoded in the V region showed an intrinsic specificity for MHC (118).

Despite this inherent specificity for MHC, the efficiency of generating a MHC-restricted TCR is very low; thus, T cells are able to continuously rearrange their TCRα-locus, which is structured in a way that allows for multiple rearrangements on each allele, until they are positive selected. However, DP T cells have only a certain amount of time, as they have a life span of only 3-4 days until they either have generated the right TCR or die. Several factors, such as RORγ and TCF-1, have been implicated in the regulation of survival during positive selection by up-regulating the anti-apoptotic factor Bcl-X_L (119-121). Notwithstanding all these mechanisms to increase the possibility for a DP cell to be positively selected, most developing T cells do not pass this checkpoint and die through “death by neglect” (*Figure 3*).

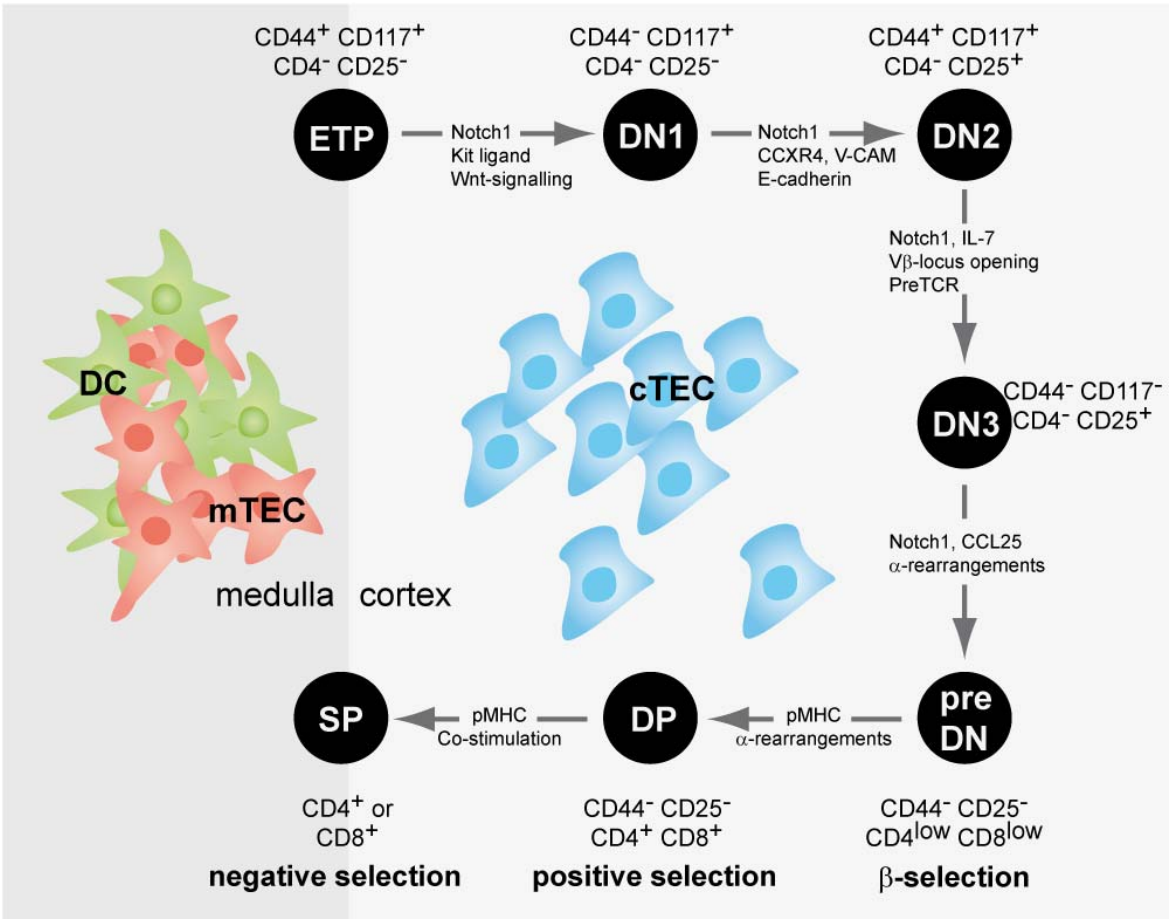


Figure 3 – T cell development. Early T cell development in the thymus proceeds along several distinct stages marked by the differential expression of CD44, CD117, CD25, CD4 and CD8. Essential factors for differentiation are depicted. Developing T cells are passing three major checkpoints, β-selection, positive selection and negative selection, at which they either die or further differentiate.

Cortical thymic epithelial cells play a central role in positive selection. Presenting self-peptide-MHC complexes to developing T cells, cTEC select those TCR fulfilling the requirements for positive selection, thereby shaping the T cell repertoire towards self-restriction. Considering the paradox that antigen recognition by DP T cells in the cortex can lead to positive selection and survival while the very same event in the medulla can trigger negative selection and cell death (see section on negative selection below), it is of great interest to identify the decisive factor(s) leading to these completely opposite outcomes.

One possibility implicates the T cell itself which might integrate signals differently depending on its differentiation stage. Changes in gene expression pattern might not only impinge on the acquisition of responses to new signals but also on the loss of response to former signals. Indeed it was shown that the threshold to discriminate between a strong and a weak engagement of the TCR is actively altered in DP T cells as compared to mature T cells, whereby the latter completely lose their ability to respond to weak TCR-binders implying an intrinsic regulation of T cell sensitivity during development (122).The molecular mechanisms underlying this phenomenon are largely unknown. It has been postulated that the signaling molecules proximal of the TCR play a fundamental role by acting as signal integrators of activating and inhibiting signals. Precisely, the TCR threshold is fine-tuned by constant inhibition via dephosphorylation involving several phosphatases like SHP-2 and PTPN22, whereby their quantitative alteration can either increase the threshold of activation or turn the cell more sensitive to TCR engagement as in the case of DP T cells (123).

A different way to explain the paradox of positive selection was the affinity and avidity models that were focusing on the TCR itself and its binding property to the pMHC complex. Supporting this, it was found that very low concentrations of an antigenic peptide could mediate survival and efficient positive selection, while high avidity interactions promoted cell death in fetal thymic organ cultures (FTOC) (111, 124).

Whereas these studies are in support of the avidity model, others clearly speak in favor of the affinity hypothesis by showing that positive selection requires pMHC complexes with antagonist or partial agonist properties (125). Generally, whether the selecting peptide was related or not, affinity studies supported the idea that the TCR affinity for ligands mediating positive selection was much lower than for negative selection.

Another mutually non-exclusive possibility was based on the assumption that peptides presented in the cortex possess unique features that are required for positive selection. This so-called “altered peptide hypothesis” suggests that there are different ligands presented by cTEC as compared to those presented in the medulla and in the periphery. Peptide elution studies, however, comparing cTEC and splenic APC did not reveal any major differences in the ligandome of these two cell types (126). In line with this, two other studies showed that even a drastically reduced ligandome, using single-ligand mouse or an H-2DM deficient mouse, was able to support the selection of a normal T-cell repertoire (127, 128). However, later it was

shown that positive selection by H-2DM$^{-/-}$ epithelium, bearing a limited peptide spectrum, was reduced threefold in the absence of negative selection by DC (129).
Whereas most of these findings would rather argue against an altered peptide hypothesis, several studies clearly speak in favor of it, all implying a differential antigen processing in cTEC: It was found that the cTEC-specific endoprotease Cathepsin L was critical for a normal CD4^{+} SP T cell compartment, presumably by generating peptides for MHCII suitable for positive selection (130). Likewise, inactivation of the thymus specific serine protease TSSP lead to decreased positive selection of several transgenic TCRs (131). Furthermore, another study showed the importance of macroautophagy, a peptide degradation process, in the selection of the TCR repertoire as positive selection of several TCR transgenic T cells was altered in autophagy-deficient thymi (132). There are also some indications for a differential MHCI peptide generation in the cortex. Recently a cTEC-specific proteasome was identified which was shown to be absolutely essential for normal CD8 T cell development beyond the DP stage, again indicating a role for the generation of “altered peptides” in the cortex (133).

1.2.3 CD4/CD8 lineage choice

The next important step immediately following positively selection is the so-called CD4/CD8-lineage choice in which thymocytes either differentiate into MHCII-restricted CD4 or MHCI-restricted CD8 single positive (SP) T cells. This decision-making process, albeit not fully understood yet, has been subject of intense investigations. Based on the fact that the extracellular domain of CD4 can only bind to the invariant domain of MHCII and CD8 can only bind to MHCI, CD4 bearing T cells recognize antigen exclusively in the context of MHCII and vice versa (134, 135). Several classical and non-classical theoretical models have been proposed trying to explain how the T cell is able to sense whether it is MHCII- or MHCI-restricted:
The classical models are either of stochastic or instructive nature and are built on the assumption that the termination of one or the other co-receptor irreversibly defines the lineage fate. While the stochastic selection model implies that CD4 or CD8 termination occurs randomly and thymocytes either receive a second TCR rescue signal in case the right receptor was repressed or die (136, 137), the instructive

model suggests that the MHCII- and MHCI-restricted TCR signals are distinct from one another, with respect to the signal strength (138). Another classical instructive model is the duration-of-signal model which also comprises that the CD4/CD8 lineage decision requires qualitatively different signals, however not the signal strength but the duration of the TCR signal determines the lineage choice (139). Accordingly, TCR signals of long duration terminate CD8 expression while short TCR signals terminate CD4 expression.

In the end all three models have been contradicted by experimental observations, however the idea of the TCR signal duration as a major CD4/CD8 lineage determinant remains still valid today and is the central feature of the kinetic signaling model (140).

With the finding that positively selected TCR-signaled DP T cells terminate CD8 expression, it became clear that $CD4^{+}CD8^{low}$ T cells are uncommitted and represent the immediate precursor of CD8 and CD4 SP T cells (141, 142). Consequently, MHCI-restricted signals are short natured whereas MHCII-restricted signals are long natured. Based on this, the kinetic signaling model suggests a scenario in which the T cell senses changes in TCR signaling after CD8 down-regulation; if the T cell is MHCII-restricted and therefore CD4-dependent, the TCR signal will not alter after termination of CD8 and the cell will be deviated into the CD4 SP lineage, however if the T cell is MHCI-restricted and consequently dependent on CD8, the TCR signal will be weaker without CD8 expression, signaling the cell to differentiate into the CD8 lineage.

Of central importance in the process of CD4/CD8 lineage choice are IL-7 as well as several transcription factors. IL-7 was shown to be crucial for CD8 lineage choice as blockade of IL-7R signaling abrogated the termination of CD4 transcription and up-regulation of CD8 transcription and hence CD8 lineage commitment (143). Recently a transcriptional network has been implicated in T lineage choice including the transcription factors ThPOK (T-helper-inducing POZ/Krueppel-like factor) and RUNX3 (Runt-related transcription factor). CD8 down-regulation is dependent on ThPOK and further it prevents RUNX3 from silencing the CD4 locus (144, 145). Moreover, TOX (Thymocyte selection-associated high mobility group box protein TOX) has been found to be responsible for maintaining or up-regulating CD4 transcription in positively selected DP T cells and finally it was observed that GATA3 blocks CD8 lineage differentiation while promoting CD4 lineage choice (146-149).

Intense studies in the last years have shed much light on the process of CD4/CD8 lineage choice, however the exact sequence of events and the molecular players involved still remain to be determined (*Figure 4*).

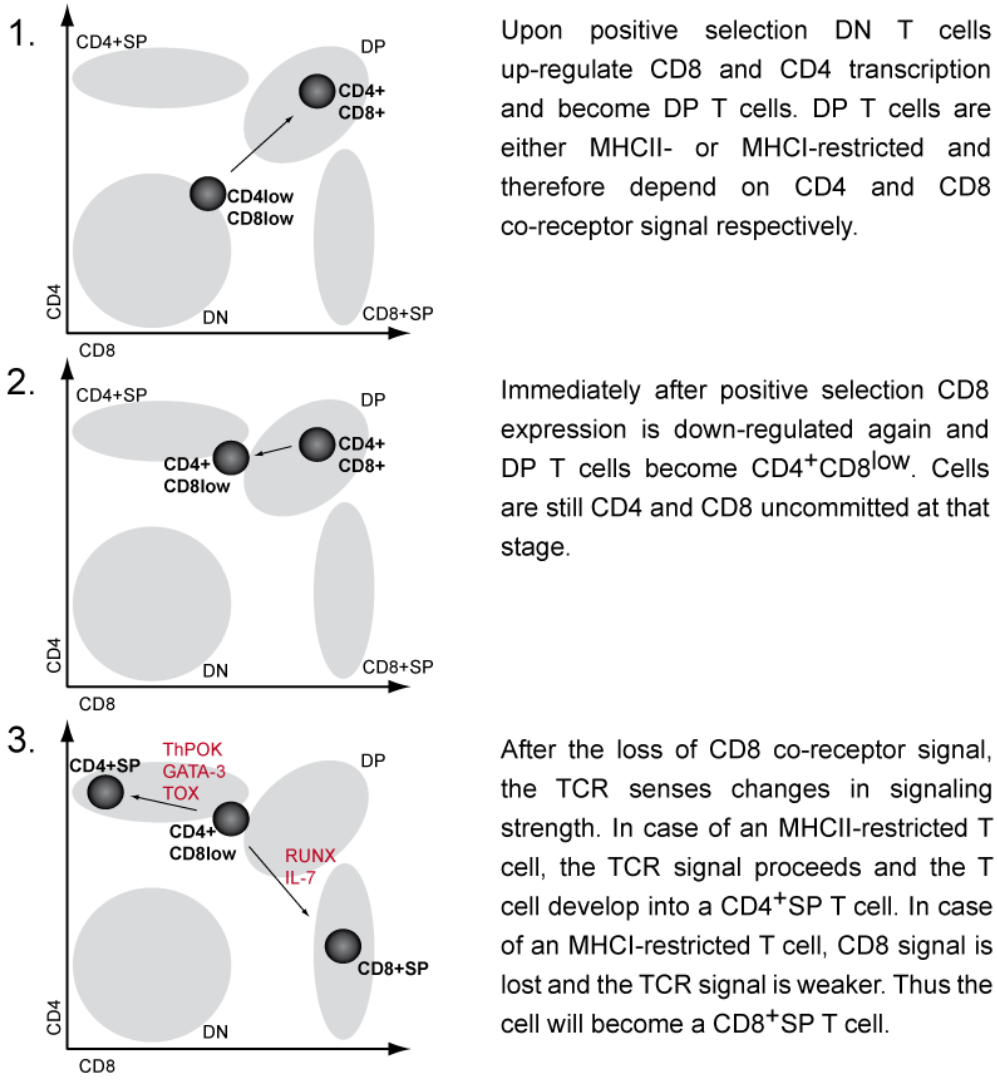


Figure 4 - Kinetic signaling model. Depicted are the three sequential steps in the CD4/CD8 lineage decision process. Surface molecules indicative of different maturation stages as well as factors necessary for CD4 or CD8 lineage choice are shown.

1.2.4 Central tolerance – a historical overview

Early concepts of immunity date back to the beginning of the 20th century and the theory of Paul Ehrlich on antigen recognition. To enlighten the conundrum how the body can respond to an enormously wide range of pathogens, he proposed the "side-chain theory". He believed that every naturally occurring antigen can be recognized by at least one chemical side chain expressed on the surface of cells. A side chain can form a link with the appropriate antigen which leads to the production of high amounts of the very same chain and its secretion into the blood as an "antibody".
Ehrlich´s selective theory of antibody production had soon been challenged by the work of Karl Landsteiner and Merill Chase, showing that antibodies could be raised against any chemical structure – even synthetic structures. Thus they introduced the "template instructive hypothesis" that dominated the first half of the 20th century. The common believe was that an antigen interacts with a globulin in the early stage of globulin formation and thereby helps to form a mature globulin molecule specific for the antigen. Even though this concept could explain how antibodies can be formed against any synthetic structure, it failed to account for the rapid rise in antibody production after immunization as well as the phenomenon of memory formation.
Niels Jerne, in 1955, dealt with this problem. He picked up Ehrlich´s initial ideas and proposed the "natural selection theory" of antibody formation. According to his theory, an antigen binds to an antibody by chance, and triggers replication of the very same antibody (150).
Jerne´s selection theory paved the way for Frank Macfarlane Burnett´s "clonal selection theory", a seminal landmark modern immunology (150). While Jerne considered the antibody as the replicating unit, Burnett for the first time positioned the cell itself in the center of interest, being the replicating unit. According to this, antibodies would function as cell surface receptors that can eventually bind to an antigen, whereby the cell is activated and gives rise to numerous clones that massively produce and secrete antibodies of the very same specificity. Consequently the cell became the important unit being the selectable element in immunity. Burnett also solved the problem of memory formation as well as affinity maturation suggesting that mutations in the process of replication would eventually result in an increased affinity to a particular antigen. Most importantly he proposed a model for the phenomenon of tolerance, for which he together with Peter Medawar shared the

Nobel Prize for physiology and medicine in 1960 and which is, at least in part, still valid today (151). He was convinced that as a consequence of random antibody generation, the immune system had to be purged from auto-reactive cells during embryogenesis and early after birth when the cells are exclusively exposed to self antigens.

It took over 20 years and great advances in cellular and molecular immunology until the paradigm of “developmental tolerance” was again disproven by the work of Nicole Le Dourain and colleagues. They found that embryonic tissues from quail engrafted into age-matched chickens were rejected soon after birth (152, 153) and importantly, this graft rejection could be prevented by solely transplanting thymic rudiments from the graft donor. This certainly highly unexpected finding revealed two important points: first, it was not sufficient to simply “see” antigen during embryogenesis in order to acquire self-tolerance and second, peripheral, tissue-specific tolerance was established by pure thymic epithelium that was completely devoid of hematopoietic cells. The mechanism of how thymic epithelium can induce tolerance to the whole spectrum of peripheral antigens was still not understood at that time. However, a few years later, in 1989 Linsk et al. proposed a model according to which the thymus represents a patch quilt of ectopically expressed genes (154). Direct proof followed with the finding that “promiscuous gene expression” (pGE) of tissue-restricted antigens (TRA) was a specific attribute of medullary thymic epithelial cells (mTEC) and that this phenomenon was largely regulated by AIRE (45, 46) and absolutely crucial for central tolerance.

The mechanism of how self-reactive T cells are purged from the body became clear in the middle of the 80s when B and T cell receptors were discovered and the first T cell receptor transgenic mouse was generated, making it possible to follow and visualize T cells during development (155). Self-reactive T cells were found to be deleted upon antigen encounter in the thymus. However, in all these systems, deletion was rarely complete raising the question whether there might be additional mechanisms of tolerance.

The missing link was provided a few years later by the identification of a T cell subset that was essential for the prevention of autoimmunity. Belonging to the CD4 T cell lineage, these cells expressed the high affinity interleukin-2 receptor and functioned by actively suppressing other potentially dangerous T cells (156-159). A new

mechanism of tolerance was finally established, termed dominant tolerance, which should dominate the field of immunology ever since.

1.2.5 Negative selection

Negative selection represents the third and last critical checkpoint during T cell development and is operative in removing potentially dangerous self-reactive T cells. Even though Le Dourain most convincingly established the importance of the thymus in central tolerance, the biological mechanism of how auto-reactive T cells are rendered innocuous was only speculative. Two scenarios were favored, proposing that potentially harmful T cells are either silenced by undergoing anergy or are purged from the repertoire by clonal deletion. The outstanding work of Marrack et al. finally shed more light on this issue by showing that T cells, that recognized antigen in the thymus were eliminated, a finding that was clearly in support of the clonal deletion model (160). It was found that T cells carrying the Vβ17a TCR chain were deleted in animals expressing superantigens derived from the mouse mammary tumor virus and presented in the context of I-E MHC molecules, while the same T cells normally matured and migrated to the periphery in the absence of superantigens. Moreover, it became clear that there are various superantigens (Mtv-8,-9) depending on the mouse strain that are recognized by particular Vβ segments (Vβ17a, Vβ5, Vβ11) (161, 162).

Later, the clonal deletion model was further validated in numerous TCR transgenic mouse models expressing a receptor for a self antigen along with the self antigen. In most of these systems the antigen was transgenically expressed (e.g. Hemagglutinin, HA), in some others however TCR-transgenic mice were generated that recognized a naturally expressed antigen (e.g. H-Y) (reviewed in (3)). These mice provided an excellent tool for studying negative selection, with respect to the anatomical location, the relevant APC and the molecular signaling events involved.

It has been a longstanding issue where and when self-reactive T cells are removed from the thymus. Intuitively, the medulla is the likely site for negative selection, as it provides the most complex ligandome. Unlike the cortex it is heavily colonized by hematopoietic APC, presumably carrying all kinds of peripheral blood-born antigens and furthermore, the medulla is composed of mTEC, providing a wide range of rare

tissue-restricted antigens. In the same line, clonal deletion most likely occurs rather late in T cell development as DN T cells not yet express an intact TCR which is prerequisite for negative selection. The problem appears, however, that the medulla contains mainly mature T cells that are resistant to negative selection; nevertheless it can be assumed that mature SP T cells upon migration to the medulla remain susceptible to deletion for a certain time span. Indeed it was found that the medulla contains a substantial fraction of semi-mature, HSA^{high} $CD4^{+}$ SP T cells that were still sensitive to tolerance induction (163).

The process of negative selection was found to be remarkably efficient, as already minute amounts of DC were sufficient to switch from no deletion to full scale deletion of polyclonal T cells in an *in vitro* RTOC system (64, 164). Ligands inducing negative selection are unlike positively selecting ligands highly stimulatory to mature T cells. Thus negative selection of T cells is generally initiated by high affinity pMHC-TCR interactions that induce strong signaling through the TCR, albeit TCR triggering alone was shown to be insufficient for negative selection. Several co-stimulatory cell surface molecules have been described to play a role in the induction of negative selection; among these are CD28, CD5, CD43 and Fas (165, 166). In addition it was found that mice deficient for the co-stimulatory receptor CD40 were also found to be impaired in negative selection (167).

Even though the proximal TCR signaling events overall appear to be similar in positive and negative selection, some molecules have been uniquely attributed to negative selection (3). In particular the mitogen activated protein kinase (MAPK) family members Jun N-terminal kinase (JNK) and p38 are central players in the initiation of negative selection as deficiency in either one of these pathways leads to defective induction of apoptosis in self-reactive T cells (168). Early TCR proximal signaling events involved the adaptor protein Grb-2 which recruits the Ras-activator SOS1 to the membrane and ultimately leads to the activation the JNK/p38 pathways (168). Recently another important activator of the JNK/p38 kinases, the Misshapen-Nck-interacting kinase-related kinase (MINK), has been identified to play a fundamental role in negative selection (169). Its expression is highly regulated in T cell development, being low in DN and strongly up-regulated in DP thymocytes. Accordingly, knock down of MINK had no effect on positive selection, while it specifically interfered with negative selection by crippling the apoptosis pathway (170).

Apoptosis has a central function in tolerance induction by being essential for the deletion of auto-reactive T cells. The executor molecules in this process are primarily members of the Bcl-2 family, like BIM as well as Bax and Bak that are pro-apoptotic family members. Accordingly, deficiency of BIM as well as combined deficiency of its down-stream effectors Bax and Bak hampered negative selection (170, 171). Furthermore, it was shown that BIM activation crucially depends on the JNK and p38 kinase pathways, whereby expression of BIM is directly regulated by the transcription factor Nur77 which belongs to the orphan steroid receptor family. Interestingly, in addition to its function as a transcription factor, Nur77 has been found to directly bind to the anti-apoptotic factor Bcl-2, thereby converting it into a pro-apoptotic molecule. Thus in the presence of Nur77, Bcl-2 is unable to inhibit BIM (172).
Taken together, negative selection involves several, in part synergistic events including the activation of the JNK/p38 pathways by Grb-2 and MINK, which in turn leads to the induction of the pro-apoptotic factors BIM, Bax and Bak. Furthermore, besides activating BIM expression, Nur77 inhibits the anti-apoptotic molecule Bcl-2 ultimately leading to induced cell death which is the basis of negative selection.

1.2.6 Dominant tolerance - Regulatory T cells

Ever since its discovery, negative selection was proposed to be the ultimate mechanism mediating central tolerance. However, in the middle of the 90s it became clear that an additional branch exists, representing a yet unknown mode of tolerance, the so-called dominant tolerance. This was based on the observation that the process of negative selection in the thymus is incomplete as the normal immune system harbors T cells that are self-reactive and even capable of inducing autoimmune disease. Consistent with the notion that auto-reactive T cells are present in the periphery, a subset of $CD4^+$ T cells expressing the IL-2 high affinity receptor CD25 was found to suppress the activation and expansion of these potentially hazardous cells and therefore crucially contribute to the prevention of autoimmunity (173).
Evidence for dominant tolerance came from experiments showing that neonatal thymectomy between day 2 and 4 after birth led to severe autoimmunity (174, 175). Soon after, more light was shed on this observation by identifying the regulatory cell

subset responsible for this phenomenon. Transfer of splenic T cells depleted of $CD4^+CD25^+$ T cells and injected into athymic mice was shown to be sufficient to induce autoimmune disease. Importantly the transfer of $CD25^+$ together with $CD25^-$ $CD4^+$ T cells completely prevented disease development. Thus it was thereon accepted that $CD4^+CD25^+$ regulatory T cells (T_{reg}) are crucial mediators of tolerance by keeping auto-reactive T cells under control (176).

T_{reg} share many hallmarks with conventional activated T cells, e.g. CD25, PD-1, GITR and CTLR-4, which makes their reliable identification very difficult. With the discovery of the transcription factor Foxp3 (Forkhead Box protein P3) a true marker and moreover a master regulator of T_{reg} was finally found (177-179). Foxp3 was first identified in the "scurfy" mouse mutant strain that suffered from severe autoimmune manifestations that were caused by a complete lack of T_{reg}. Detailed analysis revealed that Foxp3 is essential for the development of T_{reg} by amplifying already established features of T_{reg} precursors in the thymus and furthermore, it was shown to be indispensible for the function and maintenance of T_{reg} (180-182).

It is well established that the induction of autoimmunity is driven by the recognition of self constituents by naive T cells in the periphery. In healthy animals these potentially harmful T cells that have escaped negative selection in the thymus are controlled and suppressed by T_{reg}. This raised the question whether T_{reg} might overlap with auto-reactive T cells with respect to their TCR specificities, implying that T_{reg} development might also depend on tolerogenic interactions with self–antigens. Evidence for an affinity model of T_{reg} development came from TCR transgenic mouse models showing that the expression of a cognate antigen in the thymus could lead to the development of TCR transgenic T_{reg} specific for the antigen (183, 184). Thus it was believed that the increased affinity of a given TCR to a self-peptide-MHC complex would favor the induction of T_{reg}. In a polyclonal system the requirement of self-peptide for T_{reg} development is difficult to prove as it is not feasible to trace the fate of individual T cells carrying a specific TCR. Studies analyzing the diversity of the TCR repertoire, however, yielded some interesting insights concerning the TCR specificity of T_{reg}. Using a TCRβ transgenic mouse with a single copy of a TCRα minilocus, the variability of the α-chain could be determined. Vα2-containing variable regions of the naïve versus the regulatory thymic and peripheral T cell pool were sequenced and compared to each other (185, 186). Strikingly, the repertoire of the two pools was highly distinct and only partially overlapping whereas the diversity was comparable.

Of note, there was a high similarity between thymic and peripheral T_{reg} as was the case for thymic and peripheral naïve T cells, again suggesting that the majority of T_{reg} arise in the thymus.

TCR signaling is essential but not alone sufficient for the development of regulatory T cells. Cytokine signaling and co-receptor signaling were found to crucially contribute to this process. Studies interfering with cytokine signaling by either making use of a common γ chain-deficient or a STAT5-deficient mouse revealed an importance of these pathways for T_{reg} generation (187-189). Attempts to nail down a particular cytokine crucial for this process failed. Neither IL-2 nor IL-15 alone was required for the generation of T_{reg} in the thymus, albeit IL-2 was found to be essential for the maintenance and survival of T_{reg} in the periphery (187, 190). Thus it is currently unknown whether a single cytokine or a combination of several common γ chain cytokines, acting in a redundant manner, is essential for the development of T_{reg}. Furthermore, interference with TGFβ signaling demonstrated a requirement of TGFβ in the first wave of T_{reg} induction, a requirement that is later compensated for by IL-2 (191). Finally CD28-B7 signaling plays a central role for the development of T_{reg}. CD28-deficient mice have a strongly reduced T_{reg} compartment in the thymus which was not a consequence of a lack of soluble factors like IL-2 but of a defective in the T cell intrinsic program induced by CD28 itself (192, 193).

In summary, the initiative step in T_{reg} development seems to be high affinity interactions with self-peptide-MHC complexes, whereby cytokines and co-receptor signaling support this differentiation process. How T_{reg} are rescued from negative selection and what particular features of APC are needed for T_{reg} induction is still poorly understood. A recent study was addressing this question and found that T_{reg} differentiation does not require a particular APC but instead underlies an intrinsically regulated mechanism whereas the capacity to differentiate into T_{reg} inversely correlates with the maturation status of the T cell (194). However, if there is an optimal window of avidity that favors T_{reg} induction which is lower than the avidity required for negative selection is still unknown and will be a prospect of this study.

1.2.7 Antigen presentation in the medulla

To understand how central tolerance is maintained and how different modes of tolerance are induced, it is indispensable to get a detailed picture of the different APC in the medulla and their specific properties regarding antigen presentation. Tolerance induction occurs mainly in the medulla which consists of two types of APC, namely mTEC and DC. DC are a rather heterogeneous population containing $CD11c^{int}$ plasmacytoid DC (pDC) and $CD11c^{high}$ conventional DC (cDC) that can be further subdivided according to SIRPα expression (195). SIRPα^- cDC were found to originate in the thymus, while about one third of cDC, characterized by the presence of SIRPα, are immigrants from peripheral sites (196-198). Of note, pDC are also highly abundant in the thymus, however, their function has yet to be established.
In general DC in the thymus were unambiguously shown to be of central importance for tolerance induction (199, 200). Accordingly, DC-deficient mice developed multiorgan autoimmune disease and moreover diminished negative selection of bulk polyclonal $CD4^+$SP T cells was observed in mice deficient of antigen presentation by DC (201, 202). In addition to that, an indispensible role of DC in negative selection was also confirmed in a TCR-transgenic mouse models of deletion (203). Another remarkable feature of DC in the thymus is their ability to efficiently take up and present extracellular antigens (204-206). Consistent with this notion, it was found that a neo-self antigen that was expressed only by mTEC could lead to the deletion of T cells carrying the respective TCR in a DC-dependant manner (203). More importantly, even TRA expressed by mTEC were shown to be transferred to and presented by DC in the thymus (207). In contrast to previous assumptions, terminally differentiated mTEC, possibly through the proapoptotic function of AIRE, have a very high turn over rate of 1 – 2 weeks (34, 208). Considering the fact that only about 1-3% of mTEC express a given TRA, dying mTEC may therefore substantially contribute to antigen spreading to DC which might be a mechanism to increase the efficacy of tolerance induction (209).
The central question therefore is whether mTEC solely serve as an antigen reservoir which would support a “division of labor” model of tolerance induction by DC and mTEC or whether mTEC themselves have a function as antigen presenters. Several observations, however, strongly speak in favor of an autonomous role of mTEC as APC.

First, mTEC are besides cTEC the only non-hematopoietic cells that constitutively express MHCII and a range of co-stimulatory molecules such as CD80 upon maturation, therefore being perfectly equipped for tolerance induction (209). Second, it was recently shown that developing T cells in the medulla engage multiple short-lived interactions with APC, thereby probably thoroughly scanning their environment for self antigens (210). Thus, despite the relatively low percentage of mTEC expressing a given TRA, it would still be feasible that mTEC themselves induce tolerance, considering the long residence time of 4 – 5 days until $CD4^+$ SP T cells leave the medulla. Third, mTEC have been shown to be poor in antigen uptake from the extracellular space, indicating that they probably focus on their intracellular milieu for antigen presentation and in that way presumably enhance tolerance induction to TRA (205). Interestingly it was found that mTEC indeed use an unconventional MHCII loading pathway termed macroautophagy. In this study it was proposed that macroautophagy is shaping the CD4 T cell repertoire by biasing the MHCII ligandome of mTEC on their intracellular compartment. Consistent with this assumption, macroautophagy-deficient mice developed severe autoimmune disease (132). Forth, Gallegos et al. convincingly proved that mTEC autonomously induced the deletion of $CD8^+$ T cells specific for a neo-self antigen that was expressed in a promiscuous fashion (203). Moreover, in support of an independent role of mTEC as APC it was shown that mTEC-specific expression of hemagglutinin (HA) led to the induction of HA-specific $CD4^+$ T_{reg} in a cell autonomous, DC-independent manner (62). These results, however, have to be interpreted with caution. Recently it was found that even intact peptide-MHC (pMHC) complexes can be transferred from mTEC to DC (207, 211), which was never taken into account when MHC-deficient bone marrow chimeras were used to prove the autonomous role of mTEC in tolerance induction.

Collectively, in addition to antigen presentation by mTEC themselves, antigens can efficiently spread in the thymic medulla, whereby mTEC-derived self antigens may be transferred to and presented by DC. This process does not only seem to involve conventional pathways of antigen spreading but also the intercellular transfer of functional MHC molecules and other TEC-specific membrane proteins. Finally antigen presentation by mTEC and DC might not differ with respect to the mode of tolerance that is induced but with respect to the selection of antigens that are

presented by a particular APC, e.g. TRA, soluble or peripheral antigens, thereby autonomously shaping the TCR repertoire (Figure 5).

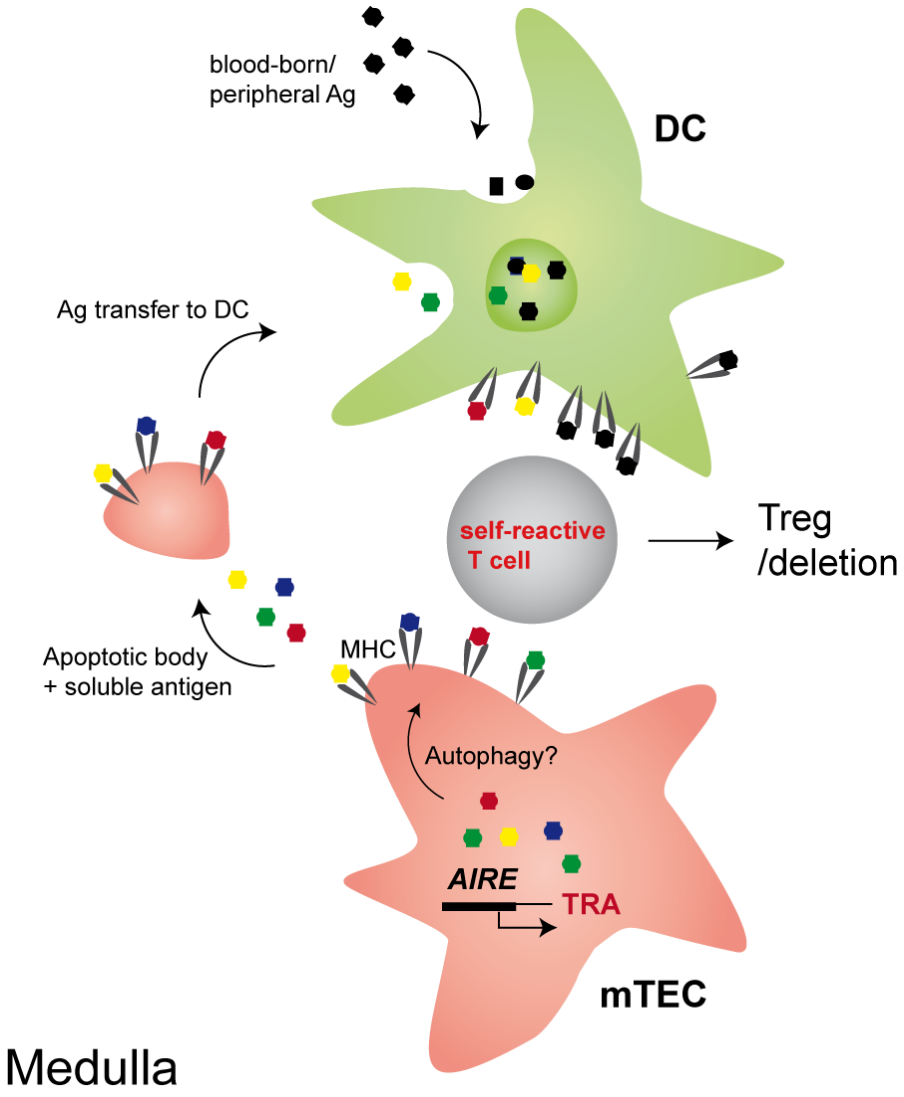


Figure 5 - Antigen presentation by medullary APC. This is a model of how self-reactive T cells might recognize antigen in the medulla. Tissue restricted antigens (TRA) are produced by mTEC in an AIRE–dependent manner and subsequently loaded on MHC molecules which might be enhanced by macroautophagy. Furthermore, TRA can also be transferred to and presented by DC, thereby possibly increasing the efficiency of tolerance induction to rare tissue restricted antigens. In addition, migratory DC might provide antigen from peripheral sites.

2. AIM OF THE STUDY

The ectopic transcription of thousands of tissue restricted antigens (TRA) is a fundamental hallmark of medullary thymic epithelial cells (mTEC) and is indispensable for tolerance induction in the thymus (33, 45). This phenomenon, termed promiscuous gene expression (pGE), is largely controlled by the autoimmune regulator (AIRE) and genetic ablation of AIRE has been shown to lead to multi-organ autoimmune disease. Thus, mTEC inarguably represent an essential source of peripheral tissue antigens for central tolerance. However, it is less clear whether mTEC, beside antigen production, also serve an important role in antigen presentation and direct tolerance induction of T cells, in particular for $CD4^+$ T cell tolerance.

Aim of this study was to address this issue by interfering with MHCII-restricted antigen presentation directly and only in mTEC. In order to do so we chose an RNA interference (RNAi) approach to tissue-specifically knock-down the Class 2 transactivator (C2TA), the master regulator of MHCII expression. Selecting C2TA instead of directly targeting MHCII enabled us to study antigen presentation by mTEC irrespective of the mouse background. In this model we aimed to assess the consequence of impaired antigen presentation by mTEC on tolerance induction of polyclonal as well as of TCR-transgenic $CD4^+$ T cells.

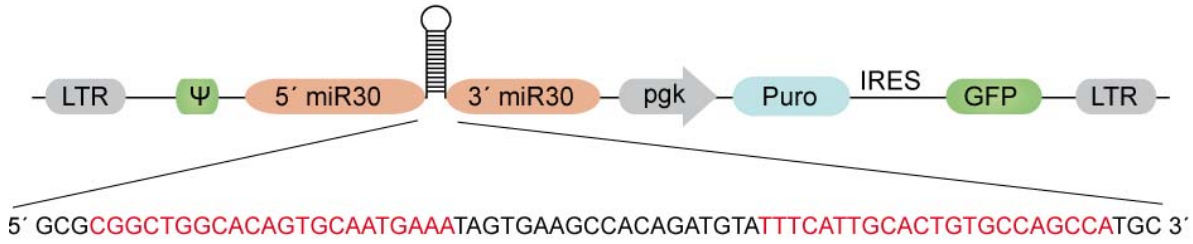
3. RESULTS

3.1 Cloning and *in vitro* evaluation of C2TA-designer miRNA

It has been previously published that a short hairpin that is integrated into the human miRNA30 backbone can be used to obtain knock-down of any gene of interest (212). Importantly, in contrast to conventional short hairpins, transcription was strictly dependent on Polymerase II, which enables tissue-specific knock-down of the gene. We took advantage of this new approach and generated five predicted designer miRNA against C2TA, the master-regulator of MHCII expression. In order to identify functional miRNA30-based C2TAsh constructs, the MHCII^{+} B cell line WEHI279.1 was infected with a retroviral vector expressing the different C2TAsh constructs and MHCII down-regulation of stable B cell clones was monitored. *Figure 6A* shows the most potent C2TAsh construct that was henceforth used in all experiments. C2TAsh#6 most strongly down-modulated MHCII mRNA expression (*Figure 6B)* and protein level (*Figure 6C*).

The functional consequence of MHCII down-modulation was tested via an antigen presenation assay using the A5 T cell hybridoma expressing the clonotypic TCR specific for HA(107-119) and GFP under the contol of IL-2 promoter elements. The results revealed a more than five fold difference in T cell stimulation capacity of C2TA knock-down B cells as compared to control-infected B cells when increasing amounts of petide were added to the co-cultures (*Figure 6D*).

A



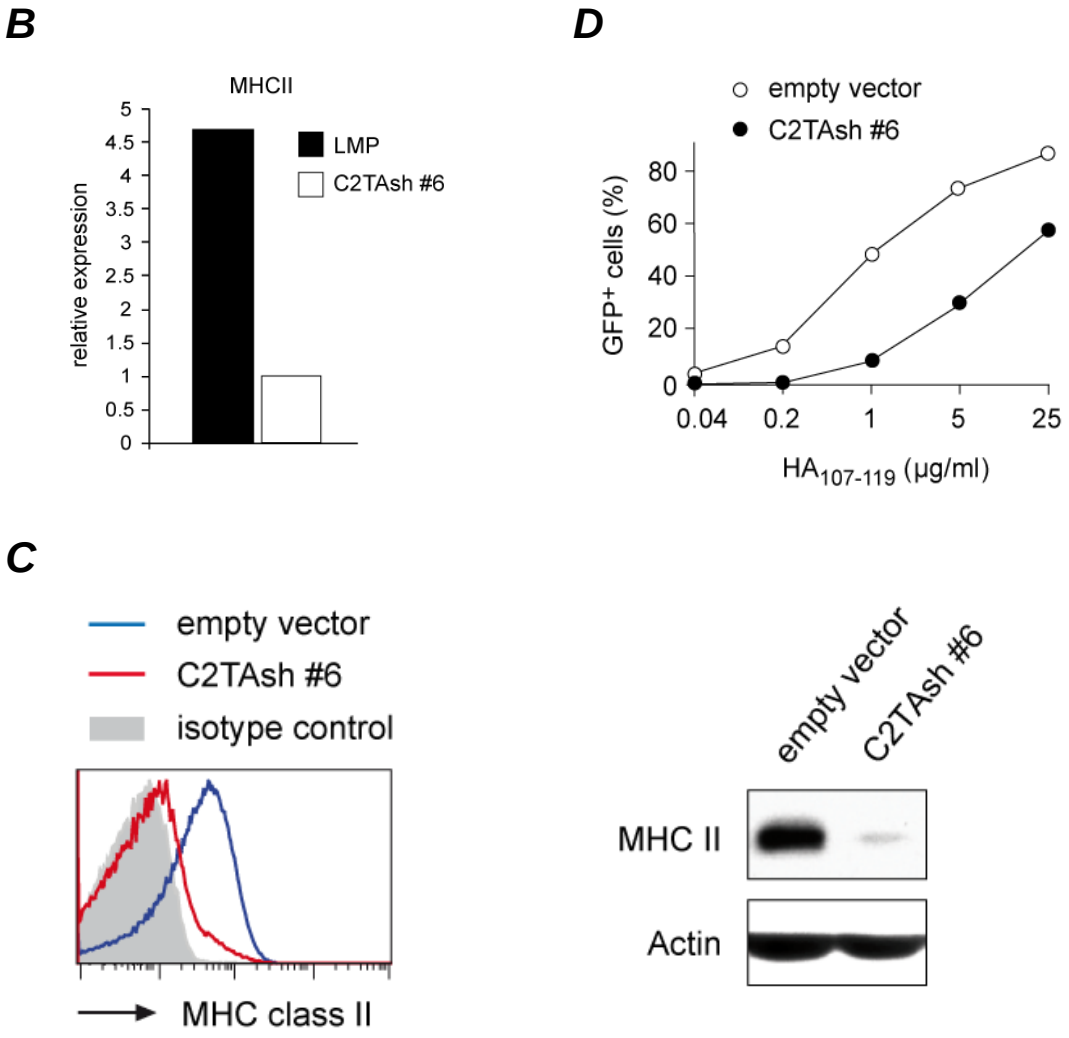


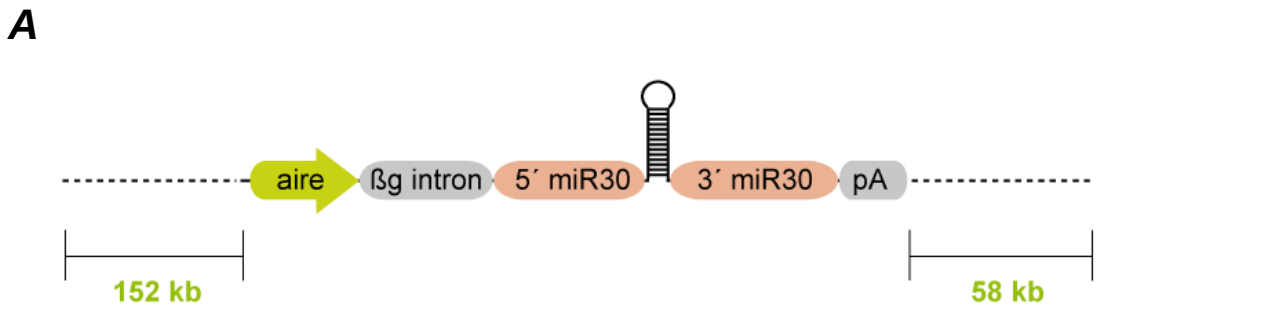
Figure 6 – Determination of knock-down efficiency of C2TA-specific designer miRNA *in vitro*. (A) Scheme of the retroviral vector (LMP) containing the C2TAsh#6. The LMP vector used for screening of C2TAsh constructs contained 5´ and 3´ regions of the primary human miRNA30 as well as the selection marker puromycin and GFP. (B) Quantitative analysis of MHCII mRNA of control and C2TAsh#6 infected B cells using real time PCR (n=3). (C) Quantification of MHCII protein levels of control and C2TAsh#6 infected B cells by FACS and western blot analysis (n=3). (D) Assessment of antigen presentation capacity. Control or C2TAsh#6 infected B cells were co-cultured with A5 hybridoma T cells in the presence of increasing amounts of HA peptide. After 19h GFP expression of hybridoma T cells was determined by FACS analysis.

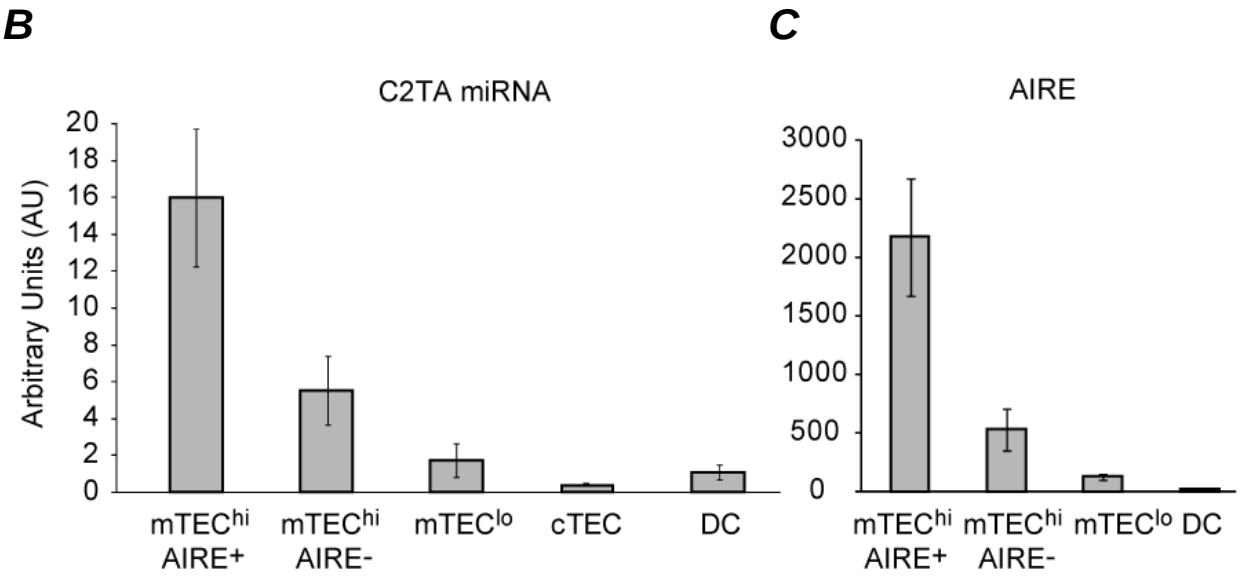
3.2 Generation of mTEC-specific C2TA knockdown mouse

To address the role of antigen presentation by mTEC in tolerance induction of $CD4^+$ T cells, we generated an mTEC-specific C2TA knock-down mouse (*C2TAkd*). A bacterial artificial chromosome transgenic construct containing 152kb upstream and 58kb downstream flanking regions of AIRE was cloned, in which the AIRE start codon was replaced by an open reading frame encoding the miRNA30-C2TAsh#6 construct (*Figure 7A*). Pronuclear injection of fertilized eggs resulted in the generation of two founder lines, both showing similar phenotypes in preliminary analyses (data not shown). One founder, termed *C2TAkd*, was used to perform all further experiments.

First the expression pattern of the miRNA30-C2TAsh#6 transgene was analyzed. To this end, various thymic stromal cell subsets were purified from *C2TAkd* animals and relative expression was determined using a custom small RNATaqMan assay. Expression of the C2TAmiRNA was exclusively restricted to mTEC, whereby $CD80^{hi}AIRE^+$ mTEC ($mTEC^{hi}$ $AIRE^+$) showed the highest level of the C2TA miRNA, followed by $CD80^{hi}AIRE^-$ mTEC (mTEC $AIRE^-$)(*Figure 7B*). Signals derived from cTEC as well as DC were below the sensitivity of the assay. These data established that the transgene was faithfully expressed, mirroring the endogenous AIRE expression (*Figure 7C*).

Next we aimed to quantify the impact of the miRNA30-C2TAsh#6 transgene expression on C2TA as well as on MHCII mRNA levels in all mTEC subsets. MHCII expression was strongly down-regulated in $CD80^{hi}AIRE^+$ mTEC ($mTEC^{hi}$ $AIRE^+$) and to lesser extend in $CD80^{hi}AIRE^-$ mTEC ($mTEC^{hi}$ $AIRE^-$) and $CD80^-$ mTEC ($mTEC^{lo}$). The same was found for C2TA expression itself, although the degree of down-modulation was lower, implying that both translational and transcriptional silencing of C2TA was induced by the C2TA miRNA (*Figure 7D*). Importantly, MHCI expression which was shown to be slightly activated by C2TA (213, 214) was not altered in *C2TAkd* animals as compared to WT. The results confirmed highly specific and efficient C2TA knockdown in *C2TAkd* mice, being exclusively restricted to mTEC and most distinct in mature mTEC.


A
aire
ßg intron
5´ miR30
3´ miR30
pA
152 kb
58 kb


B
C2TA miRNA
Arbitrary Units (AU)
20
18
16
14
12
10
8
6
4
2
0
mTEChi AIRE+
mTEChi AIRE-
mTEClo
cTEC
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AIRE
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2500
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1500
1000
500
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mTEChi AIRE+
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DC

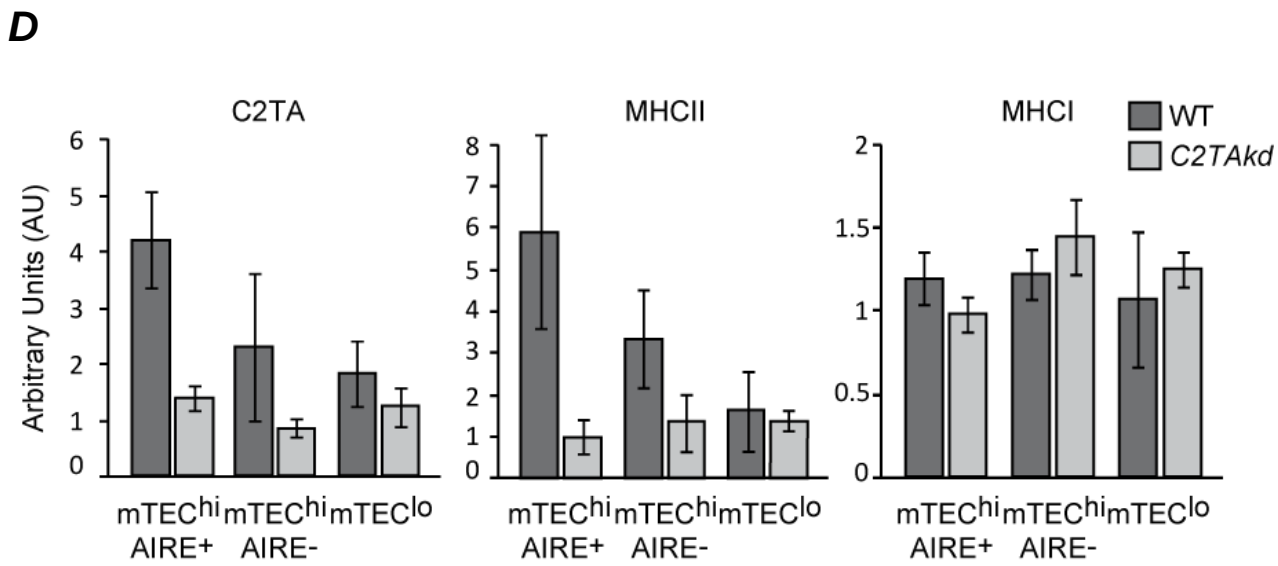

D
C2TA
MHCII
MHCI
WT
C2TAkd
Arbitrary Units (AU)
mTEChi AIRE+
mTEChi AIRE-
mTEClo

Figure 7 – Generation of *C2TAkd* mouse and analysis of transgene expression. (A) Schematic illustration of transgenic construct. The scheme shows the bacterial artificial chromosome construct containing 152kb upstream and 58kb downstream of the transcriptional start site of AIRE. The AIRE start codon is replaced by an open reading frame encoding the microRNA30-C2TAsh#6 construct cloned downstream of the β globin intron. (B) Expression analysis of the C2TA miRNA. Quantification of the C2TA miRNA in different stromal cell types ($mTEC^{hi}AIRE^{+}$, $mTEC^{hi}AIRE^{-}$, $mTEC^{lo}$, cTEC and DC) isolated from 2-3-weeks-old *C2TAkd* mice is depicted in the graph (*n=3*). (C) The graph shows the relative expression of AIRE mRNA in $mTEC^{hi}AIRE^{+}$, $mTEC^{hi}AIRE^{-}$, $mTEC^{lo}$ and DC from 5 weeks old *C2TAkd* animals (*n=3*). (D) Determination of knockdown efficiency. C2TA mRNA levels in $mTEC^{hi}AIRE^{+}$, $mTEC^{hi}AIRE^{-}$, $mTEC^{lo}$ from 5-weeks-old *C2TAkd* mice compared to WT was determined by real time PCR. Relative MHCII and MHCI expression in $mTEC^{hi}AIRE^{+}$, $mTEC^{hi}AIRE^{-}$, $mTEC^{lo}$ form 5-weeks-old *C2TAkd* and WT animals is depicted in the graph (*n=3*).

3.3 *C2TAkd* mice show highly specific and efficient down-modulation of MHCII

Next the efficiency and tissue specificity of MHCII protein down-regulation in *C2TAkd* animals was assessed. Western blot analysis of highly purified thymic stromal cell populations confirmed a strong reduction of MHCII on the protein level in mature *C2TAkd* mTEC ($mTEC^{hi}$), whereas no alteration was seen in *C2TAkd* DC. Immature mTEC ($mTEC^{lo}$) isolated from *C2TAkd* mice were also found to reduce MHCII protein levels as compared to WT. Calculations of the relative changes in MHCII protein levels showed a more than tenfold reduction of MHCII in mature mTEC (*Figure 8A*).
In addition to that, we performed flow cytometric analysis of MHCII of all major thymic stromal cell subsets, namely $Sirp\alpha^{-}$ and $Sirp\alpha^{+}$ cDC, pDC, cTEC, immature and mature mTEC. The results revealed a remarkable reduction of MHCII protein exclusively in mTEC of *C2TAkd* animals but not in any other stromal cell types analyzed (*Figure 8B*).

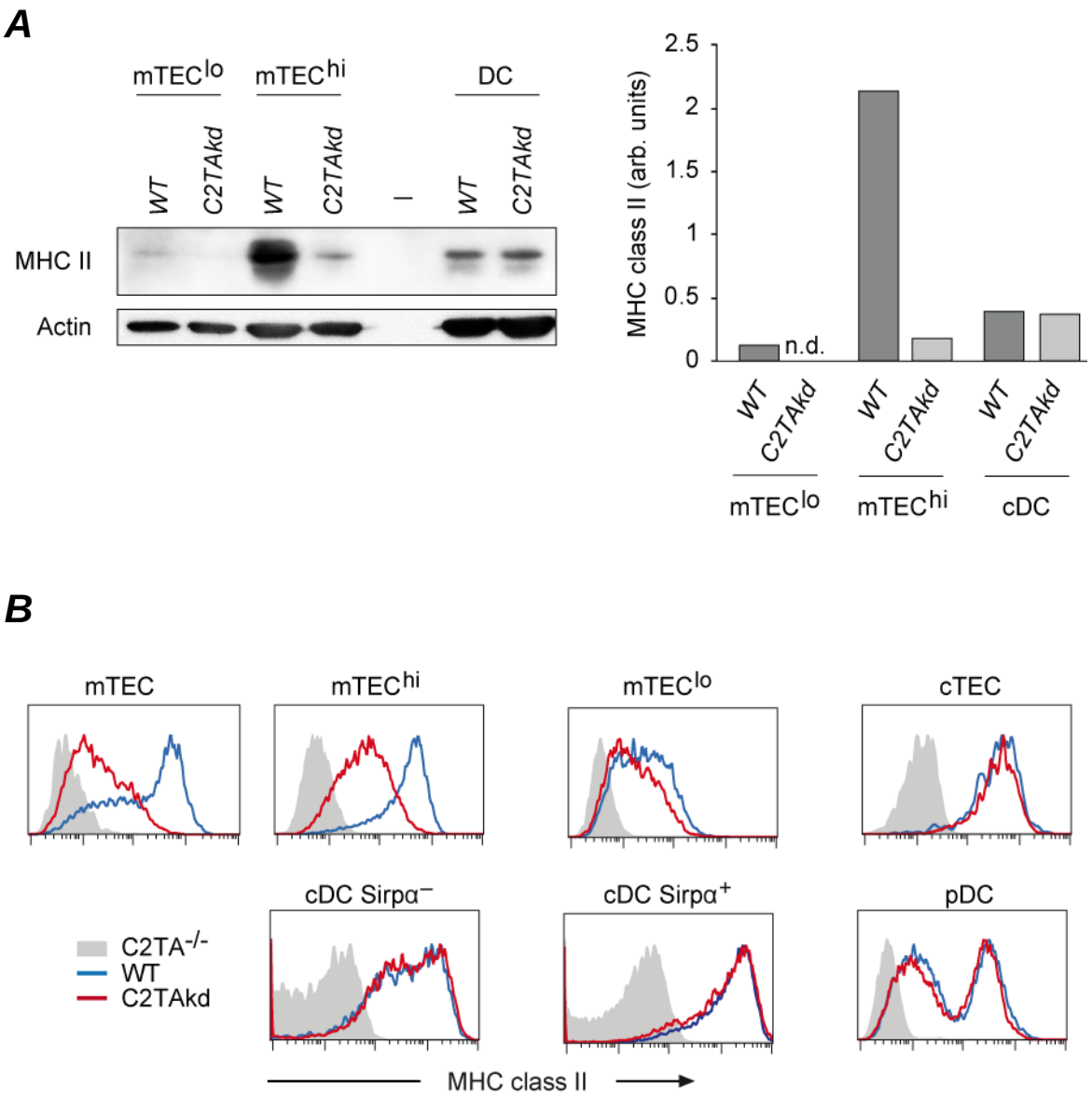


Figure 8 – Determination of C2TA knock-down efficiency and specificity in *C2TAkd* mice. (A) Immature CD80^{lo} and mature CD80^{hi} mTEC as well as DC were purified from thymic stroma of 5–6-weeks-old WT or *C2TAkd* mice. Knockdown specificity and efficiency was assessed by western blot analysis of MHCII (upper left) (n=2). Relative quantification of MHCII protein level is depicted in the upper right panel. (B) MHCII expression by different thymic stroma cell populations of WT, *C2TAkd* and C2TA$^{-/-}$thymi was determined by FACS analysis (n=3).

3.4 Diminished MHCII expression does not affect thymus integrity

A recent study proposed a crucial role of MHCII in mTEC development or maintenance (43). Therefore we carefully analyzed the overall integrity of *C2TAkd* thymi by staining sections with different TEC markers (*Figure 9*). Medullae as well as cortico-medullary junctions were properly formed in *C2TAkd* animals. Similarly, $AIRE^{+}$ mature mTEC were also normally abundant. MHCII stainings of WT thymi showed a strong positive signal in the medulla which is in accordance with an earlier study that reported a predominance of mTEC-derived cytoplasmatic MHCII signal in the thymic medulla (215). By contrast, *C2TAkd* sections stained only weakly for MHCII, whereby residual MHCII expression most likely derived from DC.

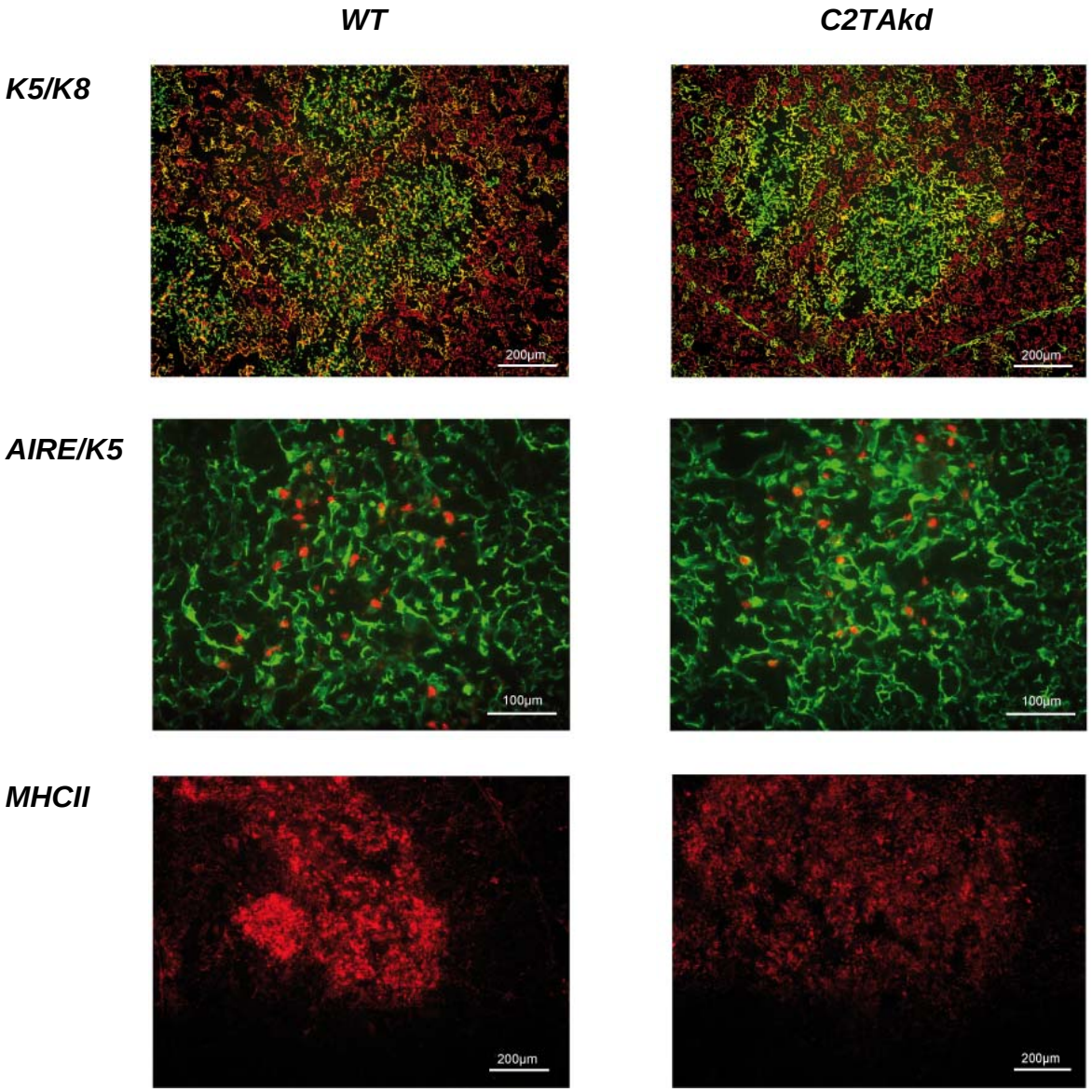


Figure 9 – Examination of medulla-cortex formation in *C2TAkd* mice. Depicted are stainings of cryosections from thymi of 6-weeks-old WT and *C2TAkd* mice for Keratin 5 (K5, green) and Keratin 8 (K8, red) to identify medulla and cortex (upper row). Middle and bottom rows show in situ detection of AIRE$^+$ (red) or MHCII$^+$ (red) cells in WT versus *C2TAkd* medullary areas (K5, green) (n=3). Scale bars are indicated.

3.5 Normal mTEC development in *C2TAkd* animals

Detailed flow cytometric analysis revealed no changes in the composition of mTEC subpopulations. About one fourth of all WT as well as *C2TAkd* mTEC expressed AIRE (24.2 ± 4.6 (*n* = 25) versus 22.2 ± 4 (*n* = 11); *P* = 0.19), the same hold true for CD80, albeit expression was slightly reduced in *C2TAkd* mTEC as compared to WT controls (60.8 ± 7 (n = 33) versus 55.8 ± 5 (n = 22); *P* = 0.004) (*Figure 10A* and *10B*). Overall, mTEC development did not seem to be affected in *C2TAkd* mice, which therefore indicates a negligible role of MHCII in this process.

To test the antigen presentation ability of *C2TAkd* and WT mice, mTEC were sorted and co-cultured with A5 GFP reporter hybridoma T cells recognizing HA(107-119) in the context of I-E^d. *C2TAkd* mTEC required at least ten times more peptide in order to have the same stimulatory effect on T cells as WT mTEC (*Figure 10C*). Taken together these results established that mTEC development and maintenance is normal in *C2TAkd* mice, while antigen presentation capacity is strongly diminished.

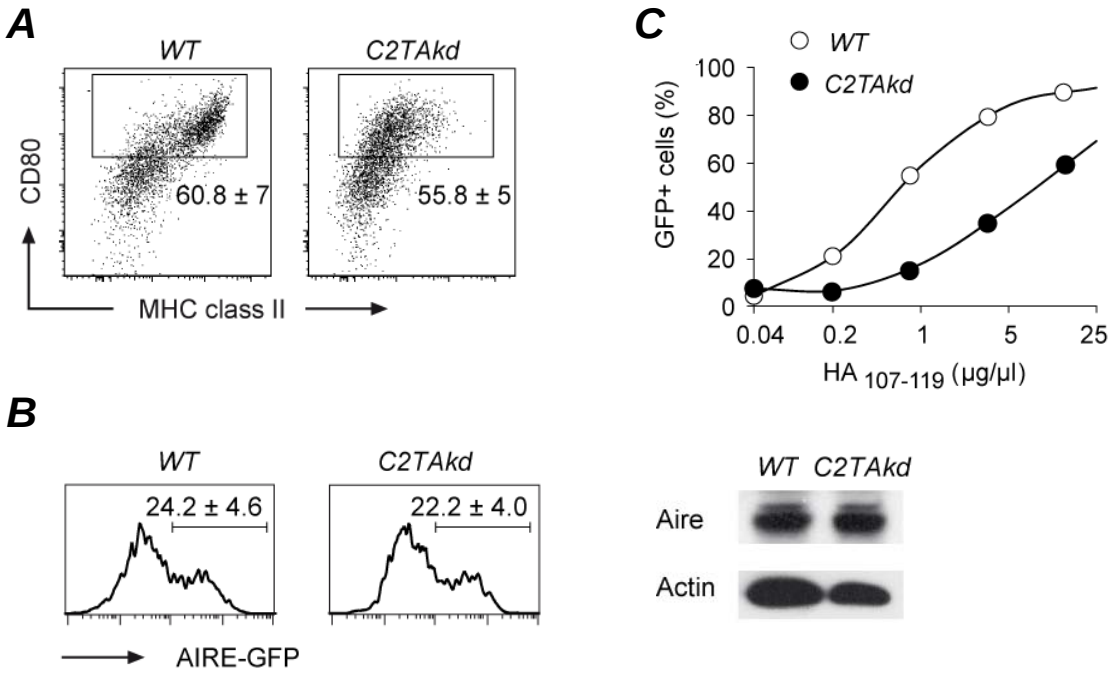


Figure 10 - Characterization of mTEC properties in *C2TAkd* mice. (A) The plots depict stainings of mTEC isolated from 5–6-weeks-old WT or *C2TAkd* animals for MHCII and CD80. (B) The histograms show Aire-GFP expression in mTEC of WT x Aire-GFP and *C2TAkd* x Aire-GFP mice as well as Aire protein quantification of *C2TAkd* mTEC versus WT mTEC by WB analysis. Numbers indicate the average frequency (± s.d.) of cells within the gates. (C) Antigen presentation capacity of *C2TAkd* mTEC. Ex-vivo isolated mTEC from WT or *C2TAkd* animals were co-cultured with A5 hybridoma T cells in the presence of increasing amounts of HA peptide. After 19h GFP expression of hybridoma T cells was determined by FACS analysis.

3.6 Defective negative selection of polyclonal CD4$^+$ SP T cells in *C2TAkd* mice

In order to assess the consequence of diminished MHCII expression in mTEC on tolerance induction of CD4$^+$ T cells, bulk T cell development was analyzed. The CD4 and CD8 profile of *C2TAkd* thymi revealed a significant increase in the CD4$^+$ SP T cell compartment (9.0 ± 1.7 (*n* = 10) versus 12.1 ± 1.7 (*n* = 9); *P* = 0.001) while the CD8$^+$ SP T cell compartment was essentially unaltered compared to WT thymi (*Figure 11*). These observations are reminiscent of earlier studies investigating the role of DC in negative selection and showing that the absence of antigen presentation by DC resulted in an enlarged CD4$^+$ SP compartment, presumably due to reduced deletion of auto-reactive T cells (201, 202). Accordingly, our results support the notion that mTEC play a non-redundant role as antigen presenting cells (APC) in negative selection of CD4$^+$ T cells.

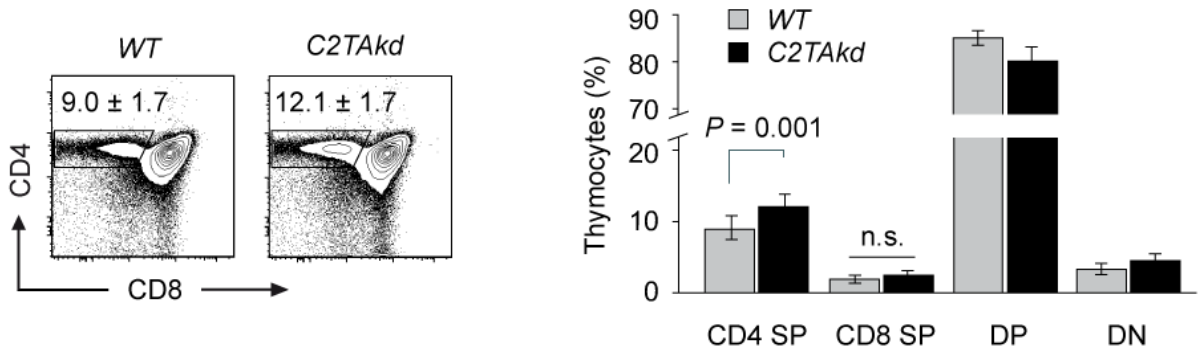


Figure 11 – Selection of $CD4^+$ and $CD8^+$ T cells in *C2TAkd* thymi. CD4 and CD8 stainings of thymocytes from 5-months-old WT and *C2TAkd* mice (left panel) (n=9). Average percentages of CD4 positive cells are indicated (± s.d.). The bar diagram depicts the relative distribution of different thymic T cell subsets of WT and *C2TAkd* mice respectively (right panel). *P* values are indicated.

3.7 A non-redundant role of mTEC and DC in negative selection

To address the contribution of DC versus mTEC in CD4 T cell tolerance induction in detail, different bone marrow chimeras were generated. Irradiated WT recipients were reconstituted with $MHCII^{-/-}$ bm resulting in the inability of DC to present antigen in the thymus. Likewise, to ascertain the impact of antigen presentation by mTEC, irradiated *C2TAkd* recipients were reconstituted with WT bone marrow. In both types of chimeras, an enlargement of the $CD4^+$ SP T cell compartment could be observed (8.5 ± 0.3 (*n* = 9) versus 12.1 ± 0.1 (*n* = 7), *P* = 0.0006 and 8.5 ± 0.3 (*n* = 9) versus 10.3 ± 0.3 (*n* = 10), *P* = 0.002) (*Figure 12A*). Importantly, an additive effect was found when both DC and mTEC were unable to present antigen to $CD4^+$ T cells in *C2TAkd* animals that were reconstituted with $MHCII^{-/-}$ bone marrow (12.1 ± 0.1 (*n* = 7) versus 15.2 ± 0.7 (*n* = 9), *P* = 0.01). Taken together, these observations speak in favor of a non-redundant role of both DC and mTEC in negative selection by autonomous presentation of antigens to $CD4^+$ T cells.

The assumption that the increased proportion of $CD4^+$ SP T cells is caused by defective negative selection would consequently also imply that these cells have an auto-reactive potential. To test this hypothesis, an *in vitro* proliferation assay was performed using peripheral lymph node (LN) DC from WT animals as APC and $CD4^+$ SP T cells from the above described bone marrow chimeras as responders. The results demonstrated that $CD4^+$ SP T cells derived from thymi either devoid of antigen presentation by DC or mTEC or both contain a fraction of cells recognizing self-peptide MHC complexes (*Figure 12B*). Of note, again an additive effect was observed when both, DC and mTEC were unable to present antigen.

Importantly, we did not find any significant changes in T_{reg} development with respect to the overall size of the $Foxp3^+$ compartment (*Figure 12C*). However, it is still possible that the TCR repertoire of the T_{reg} pool might be altered and some TCR specificities might therefore be missing in *C2TAkd* mice.

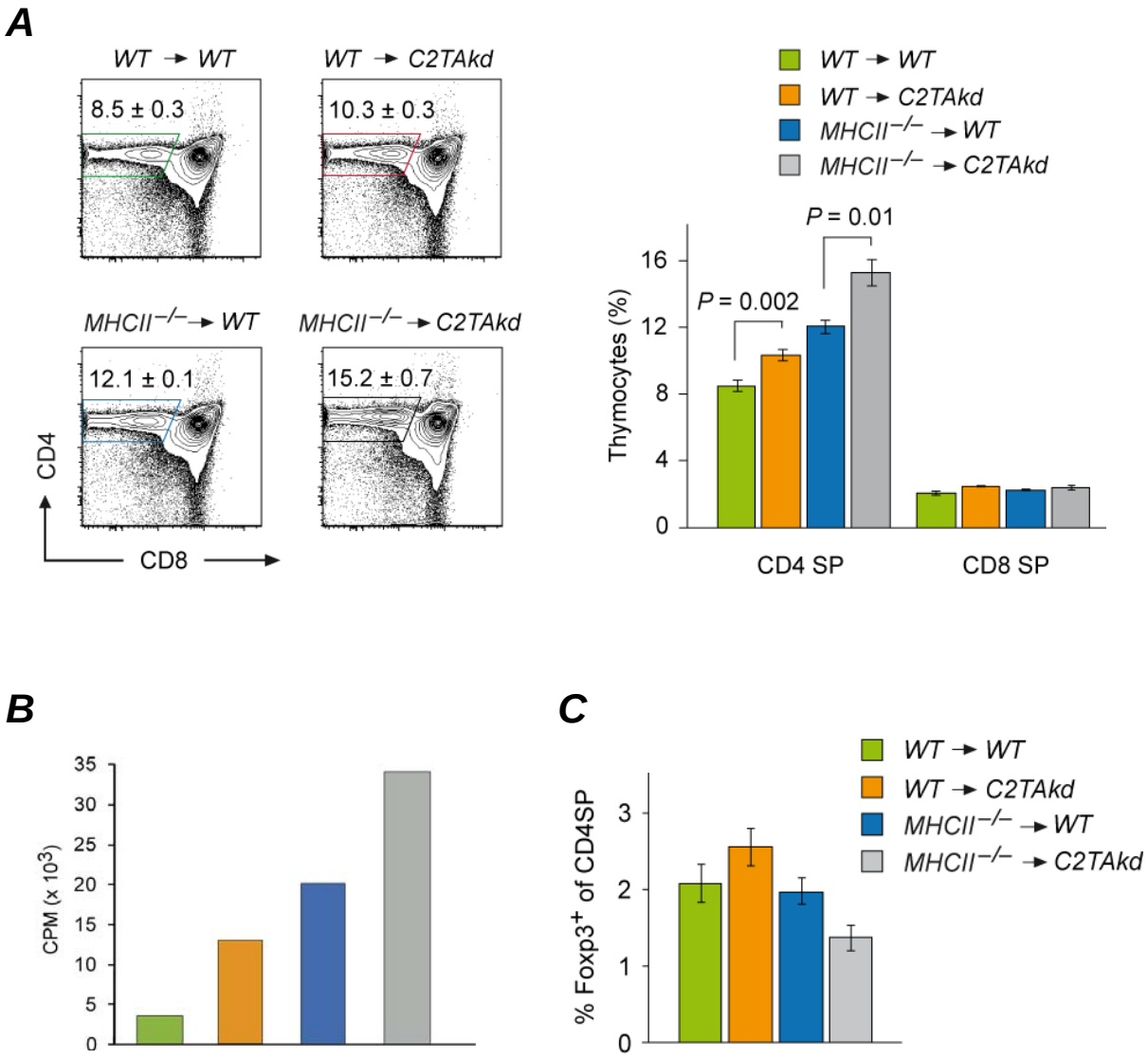
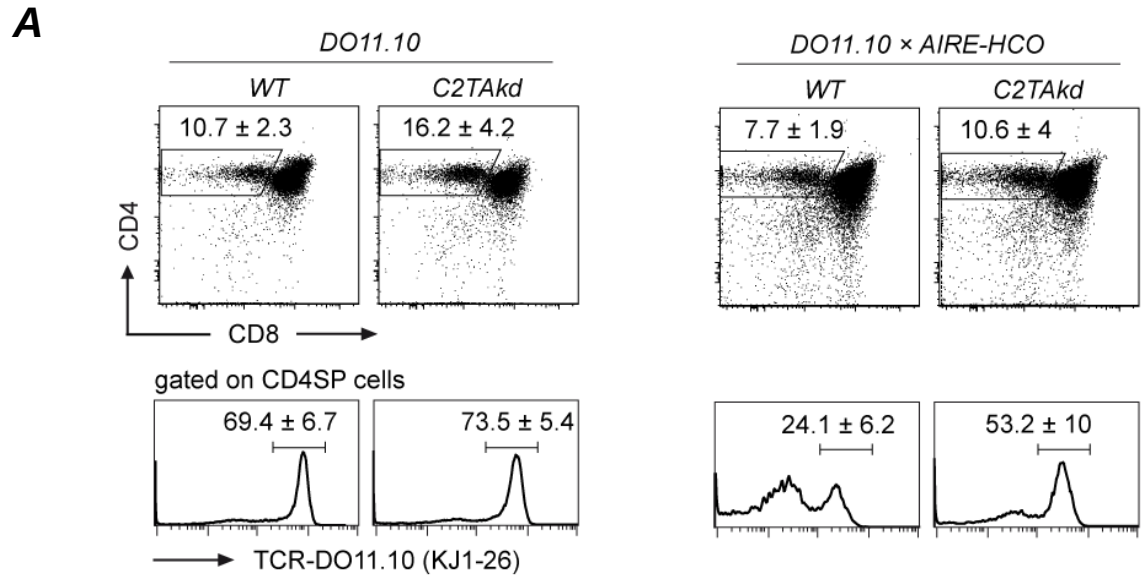


Figure 12 – CD4$^+$ T cell selection by DC versus mTEC. (A) CD4$^+$ T cell development in WT or *C2TAkd* recipients of WT or MHCII$^{-/-}$ bone marrow. The plots depict CD4 and CD8 profiles of WT or *C2TAkd* animals reconstituted with WT bone marrow and analyzed 6 weeks later (upper left panel). Lower left panel, WT or *C2TAkd* animals reconstituted with MHCII$^{-/-}$ bone marrow. Average percentages of CD4$^+$ SP T cells are shown (± SEM). The bar diagram in the upper right panel shows the percentages of CD4$^+$ T cells versus CD8$^+$ T cells in the four different bone marrow chimeras (*P* values are indicated). (B) Self-reactive potential of CD4$^+$ SP T cells that developed in the absence of antigen presentation by DC or mTEC or both. H_3 thymidin incorporation of CD4$^+$ SP T cells isolated from the four different combinations of bone marrow chimeras 6 weeks after reconstitution (see A) and cultivated with WT lymph node DC for 4 days is shown. (C) Unaltered percentage of T_{reg}. The graph depicts percentages of CD4$^+$ Foxp3$^+$ T cells in WT or *C2TAkd* recipients reconstituted with either WT or MHCII$^{-/-}$ bone marrow (*P* = *n.s.*).

3.8 Impaired negative selection of Ovalbumin-specific T cells

Next we aimed to directly address the role of antigen presentation by mTEC in negative selection versus T_{reg} development by taking advantage of a TCR transgenic approach which would enable us to study tolerance induction of T cells with known specificity. We used a previously described model system in which mTEC-specific expression of Ovalbumin (Ova) in AIRE-HCO mice led to massive deletion of Ova-specific T cells (DO11.10) (62, 194). In the absence of Ova, DO11.10 transgenic T cells were normally selected and represented the majority of naïve $CD4^+$ SP T cells in WT as well as in *C2TAkd* thymi (69.4 ± 6.7 (*n* = 10) versus 73.5 ± 5.4 (*n* = 10); *P* = 0.14) (*Figure 13A*). In the presence of cognate antigen in AIRE-HCO x DO11.10 animals a large proportion of $DO11.10^+$ T cells was lost. Importantly, the deletion of $DO11.10^+$ T cells was nearly reversed when mTEC were impaired to present the cognate antigen in *C2TAkd* x AIRE-HCO x DO11.10 mice (24.1 ± 6.2 (*n* = 12) versus 53.2 ± 10 (*n* = 14); $P = 4 \times 10^{-9}$) (*Figure 13A and 13B*).

To exclude the possibility that altered HCO expression in *C2TAkd* mice might have led to the impaired deletion of $DO11.10^+$ T cells, expression analysis of HCO in WT versus *C2TAkd* mice was performed. Results clearly showed comparable HCO levels in *C2TAkd* x AIRE-HCO and AIRE-HCO thymi (*Figure 13C*).



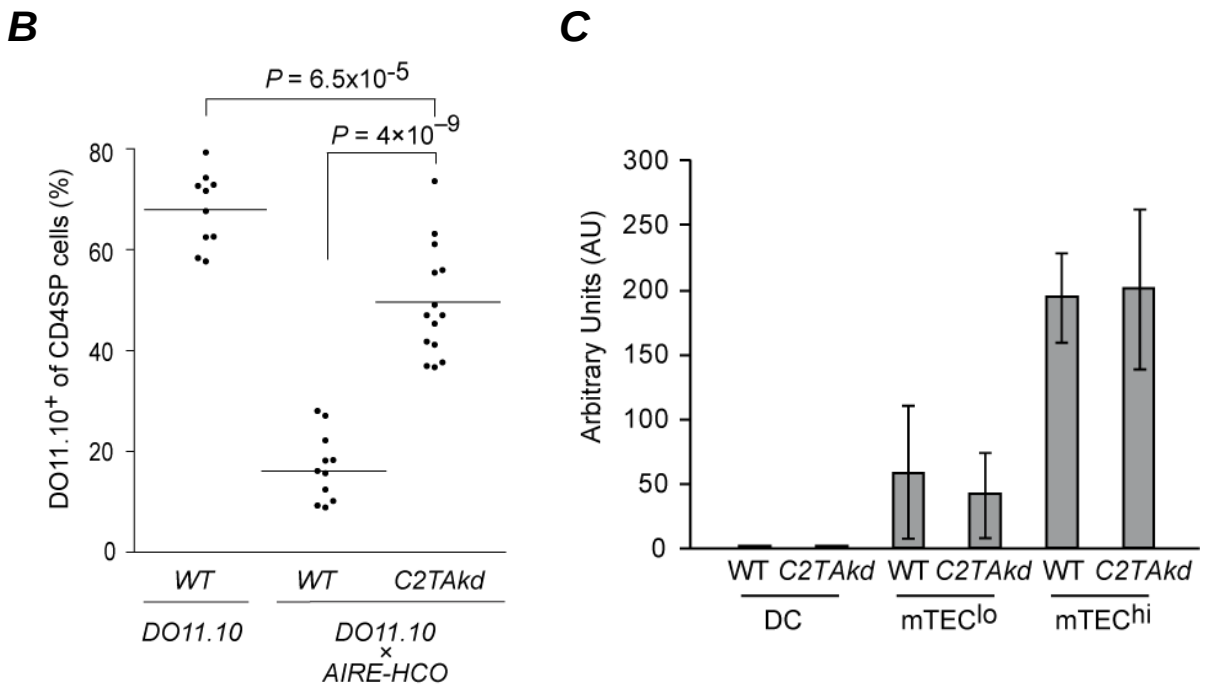


Figure 13 – Reduced negative selection of DO11.10$^+$ T cells in DO11.10 × AIRE-HCO × *C2TAkd* mice. (A) Altered deletion in DO11.10 × AIRE-HCO × *C2TAkd* mice. The plots depict CD4 and CD8 stainings of thymocytes of 6-weeks-old DO11.10 and DO11.10 x *C2TAkd* mice (upper left) or DO11.10 x AIRE-HCO and DO11.10 x AIRE-HCO x *C2TAkd* (upper right) mice. In the respective histograms below CD4$^+$ SP T cells stained for DO11.10 are shown. Numbers indicate the average frequency (± s.d.) of cells within these areas. (B) The diagram depicts the percentage of DO11.10$^+$ cells of CD4$^+$ SP T cells of DO11.10, DO11.10 × AIRE-HCO and DO11.10 × AIRE-HCO × *C2TAkd* mice (*P* values are shown). (C) Quantification of HCO-expression. Quantification of relative HCO expression in CD80^{hi} and CD80^{lo} mTEC as well as DC isolated from AIRE-HCO or AIRE-HCO x *C2TAkd* animals is depicted in the graph.

3.9 Enhanced induction of DO11.10$^+$ T_{reg} in *C2TAkd* mice

While DO11.10 single-transgenic T cells developed into naïve CD4$^+$ SP T cells, the same cells were strongly deleted in the presence of cognate antigen. The remaining cells were found to contain a substantial fraction of Ova-specific T_{reg}, identified by the expression of Foxp3. Therefore we sought to ascertain the consequence of diminished antigen presentation by mTEC on T_{reg} development. Interestingly, *C2TAkd* × DO11.10 × AIRE-HCO animals, beside diminished deletion of Ova-specific T cells, were found to exhibit a significant increase in the % of Foxp3$^+$ DO11.10$^+$ CD4$^+$ SP T cells (5.2 ± 2.1, n = 11 versus 14.3 ± 2.4, n = 13; P = 1.5 x 10^{-9}) (*Figure 14A*). This was also reflected in the absolute cell numbers of Ova-specific T_{reg} (*Figure 14B*).

Together we showed that the reduced presentation of HCO by down-modulation of MHCII on mTEC diminished deletion and at the same time favored T_{reg} induction of HCO-specific T cells. Our observations would be in accordance of an avidity model of tolerance induction, whereby the threshold for negative selection lies above the optimal avidity window for T_{reg} induction.

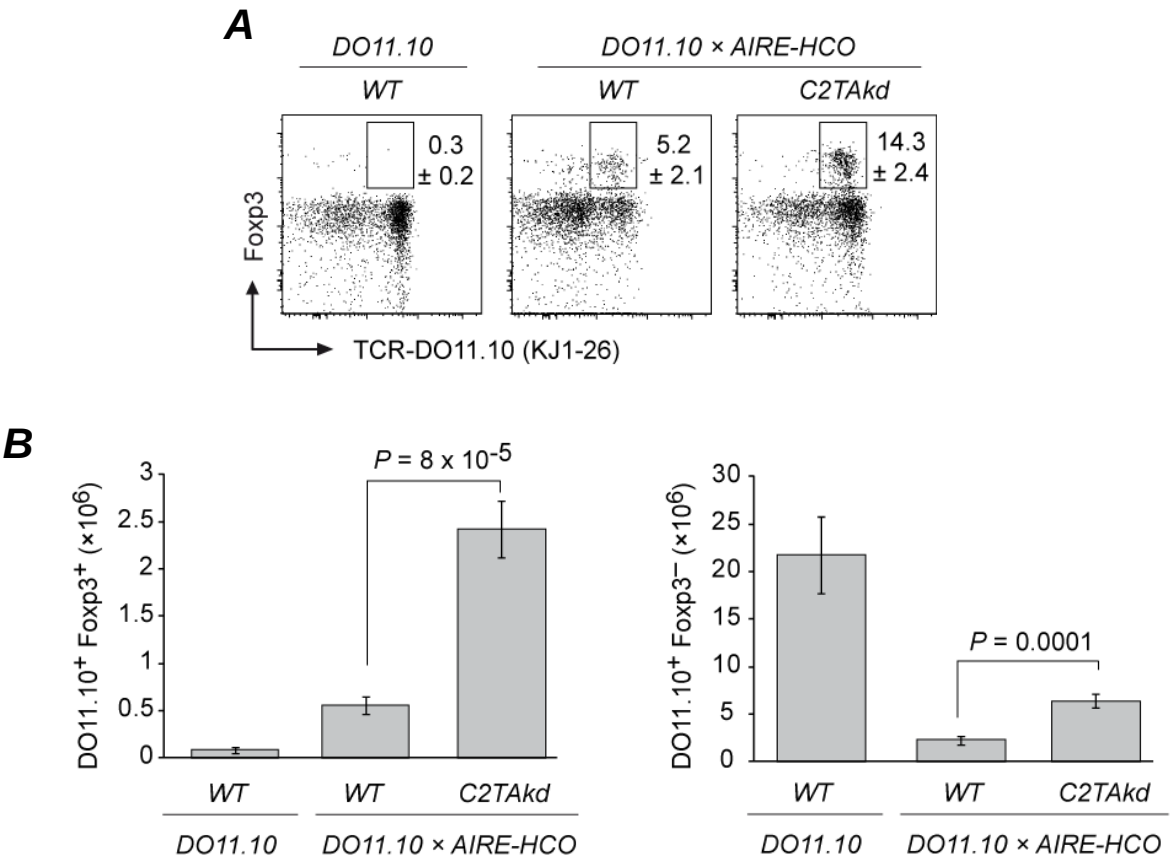


Figure 14 – Increased selection of T_{reg} in DO11.10 x AIRE-HCO x *C2TAkd* mice. (A) Increased percentage of Ova-specific T_{reg} in DO11.10 x AIRE-HCO x *C2TAkd* mice. Depicted are $CD4^+$ SP T cells of DO11.10, DO11.10 x AIRE-HCO or DO11.10 x AIRE-HCO x *C2TAkd* mice stained for Foxp3 and DO11.10. The average percentages of $Foxp3^+$ $DO11.10^+$ T cells are shown (± SEM). (B) The diagrams depict the absolute numbers of $Foxp3^+$ $DO11.10^+$ and $Foxp3^-$ $DO11.10^+$ T cells of DO11.10, DO11.10 x AIRE-HCO or DO11.10 x AIRE-HCO x *C2TAkd* mice (*P* values are indicated).

3.10 Impaired deletion and enhanced T_{reg} induction of HA-specific T cells

To exclude the possibility that the above described findings were just a particular attribute of the DO11.10 TCR, a second TCR transgenic mouse model was tested. As previously described by our lab, Hemagglutinin (HA) expression by mTEC led to partial deletion and concomitant T_{reg} induction of TCR-HA transgenic T cells (62). While TCR-HA single transgenic T cells normally developed into naïve T cells and composed roughly 30% of all $CD4^+$ SP T cells in WT as well as in *C2TAkd* animals (*Figure 15A*) (29.7 ± 4.2 (*n* = 7) versus 32.5 ± 2.8 (*n* = 8), *P* = 0.17), HA-specific T cells were deleted in AIRE-HA mice that expressed HA exclusively in mTEC (*Figure 15A*). Importantly, interfering with antigen presentation by mTEC, *C2TAkd* × TCR-HA × AIRE-HA animals exhibited reduced deletion of HA-specific T cells (5.9 ± 2.2 (*n* = 6) versus 14.5 ± 4.9 (*n* = 14), $P = 5 \times 10^{-5}$) which is in line with the results obtained by the DO11.10 model (*Figure 15A and B*).

Reminiscent of the DO11.10 system, T_{reg} development was also affected, being strongly increased in *C2TAkd* × AIRE-HA × TCR-HA mice with respect to percentages (2.9 ± 0.5 (*n* = 6) versus 7.9 ± 3.4 (*n* = 14), $P = 9 \times 10^{-5}$) as well as absolute cell numbers of HA-specific T_{reg} (*Figure 15C and D*).

Similar results were obtained using a system in which HA was ubiquitously expressed (Pgk-HA) (*Figure 16A-C*) (216).

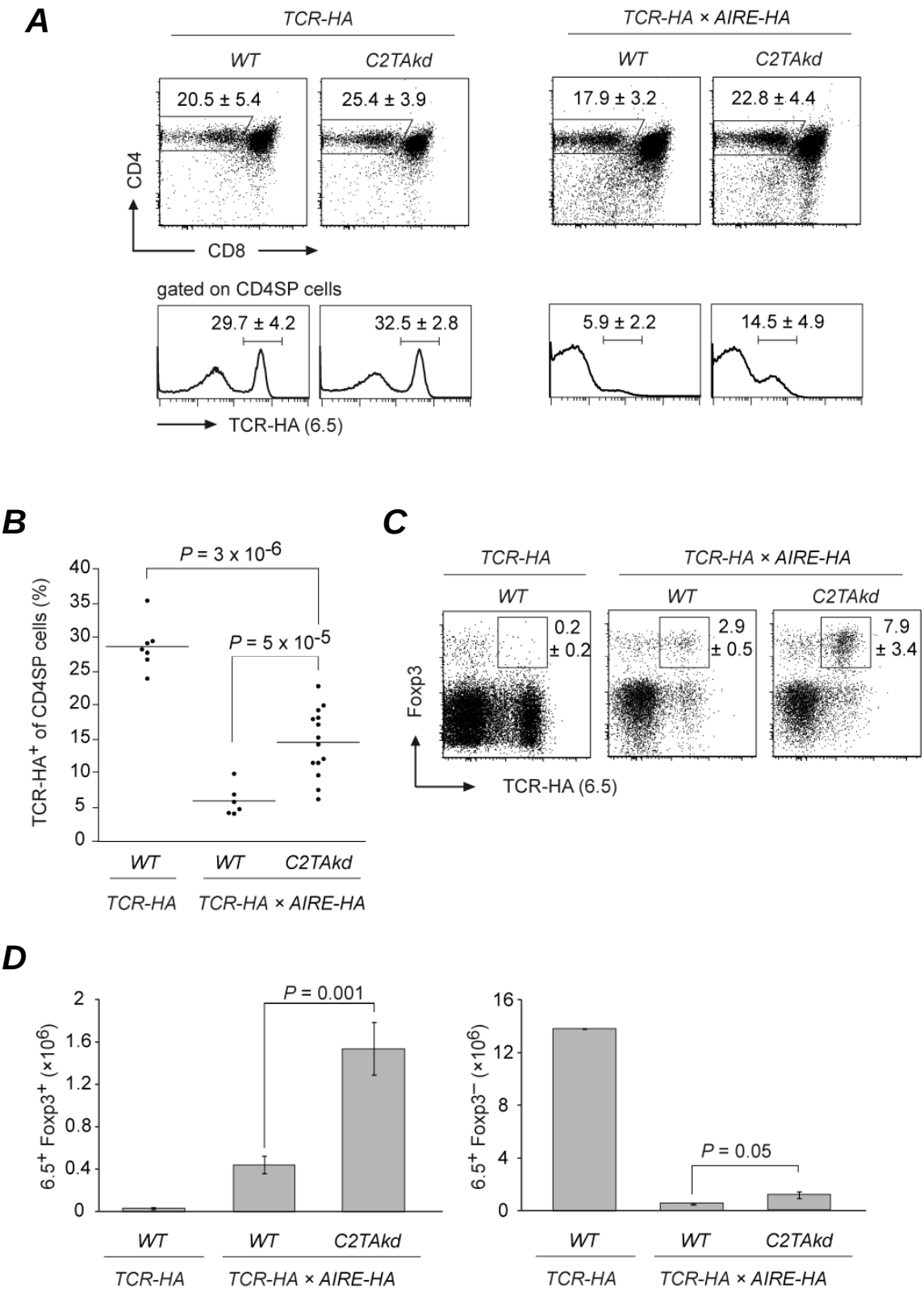

A
TCR-HA
TCR-HA × AIRE-HA
WT
C2TAkd
WT
C2TAkd
20.5 ± 5.4
25.4 ± 3.9
17.9 ± 3.2
22.8 ± 4.4
CD4
CD8
gated on CD4SP cells
29.7 ± 4.2
32.5 ± 2.8
5.9 ± 2.2
14.5 ± 4.9
TCR-HA (6.5)
B
P = 3 x 10-6
P = 5 x 10-5
TCR-HA+ of CD4SP cells (%)
40
35
30
25
20
15
10
5
0
WT
WT
C2TAkd
TCR-HA
TCR-HA × AIRE-HA
C
TCR-HA
TCR-HA × AIRE-HA
WT
WT
C2TAkd
0.2
± 0.2
2.9
± 0.5
7.9
± 3.4
Foxp3
TCR-HA (6.5)
D
P = 0.001
6.5+ Foxp3+ (×106)
2
1.6
1.2
0.8
0.4
0
WT
WT
C2TAkd
TCR-HA
TCR-HA × AIRE-HA
6.5+ Foxp3− (×106)
16
12
8
4
0
P = 0.05
WT
WT
C2TAkd
TCR-HA
TCR-HA × AIRE-HA

Figure 15 – Altered selection of HA-specific T cells in TCR-HA × AIRE-HA × *C2TAkd* mice. (A) Diminished deletion in TCR-HA × AIRE-HA × *C2TAkd* mice. Histograms depict staining of $CD4^+$ SP T cells for TCR-HA from either 6-weeks-old TCR-HA and TCR-HA × *C2TAkd* mice (lower left panel) or TCR-HA × AIRE-HA and TCR-HA × AIRE-HA × *C2TAkd* mice (lower right panel). The respective CD4 and CD8 profiles are shown above (upper row). Numbers indicate the average frequency (± s.d.) of cells within these areas. (B) The graph displays the percentages of TCR-HA^+ cells of $CD4^+$ SP T cells of individual TCR-HA, TCR-HA × AIRE-HA and TCR-HA × AIRE-HA × *C2TAkd* animals. *P* values are indicated. (C) Enhanced T_{reg} induction in TCR-HA × AIRE-HA × *C2TAkd* mice. The plots depict TCR-HA versus Foxp3 stainings of $CD4^+$ SP T cells from TCR-HA (left) and TCR-HA × AIRE-HA or TCR-HA × AIRE-HA × *C2TAkd* thymi. Numbers indicate the average frequency (± s.d.) of cells within these areas. (D) Absolute cell numbers of HA-specific Treg (left) and naïve T cells (right) from TCR-HA, TCR-HA × AIRE and TCR-HA × AIRE-HA × *C2TAkd* animals are shown in the graph, *P* values are indicated.

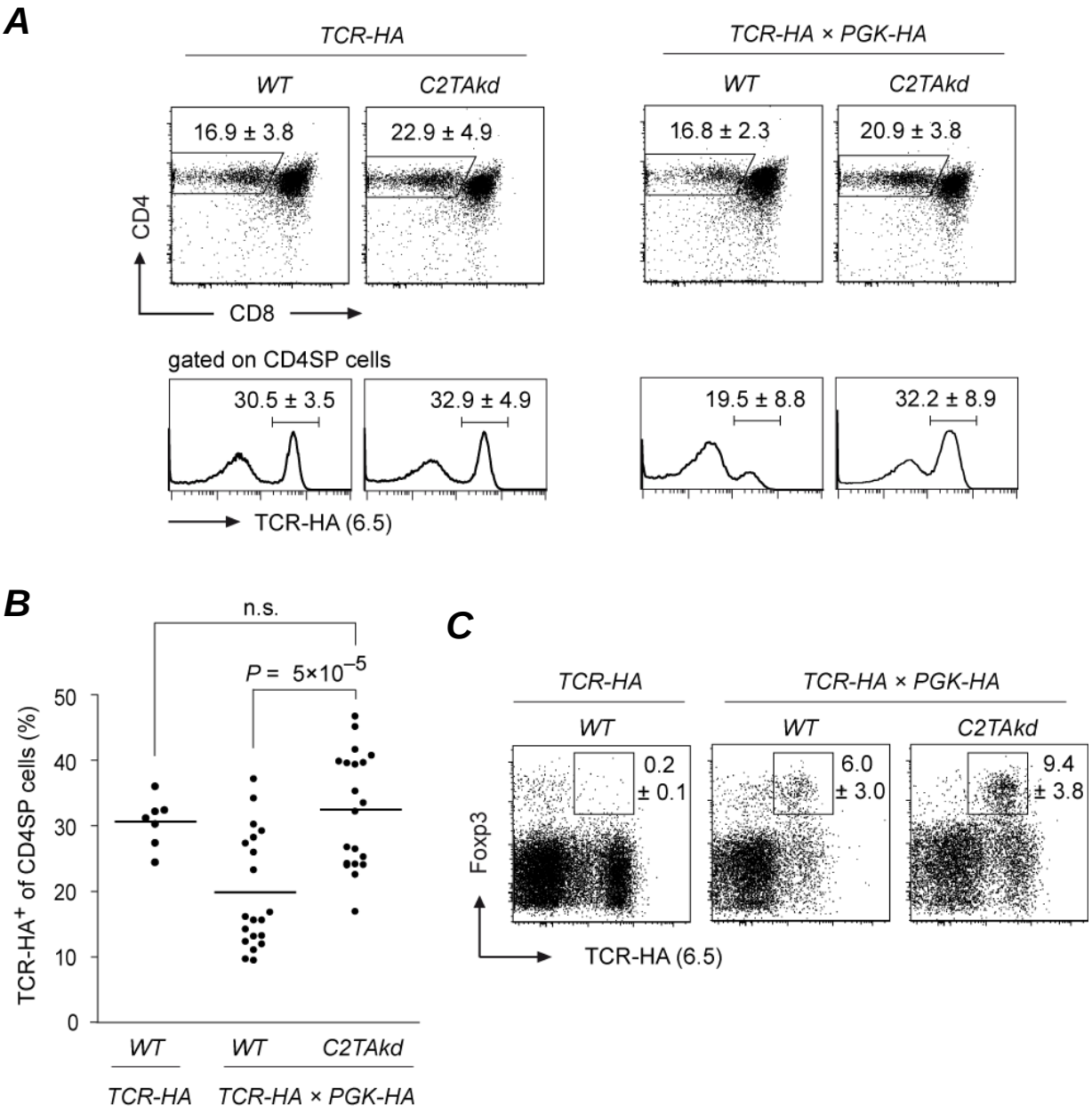


Figure 16 - Altered selection of HA-specific T cells in TCR-HA × Pgk-HA × *C2TAkd* mice. (A) Diminished deletion in TCR-HA × Pgk-HA × *C2TAkd* mice. The plots depict stainings of thymocytes of 6-week-old TCR-HA × Pgk-HA or TCR-HA × Pgk-HA × *C2TAkd* mice for CD4 and CD8 (upper row). The respective histograms below show stainings of $CD4^+$ SP T cells from TCR-HA × Pgk-HA and TCR-HA × Pgk-HA × *C2TAkd* mice for TCR-HA (lower row). Numbers indicate the average frequency (± s.d.) of cells within these areas. (B) The percentages of $TCR\text{-}HA^+$ cells of $CD4^+$ SP T cells of individual TCR-HA, TCR-HA × Pgk-HA and TCR-HA × Pgk-HA × *C2TAkd* animals are shown in the graph. *P* values are indicated. (C) Increased T_{reg} induction in TCR-HA × Pgk-HA × *C2TAkd* mice. $CD4^+$ SP T cells form TCR-HA and TCR-HA × Pgk-HA or TCR-HA × Pgk-HA × *C2TAkd* mice were stained for TCR-HA and Foxp3. Numbers indicate the average frequency of cells within these areas (± s.d.).

3.11 Dendritic cells are dispensable for the deletion and T_{reg} induction of Ova-specific T cells

Our observations clearly suggest an autonomous role of mTEC in CD4 T cell tolerance not only as antigen producer but also as antigen presenter. However, considering the recent finding that intact peptide-MHC complexes can be efficiently transferred to and presented by DC, an alternative explanation needed to be considered (207, 211). Accordingly, the altered selection of $CD4^+$ T cells might as well be a secondary effect of the C2TA knock-down and might be a consequence of diminished pMHC complex transfer to DC.

Thus we aimed to address this issue and ascertain the role of DC in the two TCR-transgenic models used, by removing DC from the system. To this end, we took advantage of mice that expressed diphtheria toxin A (DTA) under the control of a loxP-flanked neomycin resistance (neo^R) cassette from the ROSA26 locus and crossed them to CD11c-Cre mice leading to the physical ablation of DC (∆DC) (201). To ascertain the contribution of DC to tolerance induction in DO11.10 × AIRE-HCO mice, AIRE-HCO or AIRE-HCO × *C2TAkd* animals were reconstituted with DO11.10 or DO11.10 × ∆DC bone marrow. While $DO11.10^+$ T cells were normally selected in WT animals reconstituted with $DO11.10^+$ bone marrow, $DO11.10^+$ T cells were deleted in recipients expressing Ova in mTEC (83.0 ± 4.1 (*n* = 3) versus 37.7 ± 8.5 (*n* = 8), $P = 3 \times 10^{-6}$) (*Figure 17A*). Importantly, deletion was not affected in AIRE-HCO mice that were devoid of DC (37.7 ± 8.5 (*n* = 8) versus 24.67 ± 15.4 (*n* = 8), *P* = 0.6) implying a redundant role of DC in the deletion of Ova-specific T cells (*Figure 17A*). In contrast to this, *C2TAkd* × AIRE-HCO mice reconstituted with ∆DC bone marrow and therefore free of DC, showed strongly reduced deletion of $DO11.10^+$ T cells (*Figure 17A*) (24.67 ± 15.4 (*n* = 8) versus 70.5 ± 10.5 (*n* = 10), $P = 1 \times 10^{-5}$), which can only be a consequence of diminished Ova presentation by mTEC. In accordance with this, induction of Ova-specific T_{reg} was also unaffected by the absence of DC in AIRE-HCO mice reconstituted with DO11.10 ∆DC bone marrow, whereas interfering with antigen presentation by mTEC significantly enhanced T_{reg} induction even in the absence of DC (2.8 ± 4.9 (*n* = 8) versus 10.0 ± 4.0 (*n* = 8), *P* = 0.005) (*Figure 17B*). The same held true for the absolute cell numbers of $DO11.10^+$ $Foxp3^+$ $CD4^+$ SP T cells (*Figure 17C*). Essentially the same findings were obtained in the TCR-HA system in the absence of DC (*Figure 18*).

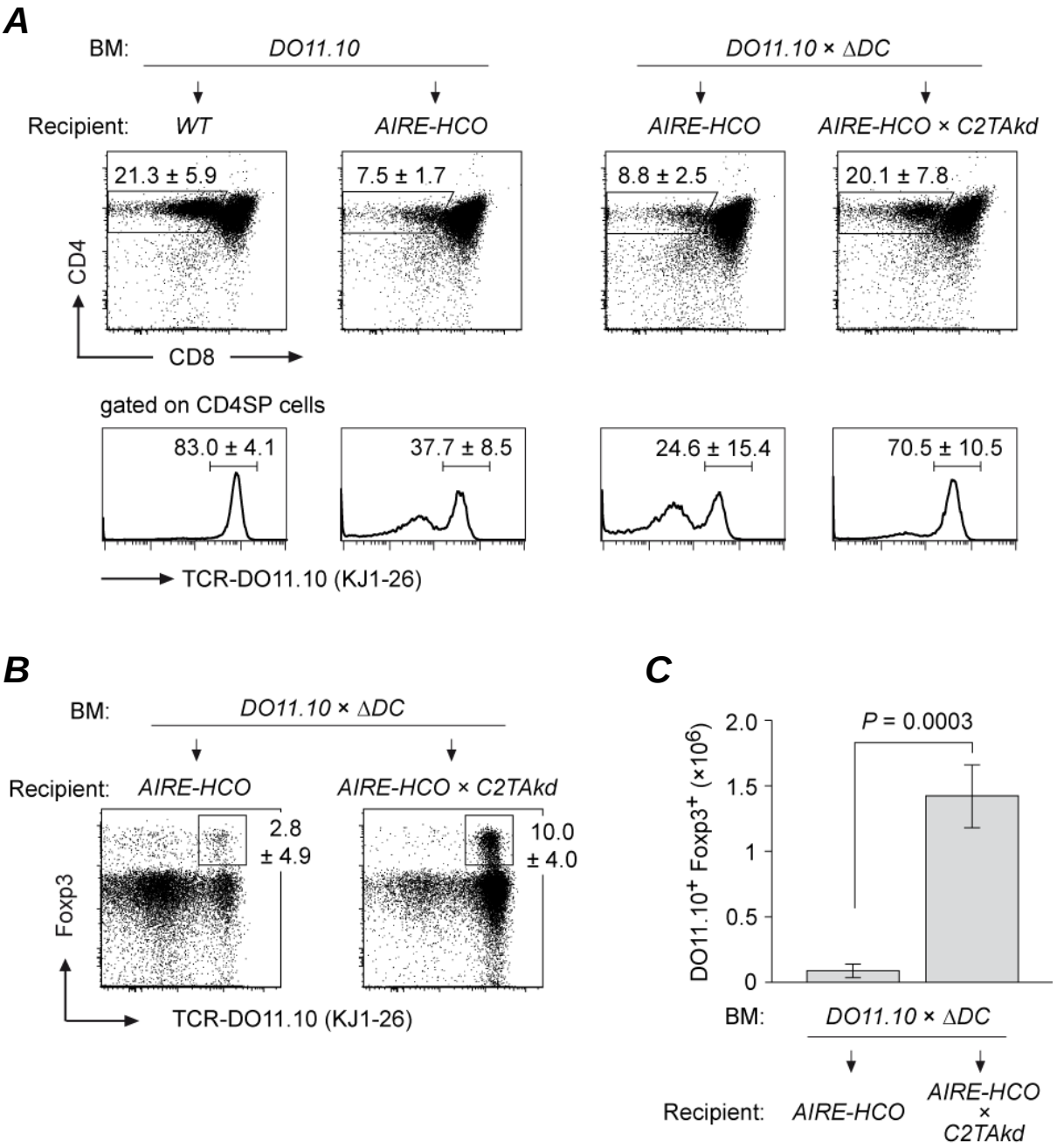


Figure 17 – Altered selection of DO11.10$^+$ T cells in DO11.10 x AIRE-HCO x *C2TAkd* mice is independent of DC. (A) Analysis of DC-deficient (ΔDC) bone marrow chimeras. WT recipients and AIRE-HCO or AIRE-HCO x *C2TAkd* recipients were either reconstituted with DO11.10 or DO11.10 x ΔDC bone marrow and CD4/CD8 T cell profiles as well as the percentage of CD4$^+$ DO11.10$^+$ cells of chimeric animals were analyzed 6 weeks later. Numbers indicate the average frequency of cells within these areas (± s.d.). (B) Plots depict DO11.10 and Foxp3 stainings of CD4$^+$ SP T cells from AIRE-HCO and AIRE-HCO x *C2TAkd* recipients reconstituted with DO11.10 x ΔDC bone marrow. Numbers indicate the average frequency of cells within these areas (± s.d.). (C) Absolute cell numbers of DO11.10$^+$ Foxp3$^+$ cells are shown in the graph. *P* values are indicated.

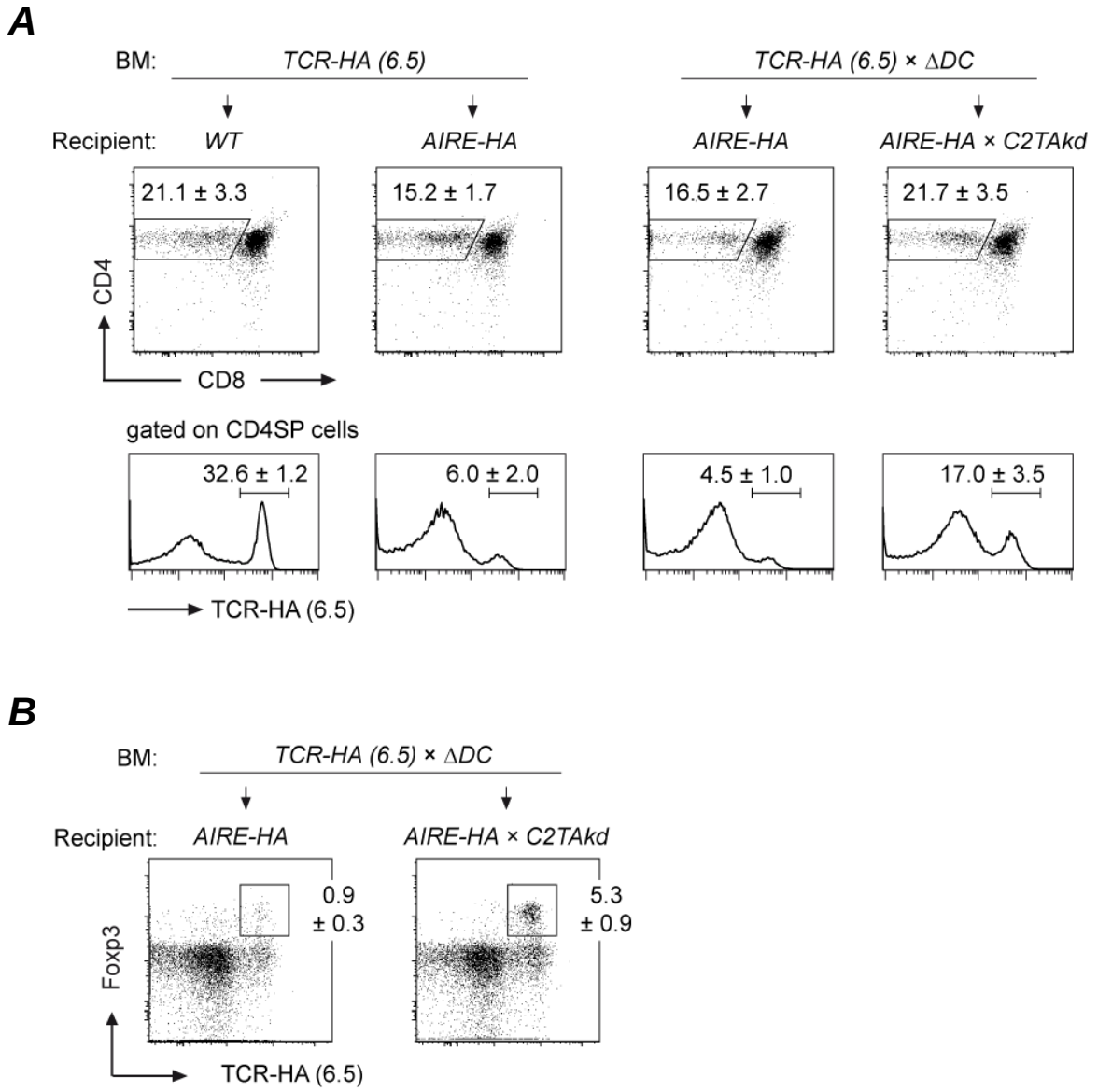


Figure 18 – Altered selection of TCR-HA$^+$ T cells in TCR-HA x AIRE-HA x *C2TAkd* mice is independent of DC. (A) Analysis of ΔDC bone marrow chimera. WT recipients and AIRE-HA or AIRE-HA x *C2TAkd* recipients were either reconstituted with TCR-HA or TCR-HA x ΔDC bone marrow and CD4/CD8 T cell profiles as well as the percentage of CD4$^+$ TCR-HA$^+$ T cells of chimeric animals were analyzed 6 weeks later. (B) Plots depict TCR-HA and Foxp3 stainings of CD4$^+$ SP T cells from AIRE-HA and AIRE-HA x *C2TAkd* recipients reconstituted with TCR-HA x ΔDC bone marrow. Numbers indicate the average frequency of cells within these areas (± s.d.).

4. DISCUSSION

Ever since the discovery of medullary thymic epithelial cells (mTEC) and their indispensable role in central tolerance induction, the mechanism(s) of how mTEC might contribute to this important task has been the center of investigations. The unique hallmark of mTEC is their capacity to express rare, otherwise tissue restricted genes thereby crucially contributing to the prevention of autoimmunity against peripheral organs. Hence, mTEC have a major function as antigen expressing cell, however, whether they also induce tolerance by direct antigen presentation to T cells is not known. We addressed this issue by generating a tissue-specific *C2TAkd* mouse and directly interfering with antigen presentation by mTEC *in vivo*.
Here, we show that mTEC serve a dual, non-redundant function in tolerance induction of $CD4^+$ T cells not only as antigen expressing cell but also as antigen presenting cell.

4.1 Transcriptional and translational RNA interference in *C2TAkd* mice?

Analysis of *C2TAkd* mice revealed efficient down regulation of MHCII mRNA and protein expression in mTEC. Quantification of C2TA mRNA, which is the actual target of the miRNA, showed a milder reduction as compared to MHCII. A plausible explanation for this finding could be the type of RNA interference (RNAi) that is induced. Generally miRNAs can regulate gene expression on two levels; first, by assembly into the RNA induced silencing complex (RISC) and the subsequent binding to and endonucleolytic cleavage of the target mRNA or second, by binding to the 3´UTR region of the target mRNA and inhibition of its translation. In other words, miRNAs can either repress the amount of mRNA and protein or only the amount of protein. Why and when a particular mode of RNAi is induced by a specific miRNA is still poorly understood. However, there are multiple examples showing either no change in mRNA level or a significant smaller reduction of the mRNA level as compared to the protein level, indicating that both transcriptional and translational silencing can be concomitantly induced by a single miRNA (217-220). Thus, it is likely that in *C2TAkd* mice mRNA and protein levels of C2TA are repressed at the same time, thereby synergistically leading to the strong reduction of MHCII.

Another possible explanation would be that the transcription of MHCII is very sensitive to alterations in the abundance of the co-activator C2TA. This would mean that already a moderate knock down of C2TA would be sufficient for a strong reduction of MHCII mRNA transcription. This scenario, however, is rather unlikely as $C2TA^{+/-}$ mice do not show significant alterations in MHCII expression as compared to WT mice (data not shown).

4.2 Specificity of C2TA for MHCII

The choice of C2TA as the miRNA target instead of MHCII itself was based on the fact that this would enable us to study antigen presentation by mTEC irrespective of the genetic background, e.g. Balb/c and BL6. Furthermore, C2TA is considered to be the master regulator of MHCII transcription. Accordingly $C2TA^{-/-}$ animals by and large recapitulate the phenotype of $MHCI^{-/-}$ mice and thus are devoid of MHCII expression (215). Indeed we found that less than 1% of all mTEC expressed MHCII at very low levels on their surface (data not shown + (43)).
Even though C2TA has a remarkable degree of specificity for MHCII and genes involved in the MHCII loading pathway (221-228), a few MHCII-unrelated genes have been reported to be influenced by C2TA. In all cases, however, expression was only very mildly regulated by C2TA and not observed *in vivo* or were subject of controversy (229, 230). Beside transcriptional repression of IL-4, FasL and Collagen α2 (225, 231-233), C2TA was also reported to up-regulate expression of MHCI and the co-stimulatory molecule Plexin A1 (213, 214, 234). Importantly, we tested all known targets of C2TA and found them either not to be expressed in mTEC or not differentially regulated in *C2TAkd* mTEC (*Figure 7* + data not shown). Thus, potential unwanted effects of *C2TAkd* on other C2TA-regulated genes than MHCII can be largely excluded.

4.3 Potential off-target effects

Endogenous, naturally occurring miRNA and their mode of action have been thoroughly investigated in the last decade. Today it is known that a given miRNA usually targets much more than one mRNA as its function does not require full

complementarity to the target (235) but instead a so called “seed region” of about 8 bases at the 5´end is essential and sufficient for effective repression of target mRNA translation. Bioinformatic predictions in mammals estimated that a few hundred miRNAs control approximately 30% of all protein-encoding genes (236).

Although the algorithm of the miRNA design program we used for the C2TA-specific miRNA design (*http://katahdin.cshl.org:9331/RNAi_web/scripts/main2.pl*) excluded sequence homologies to other genes than C2TA, it cannot be entirely excluded that the C2TA-specific miRNA represses the translation of other transcripts. This technical caveat is also known as “off-targeting”.

Therefore we carefully analyzed the phenotype of *C2TAkd* mTEC, including all known markers, e.g. MHCII, CD80, AIRE, MHCI, UEA-1, pGE (*Figure 8,9* and data not shown). Except for the down-modulation of MHCII, no alterations with respect to the markers we tested could be detected. Formally, however, this possibility still has to be considered. Future microarray analysis of mTEC from WT versus *C2TAkd* versus $C2TA^{-/-}$ as well as the generation of a second *C2TAkd* mouse, carrying a different hairpin, will uncover such off-target effects.

4.4 mTEC development in *C2TAkd* mice

It is well established that efficient TEC development depends on the cross-talk with developing T cells (237-239), involving CD40-CD40L as well as RANK-RANKL interactions (37-39). One study, however, also proposed a requirement of cognate pMHC-TCR interactions for the development of mature $AIRE^{+}$ mTEC (43). This hypothesis was based on the observation that mice expressing only one TCR and thus supposedly did not interact with peptide-MHCII (pMHCII) complexes on mTEC showed strongly reduced numbers of mTEC. Importantly, the mTEC compartment was completely restored when the cognate antigen of the TCR was expressed. The authors attributed this defect to the reduced proliferation they observed in the residual mTEC and suggested a model by which proliferating precursors in the immature mTEC population differentiate and/or expand into mature mTEC via the help of cognate self-peptide MHCII complex interactions with self-reactive $CD4^{+}$ T cells.

In consideration of these findings, we performed detailed analysis of mTEC development. Beside the normal appearance of the thymic architecture, all major mTEC subsets were normally formed. AIRE$^+$ mTEC that were notably most affected in the above mentioned study, were not reduced in *C2TAkd* animals as compared to WT. If anything at all, a slight increase of AIRE protein could be observed in *C2TAkd* mTEC. Our findings, however, do not necessarily contradict the hypothesis described above. First, even though MHCII is strongly reduced in *C2TAkd* mice, there is still some low expression level of residual MHCII, which might be sufficient and essential for the development of a full mTEC compartment because it still allows for cognate pMHC-TCR interactions. The lack of MHCII could be compensated for by increased proliferation of mTEC subsets, a possibility that we did not test for. Second, as C2TA miRNA expression is under the control of the AIRE promoter, MHCII is most strongly down-regulated in mature mTEC while immature CD80$^-$ mTEC are affected to a lesser extent. Hence, TCR – pMHC interactions between immature mTEC and T cells could still take place in *C2TAkd* animals which might be sufficient for proper mTEC development.

4.5 Negative selection in *C2TAkd* mice

The highly efficient and mTEC-specific C2TA knock down in *C2TAkd* mice, resulting in a strong reduction of MHCII, allowed us to directly investigate the consequences of impaired antigen presentation by mTEC on tolerance induction in CD4$^+$ T cell.
Analysis of bulk CD4$^+$ T cell development in *C2TAkd* mice revealed an increase of CD4$^+$ SP T cells of about 30%. This observation is in agreement with another report that found a similar enlargement of the CD4$^+$ SP T cell compartment in mice that were completely devoid of UEA$^+$ mTEC. In this study, however, impaired tolerance induction could also be explained by secondary effects, such as disrupted medulla formation and consequently hampered DC recruitment or T cell intrinsic defects (36). In contrast to this, we neither observed an altered thymic architecture nor mTEC development, but exclusively diminished MHCII expression by mTEC, leaving the thymic environment fully intact. Thus, alterations in T cell development can only be a direct result of diminished MHCII levels in mTEC.

An increase in the $CD4^+$ SP T cell compartment was also found when the role of DC in negative selection was studied. Chimeric mice reconstituted with $MHCII^{-/-}$ bone marrow to abolish antigen presentation by DC showed an enrichment of $CD4^+$ SP T cells which was supposedly caused by defective deletion of self-reactive T cells (202). This interpretation has later been strengthened by the fact that T cells from such chimeras indeed showed elevated auto-reactivity *in vitro* (240). Essentially the same conclusions were drawn from the analysis of DC knockout animals that were found to develop severe autoimmune disease, presumably as a consequence of defective negative selection (201).

Here we further clarified the role of mTEC versus DC in negative selection, using different bone marrow chimeras that were designed to eliminate antigen presentation by either mTEC or DC and found that in both cases negative selection of $CD4^+$ T cells was impaired. This became apparent by the enlargement of the $CD4^+$ SP compartment and the ability of these cells to react towards self-peptide-MHC molecules in an *in vitro* proliferation assay. The observation that the increase of $CD4^+$ SP T cells was even more pronounced if both DC and mTEC were diminished in antigen presentation is clearly in agreement with a non-redundant function of mTEC and DC as APC in the deletion of $CD4^+$ T cells.

4.6 T_{reg} induction in *C2TAkd* mice

mTEC have been implicated in the induction of regulatory T cells (T_{reg}). As previously established in our lab, expression and presentation of HA by mTEC was sufficient for the development of HA-specific T_{reg} and moreover, mTEC as well as DC were found to autonomously induce T_{reg} *in vitro* (62, 241). In contrast, here we did not observe any significant changes in the polyclonal $CD4^+$ T_{reg} compartment in mice diminished in antigen presentation by mTEC or DC, at least not with respect to the pool size. Of note, we always found a marked, yet not significant reduction of T_{reg} in mice devoid of antigen presentation by both mTEC and DC. Our findings do not necessarily imply a complete redundancy of mTEC and DC in the process of T_{reg} induction. While homeostatic mechanisms acting downstream of *bona fide* differentiation processes of T_{reg} might compensate for the absence of one or the other APC type, changes on the level of TCR specificities could still occur. Alterations in the TCR repertoire of T_{reg} can

certainly not be detected in a polyclonal system. This might as well explain why neither the complete absence of mTEC by blocking their development nor the lack of DC promoted significant perturbation in the size of the T_{reg} compartment (37, 201).
A major limitation inherent to analysis of the polyclonal T cell repertoire is that defective tolerance induction on the level of distinct TCR specificities is difficult to address. Therefore we ascertained the role of antigen presentation by mTEC in negative selection as well as in T_{reg} induction by using a TCR transgenic mouse model. As we previously published, targeted expression of Ova as well as HA to $AIRE^+$ mTEC led to the development of $CD4^+$ $Foxp3^+$ T_{reg} of the cognate TCR specificity concomitant to the partial deletion of specific TCR-transgenic $CD4^+$ T cells. Interfering with antigen presentation by mTEC in these systems resulted in diminished deletion and remarkably, increased T_{reg} development of TCR-transgenic $CD4^+$ T cells. This indicates that mTEC promote the selection of certain T_{reg} specificities, supposedly recognizing mTEC-derived antigens such as TRA, thereby crucially contributing to the repertoire formation of $CD4^+$ T_{reg}. Moreover, our observations provide direct evidence for an autonomous role of mTEC as APC not only in recessive tolerance but also the dominant tolerance induction of $CD4^+$ T cells.

4.7 Antigen transfer to DC

Antigen transfer between different types of APC has been suggested to substantially contribute to increase the efficiency of tolerance induction (242). For example it has been shown that Ova transfer to DC was not only sufficient but also essential to induce negative selection of Ova-specific TCR transgenic $CD4^+$ T cells (203). Recently much progress has been made to understand the modalities of antigen spreading within the thymic microenvironment. There appears to be a unidirectional transfer of mTEC-derived antigens, including rare tissue specific antigens, from mTEC to DC. Efficient antigen capture and presentation by DC does not only engage conventional MHCII loading pathways but also extends to the level of intercellular transfer of functional MHC molecules and other TEC-specific membrane proteins (207, 211). In view of this new observations, we addressed the possibility that reduced MHCII levels on mTEC in *C2TAkd* mice might have led to reduced capture of MHCII molecules by DC and thus to the altered tolerance induction we observed in

$CD4^+$ T cells. To this end we eliminated DC in the TCR-transgenic systems we previously used to test the importance of mTEC as antigen presenters and found that neither deletion nor T_{reg} induction of HA- or Ova-specific T cells was dependent on DC.

4.8 Avidity impinge on the mode of tolerance

Analysis of the TCR transgenic models showed that antigen presentation of HA or Ova by mTEC always led to both deletion and concomitant T_{reg} induction of $CD4^+$ T cells specific for the antigen, implying that these two different tolerogenic pathways can occur side by side. Large amounts of antigen presented by mTEC induced massive deletion of specific T cells, whereas reducing the amount of antigen on mTEC favored T_{reg} development and inhibited negative selection of T cells recognizing the cognate antigen. Thus, our findings, for the first time, provided evidence for a model by which T_{reg} induction occurs within a defined "window of avidity" which is below the threshold of negative selection, whereby the optimal antigen dose might vary between different TCR affinities as well as different APC, i.e. DC and mTEC (241). This would imply that T_{reg} induction is favored upon low avidity interactions which might lead to shaping the repertoire towards low abundance antigens such as TRA.

However, it is still unresolved why within an apparently homogenous cohort of T cells, all expressing the same TCR, one T cell develops into a T_{reg} while another T cell is deleted in one and the same antigen expressing environment. We provide several potential explanations: First, there is more and more evidence that the thymic medulla is rather heterogeneous with respect to availability of antigens, in particular with respect to low abundant antigens. Accordingly, it has been shown for promiscuously expressed genes that a given TRA was expressed by only about 3-5% of all mTEC (209). Therefore it might be a stochastic event whether a T cell runs into an APC presenting its cognate antigen. Second, T cells were shown to have a residence time of about 4 days in the medulla during which they have multiple interactions with APC thereby probably screening their environment for antigens (210). In view of this, one could speculate that a single pMHC-TCR contact might not be sufficient to induce tolerance but instead multiple consecutive interactions are

required for T_{reg} induction or deletion. Third, the medulla is a patchwork of different types of APC, all of which might have different tolerogenic properties. For example it has been recently shown that mTEC require higher optimal antigen doses for T_{reg} induction compared to DC (194). Forth, the susceptibility for tolerance induction might also be intrinsically regulated with the maturation stage. Accordingly, it was found that the capacity to deviate into the T_{reg} lineage inversely correlated with the maturation status (194). Similar rules could also apply for negative selection.

Together, several stochastic and probabilistic factors might impinge on the mode of tolerance that is induced. Depending on the encounter of different APC, the maturation status of the T cell and the availability of the cognate antigen, a T cell might be deleted or deviated into the T_{reg} lineage. It is intuitive that the amount of antigen on APC dictates the probability of interactions with T cells expressing the cognate TCR and thereby influences the mode of tolerance that is induced. Therefore there might be a balance between T_{reg} induction and negative selection, which is shifted to the one or the other direction depending on the availability of the given antigen (*Figure 19*).

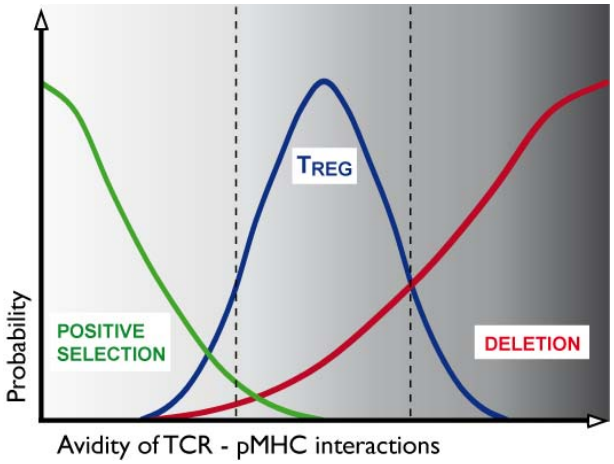


***Figure 19* - Avidity model of central tolerance induction.** The avidity of TCR - self peptide MHC interactions influences the mode of tolerance that is induced. Accordingly, high antigen doses lead to a strong susceptibility for negative selection, whereas lower antigen doses increase to probability of a T cell to be deviated into the T_{reg} lineage. Thus there is an optimal window of avidity for the development of T_{reg} that might partially overlap with positive selection or deletion. This could explain why deletion and T_{reg} induction of T cells with the very same TCR can often occur in parallel *in vivo*.

5. MATERIAL AND METHODS

Animals

TCR-HA, DO11.10, PGK-HA, C2TA$^{-/-}$ has been described elsewhere (215, 216, 243, 244). AIRE-GFP reporter mice were kindly provided by Dr. Mark Anderson (63). CD11c-Cre and DTA-floxed mice were obtained from Dr. David Vöhringer (201). C57-BL6 and Balb/c mice were purchased from Charles River. All other strains were from in-house breeding colonies. AIRE-HCO, AIRE-HA, *C2TAkd* mice were generated at the Research Institute of Molecular Pathology (62). All mice were breed in individually ventilated cages in the animal facility of the Research Institute of Molecular Pathology or in the animal facility of the Institute for Immunology of the LMU Munich. All animal studies were done according to the local regulations.

Antibodies and flow cytometry

Phycoerythrin (PE)-conjugated mAbs to CD45.1 (A20), Ly51 (BP-1), Cy-chrome-conjugated mAbs to CD8 (53-6.7), CD45 (Ly-5), CD11c (HL3), allophycocyanin (APC)-conjugated mAbs to CD45.1 (A20), phycoerythrin-Cy7 (PE-Cy7)-conjugated streptavidine, mAbs to CD25 (PC61), allophycocyanin-Cy7 (APC-Cy7)-conjugated mAbs to CD4 (GK1.5) and biotin-conjugated monoclonal antibodies (mAbs) to CD8 (53-6.7), CD4 (GK 1.5), CD80 (B7.1, 16-10A1) were purchased from Becton Dickinson. APC-conjugated EpCAM (G8.8) was purchased from BioLegend. Monoclonal Abs to EpCAM (G8.8), TCR-HA (6.5), DO11.10 (KJ1.26) and pan-MHC class II (P7.7) were purified from hybridoma supernatant and conjugated to FITC, PE or Alexa647 in our lab. 4',6-Diamidin-2'-phenylindol- dihydrochlorid (DAPI) was purchased from Invitrogen.

Intracellular staining of Foxp3 was performed according to the manufacturer's protocol using FITC–, PE- or APC-conjugated mAbs to Foxp3 (FJK-16s, eBiosciences). Surface stainings were performed according to standard procedures at a density of 1-2 x 10^6 per 50 µl and volumes were scaled up accordingly. Flow-cytometric analysis was performed on FACS Canto II (Becton Dickinson) using FACS DIVA software (Becton Dickinson) for acquisition and Flowjo (Treestar) for analysis.

Immunofluorescence

5µm frozen sections were fixed in cold acetone and permeabilized with 0.1% (vol/vol) Tween in PBS for 30 min. Sections were blocked with 10% (vol/vol) FCS in PBS for 30 min and washed 3 x 15min in 0.1% (vol/vol) Tween in PBS. Rabbit anti-keratin 5 antibody (Covance, 1:100) staining was carried out overnight at 4°C. Next day sections were washed 3 x 15min and incubated with Alexa Fluor 488-conjugated secondary anti-rabbit antibody (Molecular Probes, 1:800) for 60 min at room temperature. After washing 3 x 15 min, sections were blocked with rabbit serum (Jackson Immuno Research Laboratories, Inc.) for 30 min at room temperature and incubated with biotin anti-keratin 8 antibody (TROMA-1, Developmental Studies Hybridoma Bank, 1:100), biotin anti-AIRE antibody (5H12-2, kindly provided by Dr Hamish Scott, 1:400) or biotin anti-I-A^d (AMS-32.1, Becton Dickinson, 1:800) for 2 h at room temperature. Subsequently sections are washed 3 x 15min and the secondary reagent straptavidin-Cy3 (Jackson Immuno Research Laboratories,Inc., 1:1000) was added for 60 min at room temperature. Finally after washing 3 x 15min nuclei were counterstained with ProLong Gold antifade reagent with 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) and samples were analyzed with the fluorescence microscope BX41 (Olympus).

Western blotting

$1x10^6$ ($5x10^5$) cells were lysed in 50µl (20µl) lysis buffer at 4°C for 5 min. 1x sample buffer was added and lysate was boiled at 95°C for 4 min. Subsequently samples were loaded on a SDS-PAGE (see below).

SDS-PAGE

	Running gel (15%)	Loading gel
H_2O	4.9ml	6ml
1.5M TrisHCl+0.4% SDS (pH6.8)	5ml	2.5ml
29.2% acrylamid+0.8% bis-acrylamid	10ml	1.5ml
10% TEMED	100µl	100µl
10% ammonium-persulfat (APS)	100µl	100µl

< 10ml per 1.5mm gel

< 4ml per 1.5mm gel

Sample Buffer (3x)

H2O	ad40ml
0.5M Tris-HCl pH6.8	12 ml
Glycerol	8 ml
20% SDS	10 ml
(0.05%) Bromphenolblue	
ß-Mercaptoethanol	20 µl/ml

Lysepuffer

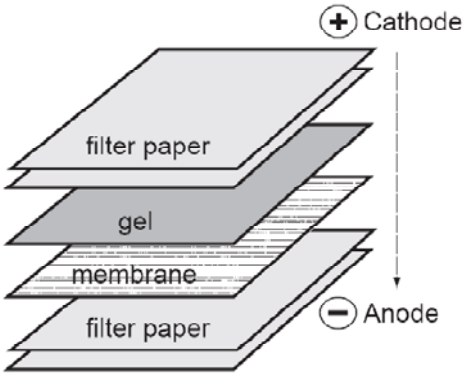
Triton X	1%
Aprotinin/ml	100KIU
Pefablock	0.16mM
Na Azid	0.1%
in PBS	

Running Buffer (10x)

Tris base	30.3 g
Glyzin	144.0 g
SDS	10g
H_2O	1l

Blotting

A semidry blotting chamber (LKB 2117 Multiphor II electrophoresis unit) was used to transfer the proteins to the membrane. The blotting procedure was carried out as depicted in the scheme below (scheme). The chamber was connected to 160mA for 2h.



Semi-dry blotting:

filter paper: GB003 (3mm), Schleicher & Schuell (soaked in buffer)

membrane: PVDF, 0.45µm, Roche (soaked in Methanol and washed with H_2O)

Electrophoresis unit: LKB2117 Multiphor II, LKB

After transfer of proteins the membrane was transferred to a blocking solution (4% milk powder) for 1h on room temperature. Thereafter the primary antibody was added and incubated over night at 4°C. Next day the membrane was thoroughly washed (8 x 5min) with wash buffer (PBS supplemented with 0.05% Tween20) before it was incubated with the secondary antibody (in 4% milk powder) for 4h on room temperature. After thoroughly washing at least 8 x 5min substrate is added for 5 min and the membrane can be exposed to the film.

Antibodies for WB

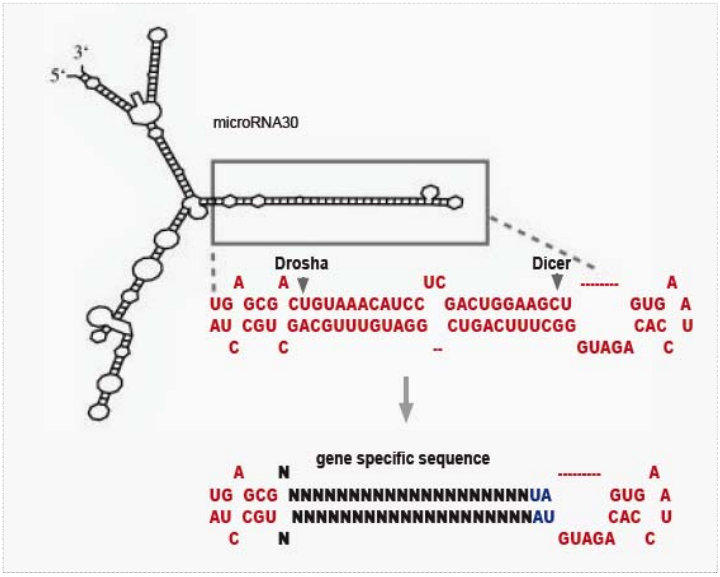
Mouse anti mouse β-actin (AC-15, Sigma-Aldrich; 1:20000)
Polyclonal rabbit anti mouse horse radish peroxidase (HRP) (P260, DAKO; 1:2000)
Rat anti mouse MHCII (H2-Ea) (IBL-5/22, Santa Cruz; 1:200)
Polyclonal mouse anti rat HRP (212-035-168, Dianova; 1:9000)
Polyclonal rabbit anti AIRE (Hamish Scott; 1:100)
Polyclonal goat anti rabbit HRP (111-035-144, Dianova; 1:70 000)
Substrate: Super Signal West Pico (Pierce)
Film: WICORex B^+ (Linhardt Röntgenbedarf)
Developer: 35 Compact (PROTEC)

Cloning

Restriction enzymes were purchased from Roche. Digestions of plasmids were performed in the appropriate buffers with 10U restriction enzyme at 37°C or 55°C for 1h. Cut vectors were separated on a 1-2% agarosegel and purified using the Gel purification Kit (Quiagen). Before ligation vector backbones were dephosphorylated with 10U alkaline phosphatase (Roche) with the appropriate buffer at 37°C for 1h. Ligation was performed using quick ligase (BioLabs) with the appropriate buffer for 5min on 25°C. Equal stoechiometric amounts of insert and backbone were used. 10µl ligation mix was incubated together with competent E.coli (DH5α), provided by the IMP service department, on ice for 20min with, followed by a heat shock of 2min at 42°C. Finally transfected bacteria were streaked on LB-Agar plates containing the appropriate antibiotic as selection marker.

Cloning of the retroviral CIITAsh vector (LMP CIITAsh)

The murine stem cell virus (MSCV)-based LMP vector was purchased from OpenBioscience and provided the flanking regions of the human miRNA30 (scheme) (245). In order to obtain the knock-down of a gene of interest, the miRNA30 can be exchanged into the sequence specific for this gene. Subsequently this “designer miRNA” will be normally processed in the miRNA processing pathway and this ultimately leads to efficient knock-down. Here we aimed to target MHCII by either using C2TA-specific sequences.



CIITA (MH3-6) specific short hairpins (97mers) were designed with the help of the website:

http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA

The RNAi oligo retriever generated the hairpin primer by selecting a ‘sense’ sequence of 22 nucleotides (nt) in length from the coding sequence of the gene of interest. Generally there are several common rules that the RNAi oligo retriever applied to the designe: The selected sequence was usually targeted to the coding sequences or 3’ UTR and contains >3 mismatches to any other gene. Furthermore SNPs were avoided as well as common exons targeted in alternatively spliced mRNAs. Importantly the 5´end of the antisense strand was thermodynamically destabilized in order to favor its incorporation into RISC.

Sequences of 97mers:

MH3 5´ TGCTGTTGACAGTGAGCGCGGGCCTCCTTGAGTGATACAATAGTGAAG CCACAGATGTATTGTATCACTCAAGGAGGCCCTTGCCTACTGCCTCGGA 3´
MH4 5´ TGCTGTTGACAGTGAGCGAGCAGCTACCTGGAACTCCTTATAGTGAAG CCACAGATGTATAAGGAGTTCCAGGTAGCTGCCTGCCTACTGCCTCGGA 3´
MH5 5´ TGCTGTTGACAGTGAGCGAGCCCAGCTACCTTGTACACTTTAGTGAAGC CACAGATGTAAAGTGTACAAGGTAGCTGGGCCTGCCTACTGCCTCGGA 3´
MH6 5´ TGCTGTTGACAGTGAGCGCGGCTGGCACAGTGCAATGAAATAGTGAAG CCACAGATGTATTTCATTGCACTGTGCCAGCCATGACTACTGCCCGGA 3´

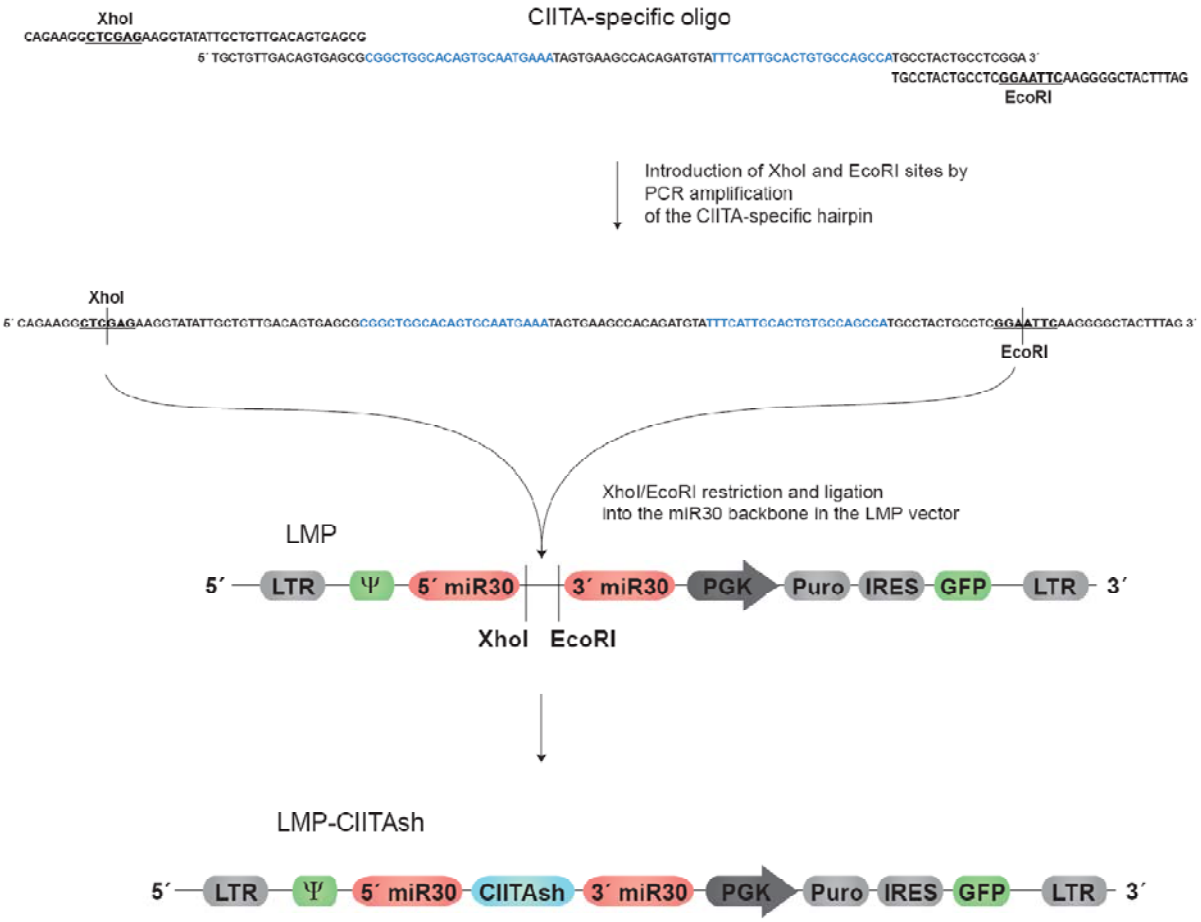
Amplification of the unique CIITA short hairpin sequence

In order to clone the CIITAsh into the miRNA30 backbone on the LMP vector, unique restriction sites were inserted by PCR amplification:

(XhoI-MH7 5´CAGAAGG**CTCGAG**AAGGTATATTGCTGTTGACAGTGAGCG 3´;
EcoRI-MH8 5´ CTAAAGTAGCCCCTT**GAATTC**CGAGGCAGTAGGCA 3´).

PCR-mix		PCR-program	
10µl	$MgCl_2$ (25mM)	94°C	1min
1µl	Advantage II	94°C	30sec
10µl	10x Buffer	54°C	30sec
10µl	dNTP (2mM)	75°C	1min
5µl	XhoI primer (10µM)	75°C	10min
5µl	EcoRI primer (10µM)	15°C	hold
5µl	GCmelt (Clontech)		
44µl	dH_2O		
10µl	97mer (0.1µM)		

The PCR product (138bp) was loaded on an agarose gel and purified with the Qiagen Gel Extraction Kit. The PCR product as well as the LMP vector was then digested with EcoRI and XhoI and again purified. After dephosphorylation of the LMP vector, the PCR product was ligated into the LMP vector (scheme). The Ligation was then transformed into DH5α bacteria that were subsequently streaked on plates with ampicillin and incubated at 37°C over night. Next day colonies were picked and incubated in 4ml LB plus ampicillin over night to perform minipreps next day. To identify clones with the proper insertion, the DNA was digested with XhoI and SacII which resulted in two products, 7019bp and 985bp if insertion was successful. Retroviral vectors were ready for testing *in vitro*.



Retroviral infection of B cell line

Transient transfection of virus producer cells (Phoenix):

Phoenix cells were seeded ($1.5x10^6$ cells per 6cm plate) and incubated over night at 37°C and 5% CO_2. Next day cells were adherent and formed a 70% confluent layer. Phoenix cells were transfected with 1-10µg vector DNA using Lipofectamin2000 (Invitrogen) according to the manufacturers protocol. DMEM medium without FCS and antibiotics was used. Transfection was repeated 24h later.

One day after the last transfection efficiency was controlled by measuring GFP expression and subsequently infection of B cells could be performed. Supernatant was cleared from cells with a 0.45µm syringe filter and polybrene was added (4µg/ml final). $0.5x10^6$ B cells (WEHI279.1) were resuspended in the supernatant containing the virus and incubated at 37°C and 5%CO_2. Infection procedure was repeated next day and infection efficiency was checked 48h after. Puromycin selection was started 48h after the last infection to obtain stable clones.

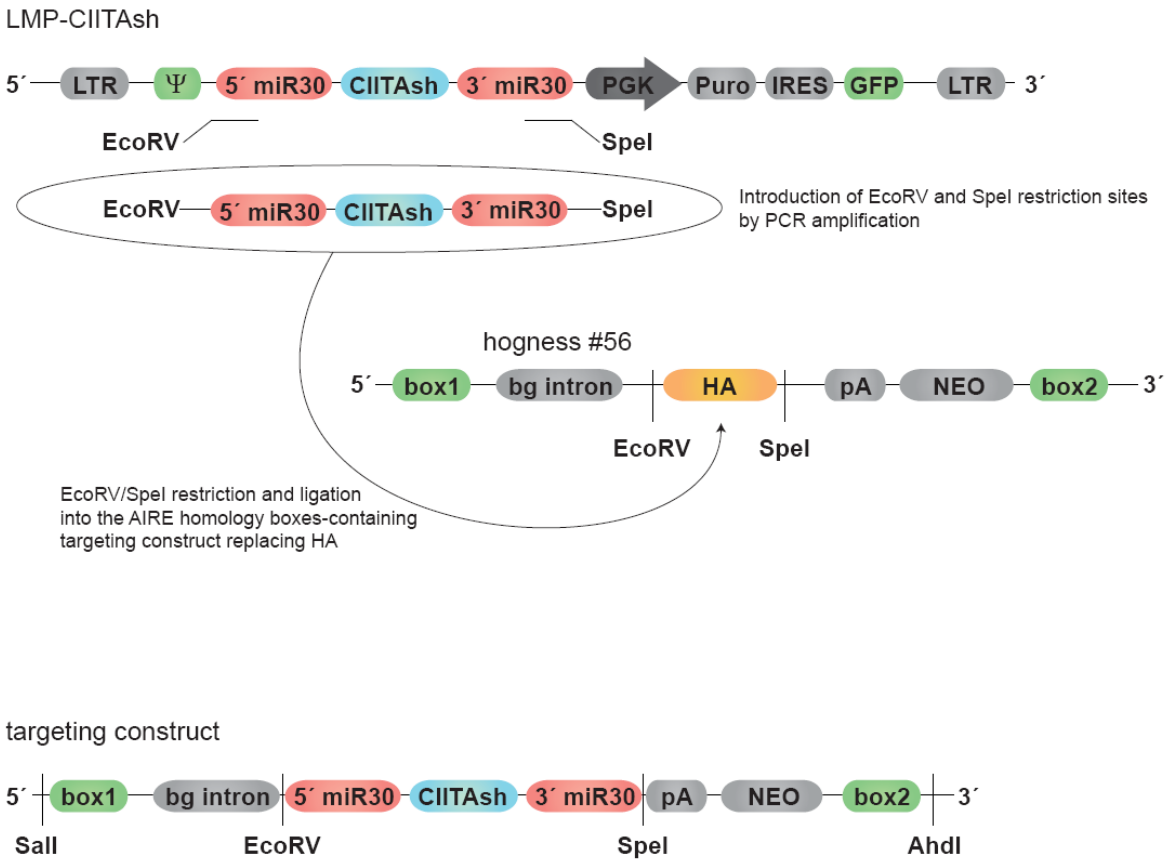
Generation of the transgenic *CIITAkd* mouse

Cloning of the AIRE targeting construct

The targeting construct used for the BAC modification was generated by PCR-amplification of the miRNA30-CIITAsh flanked by the miRNA30 5´ and 3´ flanking regions introducing EcoRV and SpeI sites (prove reading polymerase was used).

EcoRV-MH22: 5´ ATGG**GATATC**CACTCCTTCTCTAGGCGCCG 3´
SpeI-MH23: 5´ ACCGG**ACTAGT**GGAAAAGCGCCTCCCCTACC 3´

The PCR product (328bp) was gel purified and subsequently digested with EcoRV and SpeI to be ligated into the hogness #56 instead of HA (see scheme below).



The targeting construct was recombined into the mouse BAC RP23-77011 which contains 58kb downstream and 152kb upstream from the transcription start site of AIRE (BACPAC Resources Center, Oakland, USA (246). Homology boxes spanning nucleotides -379 to -2 (5': box1) and 17 to 399 (3': box2) (with A of the start-codon being +1) were used for homologous recombination of the miR30-CIITAsh into the AIRE locus.

BAC modification

Transformation of BAC with pBAD

DH10β bacteria containing the BAC RP23-77011 were grown in 4ml LB supplemented with chloramphenicol (12.5µg/ml) over night at 37°C. Bacteria were then spun down at 10000 rpm for 2min and resuspended in 1ml ice-cold 10% glycerol. This washing step was repeated twice followed by electroporation (200Ω, 25uFD, 2,5kV) of bacteria in 50µl 10% glycerol with 500ng pBAD. pBAD encodes for the recombinase required for BAC modification later on. LB was added to the

transformed bacteria and incubated for 1h at 37°C without antibiotics. After that periode, bacteria were plated on LB plates supplemented with chloramphenicol and ampicillin (100µg/ml) and incubated on 37°C over night.

Transformation and recombination of insert

3 colonies were picked next day and again incubated in 2-4ml LB plus ampicillin and chloramphenicol over night. Next day 1ml of the culture was grown in 70ml LB plus ampicillin and chloramphenicol at 37°C until OD_{600}=0.1. At that point L-arabinose was added and bacteria were further grown until an OD_{600} of 0.3-0.4 was reached. Bacteria were spun down at 7000rpm for 5min and washed 3 times with 1ml cold 10% glycerol. The supernatant was then discarded and bacteria were resuspended in remaining glycerol. 400-1000ng insert (targeting construct digested with AhdI and SalI) was added and bacteria were electroporated (see pBAD transformation). Transformed bacteria were incubated at 37°C for 1.5h. In order to select for recombination of the insert, bacteria were streaked on LB plated supplemented with kanamycin and chloramphenicol at 37°C over night.

Screening for positive clones

Next day 12 colonies were picked and incubated over night with LB plus kanamycin and chloramphenicol. From these cultures DNA was isolated using Quiagen Miniprep kit and tested for successful recombination via PCR.

Screening scheme

KA9 and KA10 identified empty vectors, MH40 and MH23 amplified positive clones and KA8 and KA10 were used to check for the loss of neomycin. TD5430 PCR program was used in all cases.

Left over of the LB culture of the positive clones was diluted 1 to 1000 and one drop was plated on kanamycinan/chloramphenicol LB plates and incubated at 37°C over

night. On the next day, 50 colonies per plate were picked and replica-plated first on plates with ampicillin and second on plates with chloramphenicol/kanamycin plates. Those clones that were growing on chloramphenicol/kanamycin plates but not under ampicillin selection already lost the recombinase plasmid and can therefore be further used.

Deletion of kanamycin resistance

In order to delete the kanamycin resistance, bacteria were transformed with the flp-plasmid carrying the flp recombinase. For that purpose 4 clones were inoculated in 2ml LB supplemented with kanamycin and chloramphenicol over night. Next day cultures were washed and transformed with approximately 300ng of flp-plasmid. Transformed bacteria were first incubated at 30°C for one hour and subsequently streaked on LB plates supplemented with tetracyclin and incubated on 30°C over night.

Next 6 clones from different tetracyclin plates were inoculated in 2ml LB without antibiotics over night on 37°C leading to the loss of the flp plasmid. To test for the deletion of the kanamycin resistance over night cultures were diluted 1 to 1000 and streaked on chloramphenicol plates and on plates containing both chloramphenicol and kanamycin plates were incubated at 37°C over night. Those clones that grow significantly less on kanamycin/chloramphenicol plates were tested for the loss of the kanamycin by PCR (see screening scheme). Those clones that were screened positive for the loss of the kanamycin resistance were again tested and replica plated on first tetracyclin second chloramphenicol and third chloramphenicol/kanamycin plates. In parallel the very same clones were inoculated in 4ml LB with chloramphenicol over night at 37°C.

The 4ml LB culture of clones that were only growing on chloramphenicol plates were used for injection.

Large scale preparation of BAC DNA

One or two clones that positively passed all screening procedures were inoculated in 2 x 1l LB with chloramphenicol at 37°C over night. Cultures were centrifuged and pellet were resuspended in buffer P1 (45ml P1 per 1l culture, Maxi Prep Quiagen). To lyse the cells 15ml of P2 was added per 45ml bacteria suspension and the lysate

was incubated for 15min at room temperature. Next 15ml buffer P3 was added followed by a 20min incubation step on ice and a centrifugation step for 30min at 4500rpm. After that the supernatant (filtered) was loaded on equilibrated columns (6 per 2l culture) and columns were washed 2x with 30ml wash buffer. BAC DNA was eluted with 15ml pre-warmed elution buffer per column and precipitated with 10.5ml isopropanol. After 1h centrifugation a small pellet was visible which was then transferred to an eppendorf tube and washed 3x with 70% Ethanol. Finally the DNA was air-dried and resuspended in 500µl TE-buffer.

BAC DNA purification – Cesiumchloride gradient

Two snap cap tubes with 4.4g of cesiumchloride were filled with either 4400µl TE-buffer or 4150µl BAC DNA and 250µl EtBr. As soon as the cesiumchloride was dissolved, solutions were transferred to Vt.65.2 Beckman centrifuge tubes, balanced and sealed. The gradient was ultra centrifuged at 58000rpm at 22°C for 17h (Vt.65.2 rotor). Next day the purified BAC DNA was recovered under the UV light. To extract the DNA, 1ml isoamylalcohole was added, the sample was mixed, spun down and supernatant was discarded. This step was repeated several times.
The DNA was dialyzed against injection buffer for several days whereas the buffer was changed every day.

10x injection buffer

1830ml H_2O
160ml TRIS (1M, pH 7.5)
4ml EDTA (0.5M)
NaCl (100mM final)

BAC DNA preparation for pronuclear injections

After dialysis BAC DNA concentration was determined by spectrophotometry. The quality was controlled on a 0.5% agarosegel. For pronuclear injections DNA was diluted in injection buffer to a concentration of 1ng/ml.

Bone marrow chimeras

Bone marrow was obtained from donor mice by isolating tibia and femur and flushing out the bone marrow mass using a syringe and a needle. Single cell suspension was made and subsequently bone marrow was depleted from T cells using biotinylated CD8 and CD4 mAbs and streptavidin MACS beads (Myltenyi) according to standard procedures. Balb/c and BL6 recipient mice were lethally irradiated with 2x450rad and 2x550rad respectively and reconstituted by injecting 6-8x10^6 bone marrow cells into the tail vein. Chimeras were analyzed 5-6 weeks after reconstitution.

Antigen-presentation assay

2 x 10^4 A5 T cell hybridoma cells were co-cultured with 1 – 2 x 10^4 antigen presenting cells (APC) in 200 µl IMDM supplemented with 1% FCS in 96-well round bottom plates. HA107-119 peptide (SVSSFERFEIFPK, recognized by the TCR-HA in the context of I-E^d). GFP expression of A5 T cells was measured by flow-cytometry 19h later.

In vitro assay

5 x 10^4 $CD4^+$SP thymocytes were co-cultered with 2.5x10^4 thymic APCs in IMDM supplemented 10% FCS, PenStrep glu?,b merc. H^3 thymidine was added to the culture on day 3 (1µCu/well) and thymidin incorporation was measured 19h later using a scintillation counter.

Media for primary T cell culture

IMDM (Gibco)
10% FCS
0.292mg/ml L-Glutamin
100U/ml Streptomycin
100U/ml Penicilin
50µM β-Mercaptoethanol

Isolation of thymic epithelium

Thymi were disenthralled from connective tissue and fat, cut into very small pieces using scissors and transferred to pre-warmed digestion medium (IMDM containing 0.2 mg/ml Collagenase (Roche), 0.2 mg/ml DispaseI (Roche), 2% FCS, 25mM HEPES (pH 7.2) and 25 µg/ml DNase I). 1ml digestion medium was used per thymus. Digestion was performed in a FACS tube at 37 °C; First the suspension was mixed using a 1000µl pipette, later on the 200µl pipette was used to digest the epithelial cells and DC out of the matrix. After about 45-50min the cell suspension was transferred to 4°C followed by the addition of EDTA (5 mM final) for 5min at RT. Cells were filtered and washed in PBS and resuspended in PercollTM (ρ 1.115; GEHealthcare). A second layer of Percoll (ρ 1.055) and a third layer of FACS buffer was carefully added on top.
The Percoll solutions were prepared as follows:

ρ 1.115	**ρ 1.055**
9x Percoll stock (ρ 1.134) 1x PBS (10x) 25mM HEPES pH=7.2 (final)	1x Percoll (ρ 1.115) 1.09x PBS (1x)

The densities were calculated according to the following formula:

$$Vy = Vi \times (Di - Dii) \,/\, (Dii - Dy)$$

Vy…volume of diluting solution (PBS)
Vi…volume of Percoll stock solution
Di…density of Percoll stock solution
Di…desired density
Dy…density of PBS (=1)

Optionally the low density Percoll solution can be decreased or increased to ρ 1.050 or ρ 1.060 respectively.

After that the gradient was centrifuged at 4°C and 1350g without acceleration and break for 30min. The upper interface, containing the desired low density cell fraction, was harvested and washed in FACS buffer. Cells were now ready for staining.

RNA isolation

Total RNA was isolated from FACS sorted thymic stromal subsets using the miRNAeasy kit (Qiagen) including an on column DNAseI (Qiagen) digest according to the manufacturers recommendations.

Quantitative PCR

RNA was reverse transcribed using the iScript cDNA synthesis kit (Biorad) according to the manufacturers recommendations.

qPCR reactions were performed in duplicates on a CFX96 realtime thermal cycler (Biorad) using the myIQ sybrgreen mastermix (Biorad) in 10µl reactions according to the manufacturers recommendations. Primers were used at 500nM each.

Cycling conditions

94°C for 180sec

94°C for 10sec
56°C for 20sec
72°C for 20sec
} 40x (plate read)

Melting curve analysis

Primers:

gene	product	sequence
actb	97bp	5´ GCCTTCCTTCTTGGGTAT 3´
		5´ GGCATAGAGGTCTTTACGG 3´
Aire	117bp	5´ CCTTATCCAGCAGGTGTTT 3´
		5´ CGGGCCTTGTTCTTCA 3´
HA	147bp	5´ GCTGGGAGGATGAACTATTA 3´
		5´ CATTGATGCGTTTGAGGT 3´
H2-IA-alpha	185bp	5´ GGCTCAGAAATAGCAAGTCA 3´
		5´ AATCTCAGGTTCCCAGTGTT 3´

C2TA	148bp	5´ CCTATGCCAACATTGCG 3´
		5´ GGCTTCTGTCCTGCTTCTA 3´
H2-K (pan MHCI)	151bp	5´ GATTACATCGCCCTGAACG 3´
		5´ GGTATCTGCGGAGCCACT 3´

Relative expression levels were calculated using the comparative Ct method using actb for normalization.

Small RNA quantification

A custom small RNA assay for the detection of UUUCAUUGCACUGUGCCAGCCA (mature C2TAsh) was designed and manufactured by Applied Biosystems.

Total RNA including the smallRNA fraction was isolated from FACS sorted thymic stromal subsets (see above) using the miRNAeasy kit (QIAGEN) including an on column DNAseI (QIAGEN) digest according to the manufacturers recommendations.

RNA equivalents of approximately 2100 cells were reverse transcribed using specific RT primers for mouse small nucleolar RNA (snoRNA202) and the mature C2TA antisense shRNA (C2TAsh) (Applied Biosystems) using the TaqMan microRNA RT kit (Applied Biosystems) according to the manufacturers recommendations.

cDNA reactions were diluted 5 fold and used as template for PCR. PCR reactions were run in duplicate using primer / probe combinations for snoRNA202 and C2TAsh (Applied Biosystems) and the TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) in 20ul reactions according to the manufacturer´s recommendations. Fluorescence was recorded at the annealing step and relative expression levels were calculated with the comparative Ct method using snoRNA202 for normalization.

Genotyping

DNA preparation

Tails were incubated in 50µl digestion buffer at 55°C for 5h. Proteinase K inactivation was carried out at 95°C for 5min.

Gitocher digestion buffer (10x)
670mM Tris pH 8.8
166mM ammonium sulfate
65mM MgCl2
0.1% Gelatin

Digestion buffer
3µl Proteinase K (10mg/ml stock)
2.5µl Triton (10% Stock)
5µl Gitocher Buffer (10x)
0.5µl β-Mercapto-ethanol
39µl H2O
Subsequently 1µl of the DNA was used for genotyping.

PCR Red-buffer (5x)
250mM KCl
50mM Tris pH 8.3
43% Glycerol
7.5mM MgCl2
2mM Cresol Red

PCR reaction
1µl tail digest
2.5mM primers (final)
200µM dNTP (final)
PCR Buffer (1x final)
Taq Polymerase
Ad 30µl with H2O

All genotyping reactions were carried out using the TD54x30 program.

TD54x30

3min at 94°N

45sec at 94°C
45sec at 60°C
60sec at 72°C
} 2 cycles

45sec at 94°C
45sec at 58°C
60sec at 72°C
} 2 cycles

45sec at 94°C
45sec at 56°C
60sec at 72°C
} 2 cycles

45sec at 94°c
45sec at 54°C
60sec at 72°C
} 30 cycles

10min at 72°C

Primers used for genotyping

gene	**primer**	**sequence**
CIITAkd	MH40 fwd	5´ TAAATTCTGGCTGGCGTGG 3´
CIITAkd	MH23 rev	5´ ACCGGACTAGTGGAAAAGCGCCTCCCCTACC 3´
TCR-HA	6.5 5´ fwd	5´ ACAAGGTGGCAGTAACAGGA 3´
TCR-HA	6.5 3´ rev	5´ ACAGTCAGTCTGGTTCCTGA 3´
AIRE-HA	KA9 fwd	5´ ACAGCCACTCCTGTCTTTGC 3´
AIRE-HA	HA3 rev	5´ CTCCGTCAGCCATAGCAAATTTCT 3´

pgk-HA	HA 5 fwd	5´ GGCTACCATGCGAACAATTCAACCG 3´
pgk-HA	HA 3 rev	5´ CTCCGTCAGCCATAGCAAATTTCTG 3´
AIRE-HCO	KA9 fwd	5´ ACAGCCACTCCTGTCTTTGC 3´
AIRE-HCO	JE12 rev	5´ GAATTGTTCGCATGGTAGCC 3´
DO11.10	DO11.10 5´ fwd	5´ CAGGAGGGATCCAGTGCCAGC 3´
DO11.10	DO11.10 3´ rev	5´ TGGCTCTACAGTGAGTTTGGT 3´
CD11c-Cre	Cre 5´ fwd	5´ CGATGCAACGAGTGATGAGG 3´
CD11c-Cre	Cre 3´ rev	5´ GCATTGCTGTCACTTGGTCGT 3´
DTA	DTA 5´ fwd	5´ TACATCGCATCTTGGCCACG 3´
DTA	DTA 3´ rev	5´ CCGACAATAAATACGACGCTG 3´
AIRE-Gfp	GFP1 fwd	5´ AAGTTCATCTGCACCACCG 3´
AIRE-Gfp	GFP2 rev	5´ TCCTTGAAGAAGATGGTGCG 3´
Rag2	Rag 5´ fwd	5´ GCAACATGTTATCCAGTAGCCGGT 3´
Rag2 rev	Rag 3´	5´ TTGGGAGGACACTCACTTGCCAGT 3´
Rag2 int	Rag int	5´ GTATGCAGCCGCCGCATTGCATCA 3´
CIITA wt	CIITA1869 fwd	5´ GATCGGAGACAAGGGTGTGT 3´
CIITA wt	CIITA1870 rev	5´ GTCAGGGAGCAGGATCTTTG 3´
CIITA ko	CIITA 158 fwd	5´ CTGAATGAACTGCAGGACGA 3´
CIITA ko	CIITA 159 rev	5´ ATACTTTCTCGGCAGGAGCA 3´
MHCII	MA295 fwd	5´ TTCGTGTACCAGTTCATGGG 3´
MHCII	MA296 int	5´ TAGTTGTGTCTGCACACCGT 3´
MHCII	MA297 rev	5´ CCTGCCGAGAAAGTATCCA 3´

6. REFERENCES

1. Cannon, J.P., R.N. Haire, J.P. Rast, and G.W. Litman. 2004. The phylogenetic origins of the antigen-binding receptors and somatic diversification mechanisms. *Immunol Rev* 200:12-22.
2. Flajnik, M.F., and L. Du Pasquier. 2004. Evolution of innate and adaptive immunity: can we draw a line? *Trends Immunol* 25:640-644.
3. Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21:139-176.
4. Nehls, M., B. Kyewski, M. Messerle, R. Waldschutz, K. Schuddekopf, A.J. Smith, and T. Boehm. 1996. Two genetically separable steps in the differentiation of thymic epithelium. *Science* 272:886-889.
5. Nehls, M., D. Pfeifer, M. Schorpp, H. Hedrich, and T. Boehm. 1994. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372:103-107.
6. Gordon, J., A.R. Bennett, C.C. Blackburn, and N.R. Manley. 2001. Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mech Dev* 103:141-143.
7. Bleul, C.C., T. Corbeaux, A. Reuter, P. Fisch, J.S. Monting, and T. Boehm. 2006. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* 441:992-996.
8. Bleul, C.C., and T. Boehm. 2000. Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol* 30:3371-3379.
9. Tsukamoto, N., M. Itoi, M. Nishikawa, and T. Amagai. 2005. Lack of Delta like 1 and 4 expressions in nude thymus anlages. *Cell Immunol* 234:77-80.
10. Blackburn, C.C., C.L. Augustine, R. Li, R.P. Harvey, M.A. Malin, R.L. Boyd, J.F. Miller, and G. Morahan. 1996. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc Natl Acad Sci U S A* 93:5742-5746.
11. Gordon, J., V.A. Wilson, N.F. Blair, J. Sheridan, A. Farley, L. Wilson, N.R. Manley, and C.C. Blackburn. 2004. Functional evidence for a single endodermal origin for the thymic epithelium. *Nat Immunol* 5:546-553.
12. Manley, N.R., and M.R. Capecchi. 1995. The role of Hoxa-3 in mouse thymus and thyroid development. *Development* 121:1989-2003.
13. Anderson, G., and E.J. Jenkinson. 2001. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* 1:31-40.
14. Naiche, L.A., Z. Harrelson, R.G. Kelly, and V.E. Papaioannou. 2005. T-box genes in vertebrate development. *Annu Rev Genet* 39:219-239.
15. Lindsay, E.A., F. Vitelli, H. Su, M. Morishima, T. Huynh, T. Pramparo, V. Jurecic, G. Ogunrinu, H.F. Sutherland, P.J. Scambler, A. Bradley, and A. Baldini. 2001. Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature* 410:97-101.
16. Manley, N.R., and M.R. Capecchi. 1998. Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands. *Dev Biol* 195:1-15.
17. Wallin, J., H. Eibel, A. Neubuser, J. Wilting, H. Koseki, and R. Balling. 1996. Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* 122:23-30.

18. Peters, H., A. Neubuser, K. Kratochwil, and R. Balling. 1998. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* 12:2735-2747.
19. Hetzer-Egger, C., M. Schorpp, A. Haas-Assenbaum, R. Balling, H. Peters, and T. Boehm. 2002. Thymopoiesis requires Pax9 function in thymic epithelial cells. *Eur J Immunol* 32:1175-1181.
20. van Ewijk, W., E.W. Shores, and A. Singer. 1994. Crosstalk in the mouse thymus. *Immunol Today* 15:214-217.
21. van Ewijk, W., G. Hollander, C. Terhorst, and B. Wang. 2000. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* 127:1583-1591.
22. Klug, D.B., C. Carter, E. Crouch, D. Roop, C.J. Conti, and E.R. Richie. 1998. Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A* 95:11822-11827.
23. Hollander, G.A., B. Wang, A. Nichogiannopoulou, P.P. Platenburg, W. van Ewijk, S.J. Burakoff, J.C. Gutierrez-Ramos, and C. Terhorst. 1995. Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* 373:350-353.
24. Rodewald, H.R., and H.J. Fehling. 1998. Molecular and cellular events in early thymocyte development. *Adv Immunol* 69:1-112.
25. Petrie, H.T., and W. Van Ewijk. 2002. Thymus by numbers. *Nat Immunol* 3:604-605.
26. Couzin, J. 2002. Immunology. Plant a few cells, sprout a thymus. *Science* 296:2120-2121.
27. Rossi, S.W., A.P. Chidgey, S.M. Parnell, W.E. Jenkinson, H.S. Scott, R.L. Boyd, E.J. Jenkinson, and G. Anderson. 2007. Redefining epithelial progenitor potential in the developing thymus. *Eur J Immunol* 37:2411-2418.
28. Rossi, S.W., W.E. Jenkinson, G. Anderson, and E.J. Jenkinson. 2006. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* 441:988-991.
29. Shakib, S., G.E. Desanti, W.E. Jenkinson, S.M. Parnell, E.J. Jenkinson, and G. Anderson. 2009. Checkpoints in the development of thymic cortical epithelial cells. *J Immunol* 182:130-137.
30. Hamazaki, Y., H. Fujita, T. Kobayashi, Y. Choi, H.S. Scott, M. Matsumoto, and N. Minato. 2007. Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat Immunol* 8:304-311.
31. Farr, A.G., J.L. Dooley, and M. Erickson. 2002. Organization of thymic medullary epithelial heterogeneity: implications for mechanisms of epithelial differentiation. *Immunol Rev* 189:20-27.
32. Derbinski, J., J. Gabler, B. Brors, S. Tierling, S. Jonnakuty, M. Hergenhahn, L. Peltonen, J. Walter, and B. Kyewski. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med* 202:33-45.
33. Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annu Rev Immunol* 24:571-606.
34. Gray, D., J. Abramson, C. Benoist, and D. Mathis. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med* 204:2521-2528.
35. Anderson, M.S., E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. *Immunity* 23:227-239.

36. Akiyama, T., S. Maeda, S. Yamane, K. Ogino, M. Kasai, F. Kajiura, M. Matsumoto, and J. Inoue. 2005. Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* 308:248-251.
37. Rossi, S.W., M.Y. Kim, A. Leibbrandt, S.M. Parnell, W.E. Jenkinson, S.H. Glanville, F.M. McConnell, H.S. Scott, J.M. Penninger, E.J. Jenkinson, P.J. Lane, and G. Anderson. 2007. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med* 204:1267-1272.
38. Akiyama, T., Y. Shimo, H. Yanai, J. Qin, D. Ohshima, Y. Maruyama, Y. Asaumi, J. Kitazawa, H. Takayanagi, J.M. Penninger, M. Matsumoto, T. Nitta, Y. Takahama, and J. Inoue. 2008. The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity* 29:423-437.
39. Hikosaka, Y., T. Nitta, I. Ohigashi, K. Yano, N. Ishimaru, Y. Hayashi, M. Matsumoto, K. Matsuo, J.M. Penninger, H. Takayanagi, Y. Yokota, H. Yamada, Y. Yoshikai, J. Inoue, T. Akiyama, and Y. Takahama. 2008. The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity* 29:438-450.
40. Chin, R.K., J.C. Lo, O. Kim, S.E. Blink, P.A. Christiansen, P. Peterson, Y. Wang, C. Ware, and Y.X. Fu. 2003. Lymphotoxin pathway directs thymic Aire expression. *Nat Immunol* 4:1121-1127.
41. Boehm, T., S. Scheu, K. Pfeffer, and C.C. Bleul. 2003. Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med* 198:757-769.
42. Martins, V.C., T. Boehm, and C.C. Bleul. 2008. Ltbetar signaling does not regulate Aire-dependent transcripts in medullary thymic epithelial cells. *J Immunol* 181:400-407.
43. Irla, M., S. Hugues, J. Gill, T. Nitta, Y. Hikosaka, I.R. Williams, F.X. Hubert, H.S. Scott, Y. Takahama, G.A. Hollander, and W. Reith. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. *Immunity* 29:451-463.
44. Vogel, A., C.P. Strassburg, P. Obermayer-Straub, G. Brabant, and M.P. Manns. 2002. The genetic background of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy and its autoimmune disease components. *J Mol Med* 80:201-211.
45. Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032-1039.
46. Anderson, M.S., E.S. Venanzi, L. Klein, Z. Chen, S.P. Berzins, S.J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395-1401.
47. Venanzi, E.S., R. Melamed, D. Mathis, and C. Benoist. 2008. The variable immunological self: genetic variation and nongenetic noise in Aire-regulated transcription. *Proc Natl Acad Sci U S A* 105:15860-15865.
48. Guerau-de-Arellano, M., D. Mathis, and C. Benoist. 2008. Transcriptional impact of Aire varies with cell type. *Proc Natl Acad Sci U S A* 105:14011-14016.

49. Villasenor, J., W. Besse, C. Benoist, and D. Mathis. 2008. Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated. *Proc Natl Acad Sci U S A* 105:15854-15859.
50. Taubert, R., J. Schwendemann, and B. Kyewski. 2007. Highly variable expression of tissue-restricted self-antigens in human thymus: implications for self-tolerance and autoimmunity. *Eur J Immunol* 37:838-848.
51. Koh, A.S., A.J. Kuo, S.Y. Park, P. Cheung, J. Abramson, D. Bua, D. Carney, S.E. Shoelson, O. Gozani, R.E. Kingston, C. Benoist, and D. Mathis. 2008. Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc Natl Acad Sci U S A* 105:15878-15883.
52. Ragvin, A., H. Valvatne, S. Erdal, V. Arskog, K.R. Tufteland, K. Breen, O.Y. AM, A. Eberharter, T.J. Gibson, P.B. Becker, and R. Aasland. 2004. Nucleosome binding by the bromodomain and PHD finger of the transcriptional cofactor p300. *J Mol Biol* 337:773-788.
53. Ferguson, B.J., C. Alexander, S.W. Rossi, I. Liiv, A. Rebane, C.L. Worth, J. Wong, M. Laan, P. Peterson, E.J. Jenkinson, G. Anderson, H.S. Scott, A. Cooke, and T. Rich. 2008. AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. *J Biol Chem* 283:1723-1731.
54. Pitkanen, J., A. Rebane, J. Rowell, A. Murumagi, P. Strobel, K. Moll, M. Saare, J. Heikkila, V. Doucas, A. Marx, and P. Peterson. 2005. Cooperative activation of transcription by autoimmune regulator AIRE and CBP. *Biochem Biophys Res Commun* 333:944-953.
55. Liiv, I., A. Rebane, T. Org, M. Saare, J. Maslovskaja, K. Kisand, E. Juronen, L. Valmu, M.J. Bottomley, N. Kalkkinen, and P. Peterson. 2008. DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. *Biochim Biophys Acta* 1783:74-83.
56. Oven, I., N. Brdickova, J. Kohoutek, T. Vaupotic, M. Narat, and B.M. Peterlin. 2007. AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. *Mol Cell Biol* 27:8815-8823.
57. Mathis, D., and C. Benoist. 2009. Aire. *Annu Rev Immunol* 27:287-312.
58. DeVoss, J., Y. Hou, K. Johannes, W. Lu, G.I. Liou, J. Rinn, H. Chang, R.R. Caspi, L. Fong, and M.S. Anderson. 2006. Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J Exp Med* 203:2727-2735.
59. Gavanescu, I., B. Kessler, H. Ploegh, C. Benoist, and D. Mathis. 2007. Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity. *Proc Natl Acad Sci U S A* 104:4583-4587.
60. Liston, A., D.H. Gray, S. Lesage, A.L. Fletcher, J. Wilson, K.E. Webster, H.S. Scott, R.L. Boyd, L. Peltonen, and C.C. Goodnow. 2004. Gene dosage--limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J Exp Med* 200:1015-1026.
61. Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C.C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat Immunol* 4:350-354.
62. Aschenbrenner, K., L.M. D'Cruz, E.H. Vollmann, M. Hinterberger, J. Emmerich, L.K. Swee, A. Rolink, and L. Klein. 2007. Selection of Foxp3(+) regulatory T cells specific for self antigen expressed and presented by Aire(+) medullary thymic epithelial cells. *Nat Immunol*
63. Gardner, J.M., J.J. Devoss, R.S. Friedman, D.J. Wong, Y.X. Tan, X. Zhou, K.P. Johannes, M.A. Su, H.Y. Chang, M.F. Krummel, and M.S. Anderson.

2008. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* 321:843-847.
64. Anderson, G., E.J. Jenkinson, N.C. Moore, and J.J. Owen. 1993. MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* 362:70-73.
65. Anderson, G., J.J. Owen, N.C. Moore, and E.J. Jenkinson. 1994. Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro. *J Exp Med* 179:2027-2031.
66. Goldschneider, I., K.L. Komschlies, and D.L. Greiner. 1986. Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J Exp Med* 163:1-17.
67. Wallis, V.J., E. Leuchars, S. Chwalinski, and A.J. Davies. 1975. On the sparse seeding of bone marrow and thymus in radiation chimaeras. *Transplantation* 19:2-11.
68. Foss, D.L., E. Donskoy, and I. Goldschneider. 2001. The importation of hematogenous precursors by the thymus is a gated phenomenon in normal adult mice. *J Exp Med* 193:365-374.
69. Kyewski, B.A. 1987. Seeding of thymic microenvironments defined by distinct thymocyte-stromal cell interactions is developmentally controlled. *J Exp Med* 166:520-538.
70. Scimone, M.L., I. Aifantis, I. Apostolou, H. von Boehmer, and U.H. von Andrian. 2006. A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. *Proc Natl Acad Sci U S A* 103:7006-7011.
71. Rossi, F.M., S.Y. Corbel, J.S. Merzaban, D.A. Carlow, K. Gossens, J. Duenas, L. So, L. Yi, and H.J. Ziltener. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol* 6:626-634.
72. Arroyo, A.G., J.T. Yang, H. Rayburn, and R.O. Hynes. 1996. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. *Cell* 85:997-1008.
73. Prockop, S.E., S. Palencia, C.M. Ryan, K. Gordon, D. Gray, and H.T. Petrie. 2002. Stromal cells provide the matrix for migration of early lymphoid progenitors through the thymic cortex. *J Immunol* 169:4354-4361.
74. Robertson, P., T.K. Means, A.D. Luster, and D.T. Scadden. 2006. CXCR4 and CCR5 mediate homing of primitive bone marrow-derived hematopoietic cells to the postnatal thymus. *Exp Hematol* 34:308-319.
75. Plotkin, J., S.E. Prockop, A. Lepique, and H.T. Petrie. 2003. Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. *J Immunol* 171:4521-4527.
76. Uehara, S., S.M. Hayes, L. Li, D. El-Khoury, M. Canelles, B.J. Fowlkes, and P.E. Love. 2006. Premature expression of chemokine receptor CCR9 impairs T cell development. *J Immunol* 176:75-84.
77. Zuniga-Pflucker, J.C., and M.J. Lenardo. 1996. Regulation of thymocyte development from immature progenitors. *Curr Opin Immunol* 8:215-224.
78. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu Rev Immunol* 14:29-47.
79. Han, H., K. Tanigaki, N. Yamamoto, K. Kuroda, M. Yoshimoto, T. Nakahata, K. Ikuta, and T. Honjo. 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* 14:637-645.

80. Sambandam, A., I. Maillard, V.P. Zediak, L. Xu, R.M. Gerstein, J.C. Aster, W.S. Pear, and A. Bhandoola. 2005. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol* 6:663-670.
81. Tan, J.B., I. Visan, J.S. Yuan, and C.J. Guidos. 2005. Requirement for Notch1 signals at sequential early stages of intrathymic T cell development. *Nat Immunol* 6:671-679.
82. Schmitt, T.M., and J.C. Zuniga-Pflucker. 2002. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17:749-756.
83. Porritt, H.E., K. Gordon, and H.T. Petrie. 2003. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J Exp Med* 198:957-962.
84. Shortman, K., M. Egerton, G.J. Spangrude, and R. Scollay. 1990. The generation and fate of thymocytes. *Semin Immunol* 2:3-12.
85. Massa, S., G. Balciunaite, R. Ceredig, and A.G. Rolink. 2006. Critical role for c-kit (CD117) in T cell lineage commitment and early thymocyte development in vitro. *Eur J Immunol* 36:526-532.
86. Rodewald, H.R., M. Ogawa, C. Haller, C. Waskow, and J.P. DiSanto. 1997. Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. *Immunity* 6:265-272.
87. Staal, F.J., F. Weerkamp, M.R. Baert, C.M. van den Burg, M. van Noort, E.F. de Haas, and J.J. van Dongen. 2004. Wnt target genes identified by DNA microarrays in immature CD34+ thymocytes regulate proliferation and cell adhesion. *J Immunol* 172:1099-1108.
88. Wilson, A., W. Held, and H.R. MacDonald. 1994. Two waves of recombinase gene expression in developing thymocytes. *J Exp Med* 179:1355-1360.
89. Durum, S.K., S. Candeias, H. Nakajima, W.J. Leonard, A.M. Baird, L.J. Berg, and K. Muegge. 1998. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J Exp Med* 188:2233-2241.
90. Maki, K., S. Sunaga, and K. Ikuta. 1996. The V-J recombination of T cell receptor-gamma genes is blocked in interleukin-7 receptor-deficient mice. *J Exp Med* 184:2423-2427.
91. Maraskovsky, E., M. Teepe, P.J. Morrissey, S. Braddy, R.E. Miller, D.H. Lynch, and J.J. Peschon. 1996. Impaired survival and proliferation in IL-7 receptor-deficient peripheral T cells. *J Immunol* 157:5315-5323.
92. Vissinga, C.S., D.J. Fatur-Saunders, and F. Takei. 1992. Dual role of IL-7 in the growth and differentiation of immature thymocytes. *Exp Hematol* 20:998-1003.
93. Schmitt, T.M., M. Ciofani, H.T. Petrie, and J.C. Zuniga-Pflucker. 2004. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* 200:469-479.
94. Taghon, T.N., E.S. David, J.C. Zuniga-Pflucker, and E.V. Rothenberg. 2005. Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. *Genes Dev* 19:965-978.
95. Petrie, H.T., F. Livak, D. Burtrum, and S. Mazel. 1995. T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. *J Exp Med* 182:121-127.
96. Muegge, K., M.P. Vila, and S.K. Durum. 1993. Interleukin-7: a cofactor for V(D)J rearrangement of the T cell receptor beta gene. *Science* 261:93-95.

97. Capone, M., R.D. Hockett, Jr., and A. Zlotnik. 1998. Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. *Proc Natl Acad Sci U S A* 95:12522-12527.
98. Livak, F., M. Tourigny, D.G. Schatz, and H.T. Petrie. 1999. Characterization of TCR gene rearrangements during adult murine T cell development. *J Immunol* 162:2575-2580.
99. Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H.J. Fehling, and H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266:1208-1212.
100. von Boehmer, H., and H.J. Fehling. 1997. Structure and function of the pre-T cell receptor. *Annu Rev Immunol* 15:433-452.
101. Falk, I., and K. Eichmann. 2002. Heterogeneity of the DN4 (CD44-CD25-) subset of CD4-CD8- double negative thymocytes; dependence on CD3 signaling. *Immunol Lett* 82:123-130.
102. Vasseur, F., A. Le Campion, and C. Penit. 2001. Scheduled kinetics of cell proliferation and phenotypic changes during immature thymocyte generation. *Eur J Immunol* 31:3038-3047.
103. Petrie, H.T., M. Pearse, R. Scollay, and K. Shortman. 1990. Development of immature thymocytes: initiation of CD3, CD4, and CD8 acquisition parallels down-regulation of the interleukin 2 receptor alpha chain. *Eur J Immunol* 20:2813-2815.
104. Tanigaki, K., M. Tsuji, N. Yamamoto, H. Han, J. Tsukada, H. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* 20:611-622.
105. Moore, T.A., and A. Zlotnik. 1995. T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood* 86:1850-1860.
106. Benz, C., K. Heinzel, and C.C. Bleul. 2004. Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. *Eur J Immunol* 34:3652-3663.
107. Petrie, H.T., F. Livak, D.G. Schatz, A. Strasser, I.N. Crispe, and K. Shortman. 1993. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med* 178:615-622.
108. Penit, C. 1988. Localization and phenotype of cycling and post-cycling murine thymocytes studied by simultaneous detection of bromodeoxyuridine and surface antigens. *J Histochem Cytochem* 36:473-478.
109. Irving, B.A., F.W. Alt, and N. Killeen. 1998. Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. *Science* 280:905-908.
110. Yamasaki, S., E. Ishikawa, M. Sakuma, K. Ogata, K. Sakata-Sogawa, M. Hiroshima, D.L. Wiest, M. Tokunaga, and T. Saito. 2006. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat Immunol* 7:67-75.
111. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1995. Positive selection of thymocytes. *Annu Rev Immunol* 13:93-126.
112. Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248:701-702.

113. Singer, A., K.S. Hathcock, and R.J. Hodes. 1981. Self recognition in allogeneic radiation bone marrow chimeras. A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. *J Exp Med* 153:1286-1301.
114. Marrack, P., J.P. Scott-Browne, S. Dai, L. Gapin, and J.W. Kappler. 2008. Evolutionarily conserved amino acids that control TCR-MHC interaction. *Annu Rev Immunol* 26:171-203.
115. Blackman, M., J. Yague, R. Kubo, D. Gay, C. Coleclough, E. Palmer, J. Kappler, and P. Marrack. 1986. The T cell repertoire may be biased in favor of MHC recognition. *Cell* 47:349-357.
116. Zerrahn, J., W. Held, and D.H. Raulet. 1997. The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell* 88:627-636.
117. Merkenschlager, M., D. Graf, M. Lovatt, U. Bommhardt, R. Zamoyska, and A.G. Fisher. 1997. How many thymocytes audition for selection? *J Exp Med* 186:1149-1158.
118. Scott-Browne, J.P., J. White, J.W. Kappler, L. Gapin, and P. Marrack. 2009. Germline-encoded amino acids in the alphabeta T-cell receptor control thymic selection. *Nature* 458:1043-1046.
119. Gounari, F., I. Aifantis, K. Khazaie, S. Hoeflinger, N. Harada, M.M. Taketo, and H. von Boehmer. 2001. Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. *Nat Immunol* 2:863-869.
120. Ioannidis, V., F. Beermann, H. Clevers, and W. Held. 2001. The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. *Nat Immunol* 2:691-697.
121. Sun, Z., D. Unutmaz, Y.R. Zou, M.J. Sunshine, A. Pierani, S. Brenner-Morton, R.E. Mebius, and D.R. Littman. 2000. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* 288:2369-2373.
122. Davey, G.M., S.L. Schober, B.T. Endrizzi, A.K. Dutcher, S.C. Jameson, and K.A. Hogquist. 1998. Preselection thymocytes are more sensitive to T cell receptor stimulation than mature T cells. *J Exp Med* 188:1867-1874.
123. Li, Q.J., J. Chau, P.J. Ebert, G. Sylvester, H. Min, G. Liu, R. Braich, M. Manoharan, J. Soutschek, P. Skare, L.O. Klein, M.M. Davis, and C.Z. Chen. 2007. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129:147-161.
124. Ashton-Rickardt, P.G., A. Bandeira, J.R. Delaney, L. Van Kaer, H.P. Pircher, R.M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76:651-663.
125. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.
126. Marrack, P., L. Ignatowicz, J.W. Kappler, J. Boymel, and J.H. Freed. 1993. Comparison of peptides bound to spleen and thymus class II. *J Exp Med* 178:2173-2183.
127. Ignatowicz, L., J. Kappler, and P. Marrack. 1996. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* 84:521-529.
128. Tourne, S., T. Miyazaki, A. Oxenius, L. Klein, T. Fehr, B. Kyewski, C. Benoist, and D. Mathis. 1997. Selection of a broad repertoire of CD4+ T cells in H-2Ma0/0 mice. *Immunity* 7:187-195.

129. Anderson, G., K.M. Partington, and E.J. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J Immunol* 161:6599-6603.
130. Nakagawa, T., W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J.A. Villadangos, H. Ploegh, C. Peters, and A.Y. Rudensky. 1998. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* 280:450-453.
131. Gommeaux, J., C. Gregoire, P. Nguessan, M. Richelme, M. Malissen, S. Guerder, B. Malissen, and A. Carrier. 2009. Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes. *Eur J Immunol* 39:956-964.
132. Nedjic, J., M. Aichinger, N. Mizushima, and L. Klein. 2009. Macroautophagy, endogenous MHC II loading and T cell selection: the benefits of breaking the rules. *Curr Opin Immunol* 21:92-97.
133. Murata, S., K. Sasaki, T. Kishimoto, S. Niwa, H. Hayashi, Y. Takahama, and K. Tanaka. 2007. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* 316:1349-1353.
134. Doyle, C., and J.L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* 330:256-259.
135. Norment, A.M., R.D. Salter, P. Parham, V.H. Engelhard, and D.R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* 336:79-81.
136. Chan, S.H., D. Cosgrove, C. Waltzinger, C. Benoist, and D. Mathis. 1993. Another view of the selective model of thymocyte selection. *Cell* 73:225-236.
137. Davis, C.B., N. Killeen, M.E. Crooks, D. Raulet, and D.R. Littman. 1993. Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* 73:237-247.
138. Itano, A., P. Salmon, D. Kioussis, M. Tolaini, P. Corbella, and E. Robey. 1996. The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. *J Exp Med* 183:731-741.
139. Yasutomo, K., C. Doyle, L. Miele, C. Fuchs, and R.N. Germain. 2000. The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. *Nature* 404:506-510.
140. Brugnera, E., A. Bhandoola, R. Cibotti, Q. Yu, T.I. Guinter, Y. Yamashita, S.O. Sharrow, and A. Singer. 2000. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* 13:59-71.
141. Lundberg, K., W. Heath, F. Kontgen, F.R. Carbone, and K. Shortman. 1995. Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4-8+TCRhi thymocytes. *J Exp Med* 181:1643-1651.
142. Suzuki, H., J.A. Punt, L.G. Granger, and A. Singer. 1995. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. *Immunity* 2:413-425.
143. Yu, Q., B. Erman, A. Bhandoola, S.O. Sharrow, and A. Singer. 2003. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. *J Exp Med* 197:475-487.

144. He, X., V.P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B.A. Roe, and D.J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433:826-833.
145. Sun, G., X. Liu, P. Mercado, S.R. Jenkinson, M. Kypriotou, L. Feigenbaum, P. Galera, and R. Bosselut. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat Immunol* 6:373-381.
146. Aliahmad, P., and J. Kaye. 2008. Development of all CD4 T lineages requires nuclear factor TOX. *J Exp Med* 205:245-256.
147. Hedrick, S.M. 2008. Thymus lineage commitment: a single switch. *Immunity* 28:297-299.
148. Hendriks, R.W., M.C. Nawijn, J.D. Engel, H. van Doorninck, F. Grosveld, and A. Karis. 1999. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. *Eur J Immunol* 29:1912-1918.
149. Nawijn, M.C., R. Ferreira, G.M. Dingjan, O. Kahre, D. Drabek, A. Karis, F. Grosveld, and R.W. Hendriks. 2001. Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice. *J Immunol* 167:715-723.
150. Hodgkin, P.D., W.R. Heath, and A.G. Baxter. 2007. The clonal selection theory: 50 years since the revolution. *Nat Immunol* 8:1019-1026.
151. Billingham, R.E., L. Brent, and P.B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature* 172:603-606.
152. Ohki, H., C. Martin, M. Coltey, and N.M. Le Douarin. 1988. Implants of quail thymic epithelium generate permanent tolerance in embryonically constructed quail/chick chimeras. *Development* 104:619-630.
153. Ohki, H., C. Martin, C. Corbel, M. Coltey, and N.M. Le Douarin. 1987. Tolerance induced by thymic epithelial grafts in birds. *Science* 237:1032-1035.
154. Linsk, R., M. Gottesman, and B. Pernis. 1989. Are tissues a patch quilt of ectopic gene expression? *Science* 246:261.
155. Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell* 52:831-841.
156. Modigliani, Y., A. Coutinho, P. Pereira, N. Le Douarin, V. Thomas-Vaslin, O. Burlen-Defranoux, J. Salaun, and A. Bandeira. 1996. Establishment of tissue-specific tolerance is driven by regulatory T cells selected by thymic epithelium. *Eur J Immunol* 26:1807-1815.
157. Fowell, D., and D. Mason. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med* 177:627-636.
158. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.
159. Modigliani, Y., P. Pereira, V. Thomas-Vaslin, J. Salaun, O. Burlen-Defranoux, A. Coutinho, N. Le Douarin, and A. Bandeira. 1995. Regulatory T cells in thymic epithelium-induced tolerance. I. Suppression of mature peripheral non-tolerant T cells. *Eur J Immunol* 25:2563-2571.

160. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273-280.
161. Woodland, D.L., F.E. Lund, M.P. Happ, M.A. Blackman, E. Palmer, and R.B. Corley. 1991. Endogenous superantigen expression is controlled by mouse mammary tumor proviral loci. *J Exp Med* 174:1255-1258.
162. Woodland, D.L., M.P. Happ, K.J. Gollob, and E. Palmer. 1991. An endogenous retrovirus mediating deletion of alpha beta T cells? *Nature* 349:529-530.
163. Kishimoto, H., and J. Sprent. 1997. Negative selection in the thymus includes semimature T cells. *J Exp Med* 185:263-271.
164. Merkenschlager, M., C. Benoist, and D. Mathis. 1994. Evidence for a single-niche model of positive selection. *Proc Natl Acad Sci U S A* 91:11694-11698.
165. Punt, J.A., B.A. Osborne, Y. Takahama, S.O. Sharrow, and A. Singer. 1994. Negative selection of CD4+CD8+ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. *J Exp Med* 179:709-713.
166. Kishimoto, H., and J. Sprent. 1999. Several different cell surface molecules control negative selection of medullary thymocytes. *J Exp Med* 190:65-73.
167. Williams, J.A., S.O. Sharrow, A.J. Adams, and R.J. Hodes. 2002. CD40 ligand functions non-cell autonomously to promote deletion of self-reactive thymocytes. *J Immunol* 168:2759-2765.
168. Gong, Q., A.M. Cheng, A.M. Akk, J. Alberola-Ila, G. Gong, T. Pawson, and A.C. Chan. 2001. Disruption of T cell signaling networks and development by Grb2 haploid insufficiency. *Nat Immunol* 2:29-36.
169. McCarty, N., S. Paust, K. Ikizawa, I. Dan, X. Li, and H. Cantor. 2005. Signaling by the kinase MINK is essential in the negative selection of autoreactive thymocytes. *Nat Immunol* 6:65-72.
170. Bouillet, P., J.F. Purton, D.I. Godfrey, L.C. Zhang, L. Coultas, H. Puthalakath, M. Pellegrini, S. Cory, J.M. Adams, and A. Strasser. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415:922-926.
171. Rathmell, J.C., T. Lindsten, W.X. Zong, R.M. Cinalli, and C.B. Thompson. 2002. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nat Immunol* 3:932-939.
172. Calnan, B.J., S. Szychowski, F.K. Chan, D. Cado, and A. Winoto. 1995. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* 3:273-282.
173. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531-562.
174. Bonomo, A., and P. Matzinger. 1993. Thymus epithelium induces tissue-specific tolerance. *J Exp Med* 177:1153-1164.
175. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-396.
176. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162:5317-5326.

177. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
178. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
179. Khattri, R., T. Cox, S.A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4:337-342.
180. Williams, L.M., and A.Y. Rudensky. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8:277-284.
181. Gavin, M.A., J.P. Rasmussen, J.D. Fontenot, V. Vasta, V.C. Manganiello, J.A. Beavo, and A.Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445:771-775.
182. Lin, W., D. Haribhai, L.M. Relland, N. Truong, M.R. Carlson, C.B. Williams, and T.A. Chatila. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8:359-368.
183. Jordan, M.S., A. Boesteanu, A.J. Reed, A.L. Petrone, A.E. Holenbeck, M.A. Lerman, A. Naji, and A.J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2:301-306.
184. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 3:756-763.
185. Hsieh, C.S., Y. Liang, A.J. Tyznik, S.G. Self, D. Liggitt, and A.Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 21:267-277.
186. Hsieh, C.S., Y. Zheng, Y. Liang, J.D. Fontenot, and A.Y. Rudensky. 2006. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* 7:401-410.
187. Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.
188. Yao, Z., Y. Kanno, M. Kerenyi, G. Stephens, L. Durant, W.T. Watford, A. Laurence, G.W. Robinson, E.M. Shevach, R. Moriggl, L. Hennighausen, C. Wu, and J.J. O'Shea. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109:4368-4375.
189. Antov, A., L. Yang, M. Vig, D. Baltimore, and L. Van Parijs. 2003. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. *J Immunol* 171:3435-3441.
190. D'Cruz, L.M., and L. Klein. 2005. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 6:1152-1159.
191. Liu, Y., P. Zhang, J. Li, A.B. Kulkarni, S. Perruche, and W. Chen. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol* 9:632-640.
192. Tang, Q., K.J. Henriksen, E.K. Boden, A.J. Tooley, J. Ye, S.K. Subudhi, X.X. Zheng, T.B. Strom, and J.A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171:3348-3352.
193. Sansom, D.M., and L.S. Walker. 2006. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunol Rev* 212:131-148.

194. Wirnsberger, G., F. Mair, and L. Klein. 2009. Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control. *Proc Natl Acad Sci U S A*
195. Wu, L., and K. Shortman. 2005. Heterogeneity of thymic dendritic cells. *Semin Immunol* 17:304-312.
196. Donskoy, E., D. Foss, and I. Goldschneider. 2003. Gated importation of prothymocytes by adult mouse thymus is coordinated with their periodic mobilization from bone marrow. *J Immunol* 171:3568-3575.
197. Li, J., J. Park, D. Foss, and I. Goldschneider. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J Exp Med* 206:607-622.
198. Proietto, A.I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D'Amico, R.J. Steptoe, S.H. Naik, M.H. Lahoud, Y. Liu, P. Zheng, K. Shortman, and L. Wu. 2008. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A* 105:19869-19874.
199. Jenkinson, E.J., G. Anderson, and J.J. Owen. 1992. Studies on T cell maturation on defined thymic stromal cell populations in vitro. *J Exp Med* 176:845-853.
200. Volkmann, A., T. Zal, and B. Stockinger. 1997. Antigen-presenting cells in the thymus that can negatively select MHC class II-restricted T cells recognizing a circulating self antigen. *J Immunol* 158:693-706.
201. Ohnmacht, C., A. Pullner, S.B. King, I. Drexler, S. Meier, T. Brocker, and D. Voehringer. 2009. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med* 206:549-559.
202. van Meerwijk, J.P., S. Marguerat, R.K. Lees, R.N. Germain, B.J. Fowlkes, and H.R. MacDonald. 1997. Quantitative impact of thymic clonal deletion on the T cell repertoire. *J Exp Med* 185:377-383.
203. Gallegos, A.M., and M.J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med* 200:1039-1049.
204. Humblet, C., A. Rudensky, and B. Kyewski. 1994. Presentation and intercellular transfer of self antigen within the thymic microenvironment: expression of the E alpha peptide-I-Ab complex by isolated thymic stromal cells. *Int Immunol* 6:1949-1958.
205. Klein, L., B. Roettinger, and B. Kyewski. 2001. Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. *Eur J Immunol* 31:2476-2486.
206. Viret, C., A.K. Barlow, and C.A. Janeway, Jr. 1999. On the intrathymic intercellular transfer of self-determinants. *Immunol Today* 20:8-10.
207. Koble, C., and B. Kyewski. 2009. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med* 206:1505-1513.
208. Gabler, J., J. Arnold, and B. Kyewski. 2007. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. *Eur J Immunol* 37:3363-3372.
209. Derbinski, J., S. Pinto, S. Rosch, K. Hexel, and B. Kyewski. 2008. Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. *Proc Natl Acad Sci U S A* 105:657-662.

210. Le Borgne, M., E. Ladi, I. Dzhagalov, P. Herzmark, Y.F. Liao, A.K. Chakraborty, and E.A. Robey. 2009. The impact of negative selection on thymocyte migration in the medulla. *Nat Immunol* 10:823-830.
211. Millet, V., P. Naquet, and R.R. Guinamard. 2008. Intercellular MHC transfer between thymic epithelial and dendritic cells. *Eur J Immunol* 38:1257-1263.
212. Zeng, Y., E.J. Wagner, and B.R. Cullen. 2002. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* 9:1327-1333.
213. Martin, B.K., K.C. Chin, J.C. Olsen, C.A. Skinner, A. Dey, K. Ozato, and J.P. Ting. 1997. Induction of MHC class I expression by the MHC class II transactivator CIITA. *Immunity* 6:591-600.
214. Gobin, S.J., A. Peijnenburg, V. Keijsers, and P.J. van den Elsen. 1997. Site alpha is crucial for two routes of IFN gamma-induced MHC class I transactivation: the ISRE-mediated route and a novel pathway involving CIITA. *Immunity* 6:601-611.
215. Chang, C.H., S. Guerder, S.C. Hong, W. van Ewijk, and R.A. Flavell. 1996. Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. *Immunity* 4:167-178.
216. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100:8886-8891.
217. Brennecke, J., A. Stark, R.B. Russell, and S.M. Cohen. 2005. Principles of microRNA-target recognition. *PLoS Biol* 3:e85.
218. Chen, X. 2004. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* 303:2022-2025.
219. Poy, M.N., L. Eliasson, J. Krutzfeldt, S. Kuwajima, X. Ma, P.E. Macdonald, S. Pfeffer, T. Tuschl, N. Rajewsky, P. Rorsman, and M. Stoffel. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432:226-230.
220. Cimmino, A., G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, and C.M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 102:13944-13949.
221. LeibundGut-Landmann, S., J.M. Waldburger, M. Krawczyk, L.A. Otten, T. Suter, A. Fontana, H. Acha-Orbea, and W. Reith. 2004. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *Eur J Immunol* 34:1513-1525.
222. Steimle, V., L.A. Otten, M. Zufferey, and B. Mach. 1993. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell* 75:135-146.
223. Westerheide, S.D., P. Louis-Plence, D. Ping, X.F. He, and J.M. Boss. 1997. HLA-DMA and HLA-DMB gene expression functions through the conserved S-X-Y region. *J Immunol* 158:4812-4821.
224. Taxman, D.J., D.E. Cressman, and J.P. Ting. 2000. Identification of class II transcriptional activator-induced genes by representational difference analysis: discoordinate regulation of the DN alpha/DO beta heterodimer. *J Immunol* 165:1410-1416.
225. Nagarajan, U.M., J. Lochamy, X. Chen, G.W. Beresford, R. Nilsen, P.E. Jensen, and J.M. Boss. 2002. Class II transactivator is required for maximal expression of HLA-DOB in B cells. *J Immunol* 168:1780-1786.

226. Khalil, H., F. Deshaies, A. Bellemare-Pelletier, A. Brunet, A. Faubert, G.A. Azar, and J. Thibodeau. 2002. Class II transactivator-induced expression of HLA-DO(beta) in HeLa cells. *Tissue Antigens* 60:372-382.
227. Ting, J.P., and J. Trowsdale. 2002. Genetic control of MHC class II expression. *Cell* 109 Suppl:S21-33.
228. Boss, J.M., and P.E. Jensen. 2003. Transcriptional regulation of the MHC class II antigen presentation pathway. *Curr Opin Immunol* 15:105-111.
229. Annacker, O., C. Asseman, S. Read, and F. Powrie. 2003. Interleukin-10 in the regulation of T cell-induced colitis. *J Autoimmun* 20:277-279.
230. Sisk, T.J., T. Gourley, S. Roys, and C.H. Chang. 2000. MHC class II transactivator inhibits IL-4 gene transcription by competing with NF-AT to bind the coactivator CREB binding protein (CBP)/p300. *J Immunol* 165:2511-2517.
231. Zhu, X.S., and J.P. Ting. 2001. A 36-amino-acid region of CIITA is an effective inhibitor of CBP: novel mechanism of gamma interferon-mediated suppression of collagen alpha(2)(I) and other promoters. *Mol Cell Biol* 21:7078-7088.
232. Yee, C.S., Y. Yao, P. Li, M.J. Klemsz, J.S. Blum, and C.H. Chang. 2004. Cathepsin E: a novel target for regulation by class II transactivator. *J Immunol* 172:5528-5534.
233. Gourley, T.S., D.R. Patel, K. Nickerson, S.C. Hong, and C.H. Chang. 2002. Aberrant expression of Fas ligand in mice deficient for the MHC class II transactivator. *J Immunol* 168:4414-4419.
234. Wong, A.W., W.J. Brickey, D.J. Taxman, H.W. van Deventer, W. Reed, J.X. Gao, P. Zheng, Y. Liu, P. Li, J.S. Blum, K.P. McKinnon, and J.P. Ting. 2003. CIITA-regulated plexin-A1 affects T-cell-dendritic cell interactions. *Nat Immunol* 4:891-898.
235. Doench, J.G., and P.A. Sharp. 2004. Specificity of microRNA target selection in translational repression. *Genes Dev* 18:504-511.
236. Lewis, B.P., C.B. Burge, and D.P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15-20.
237. Rodewald, H.R. 2008. Thymus organogenesis. *Annu Rev Immunol* 26:355-388.
238. Shores, E.W., W. Van Ewijk, and A. Singer. 1991. Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur J Immunol* 21:1657-1661.
239. Surh, C.D., B. Ernst, and J. Sprent. 1992. Growth of epithelial cells in the thymic medulla is under the control of mature T cells. *J Exp Med* 176:611-616.
240. van Meerwijk, J.P., and H.R. MacDonald. 1999. In vivo T-lymphocyte tolerance in the absence of thymic clonal deletion mediated by hematopoietic cells. *Blood* 93:3856-3862.
241. Wirnsberger, G., F. Mair, and L. Klein. 2009. Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control. *Proc Natl Acad Sci U S A* 106:10278-10283.
242. Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844.

243. Kirberg, J., A. Baron, S. Jakob, A. Rolink, K. Karjalainen, and H. von Boehmer. 1994. Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. *J Exp Med* 180:25-34.
244. Murphy, D.B., D. Lo, S. Rath, R.L. Brinster, R.A. Flavell, A. Slanetz, and C.A. Janeway, Jr. 1989. A novel MHC class II epitope expressed in thymic medulla but not cortex. *Nature* 338:765-768.
245. Paddison, P.J., M. Cleary, J.M. Silva, K. Chang, N. Sheth, R. Sachidanandam, and G.J. Hannon. 2004. Cloning of short hairpin RNAs for gene knockdown in mammalian cells. *Nat Methods* 1:163-167.
246. Yang, X.W., P. Model, and N. Heintz. 1997. Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat Biotechnol* 15:859-865.

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Nationality: Austria

Education

Mar. 2008-present
PhD at the Ludwig-Maximilian University Munich
Supervisor: Univ. Prof. Dr. Ludger Klein
Title: Direct antigen presentation by medullary thymic epithelial cells is essential for CD4 T cell tolerance

Jan. 2006-Feb. 2008
PhD at the Institute of Molecular Pathology in Vienna
Supervisor: Univ. Prof. Dr. Ludger Klein
Title: Direct antigen presentation by medullary thymic epithelial cells is essential for CD4 T cell tolerance

Dec. 2005
Diploma examination with distinction

Oct. 2004 – Nov.2005
Diploma Work at the Institute of Immunology in Vienna Supervisor: Univ. Prof. Dr. Hannes Stockinger
Project Title: "Regulatory T cells – Identification and characterization of molecules critical for their function"

Jul. 2003 – Jan. 2004
Studies at Victoria University of Wellington/New Zealand

Since 2000
University of Vienna
Field of Study: Biology
Branch of Study: Microbiology/Genetics (since Oct. 2002)

Work experience

Jul. 2004-
Sep. 2004

Intercell AG
Dept. Antigen Identification
Molecular Biology Lab

- Identification of novel antigens for protection against infection by the respective bacterial pathogen
- Cloning, expression and purification of recombinant antigens

Apr. 2004-
May. 2004

Institute for Genetics and Animal breeding (Veterinarian University)

- Allele specific real time PCR
- Cytokine quantification in mice by real time PCR

Meetings and conferences

June 2009 — Oral presentation:
"Kyoto T cell conference" in Kyoto Japan

May 2007 — Poster presentation
"Rolduc Thymus conference"

Publication list

1. Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844.

2. Aschenbrenner, K., L.M. D'Cruz, E.H. Vollmann, M. Hinterberger, J. Emmerich, L.K. Swee, A. Rolink, and L. Klein. 2007. Selection of Foxp3(+) regulatory T cells specific for self antigen expressed and presented by Aire(+) medullary thymic epithelial cells. *Nat Immunol*

3. Drbal, K., M. Moertelmaier, C. Holzhauser, A. Muhammad, E. Fuertbauer, S. Howorka, M. Hinterberger, H. Stockinger, and G.J. Schutz. 2007. Single-molecule microscopy reveals heterogeneous dynamics of lipid raft components upon TCR engagement. *Int Immunol* 19:675-684.