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Delineating a phenotypic and functional roadmap
to regulatory T cell development
at the $CD4^+$ single positive stage

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1 SUMMARY

T cells, which are crucial effector cells of the adaptive immune system, develop in the thymus from common lymphoid progenitor cells. Each T cell carries a unique T cell receptor (TCR), generated during thymic development by random rearrangement of a number of gene fragments. Using this mechanism an enormous variety of TCR specificities can be generated, equipping T cells with the ability to react against every possible pathogen. However, with a certain probability TCRs recognizing self antigen will arise. These autoreactive T cells are mainly purged from the repertoire by the process of negative selection, but still some of them escape from the thymus to the periphery. To keep them in check, so called regulatory T cells (Treg) exist. Treg are characterized by the constitutive expression of the Interleukin-2 receptor α-chain (CD25) and the X-chromosome encoded transcription factor Foxp3 (Forkhead box P3), the latter being crucial for their function. Disruption of Foxp3 in mice results in lethal autoimmunity at the age of 3-4 weeks, while Foxp3 mutations in humans cause the severe autoimmune disease IPEX (immune dysregulation, polyendocrinopathy, X-linked).

Treg can develop from conventional T cells in the periphery under certain conditions, but most Treg are generated in the thymus. It has been shown that this process can be initiated upon interaction with peptide-MHC class II complexes on medullary thymic epithelial cells (mTECs), but the precise mechanism underlying thymic Treg development remains enigmatic.

This study was initiated to delineate the process of thymic Treg development. For this purpose a TCR transgenic mouse model was used, in which T cells specific for hemagglutin can be tracked on single cell level. In AIRE-HA × TCR-HA mice, which express hemagglutinin (HA) under control of the Aire promotor specifically in mTECs T cells expressing the hemagglutinin specific TCR are efficiently selected into the Treg lineage.

By intrathymic transfer of naïve TCR-HA^{+} RAG2$^{-/-}$ thymocytes into AIRE-HA recipients we could demonstrate that induction of CD25^{+} Foxp3^{+} Treg at the CD4^{+} single positive (SP) stage of T cell development is possible. The efficacy of Treg induction was affected both by the maturational stage of the transferred cells and the level of cognate antigen expression. Using *in vivo* intrathymic transfer and *in vitro* experiments, CD25^{-} Foxp3^{-} GITR^{+} and CD25^{+} Foxp3^{-} CD4^{+} SPs were identified as precursor cells capable of developing to Foxp3^{+} Treg without the requirement for any further TCR stimulus.

Elucidating the mechanistic details of these processes would be greatly facilitated by a suitable *in vitro* system closely mimicking the *in vivo* situation. We could show that thymic dendritic cells (DCs) and mTECs are capable of converting naïve CD4^{+} SPs to Foxp3^{+} Treg *in vitro*, providing a system in which easy manipulation is possible.

2 ZUSAMMENFASSUNG

T-Zellen sind wichtige Zellen des adaptiven Immunsystems und entwickeln sich im Thymus aus lymphoiden Vorläuferzellen. Jede T-Zelle besitzt einen einzigartigen T-Zell Rezeptor (TCR), der während der Entwicklung im Thymus durch zufälliges Umstellen bestimmter Genfragmente entsteht. Durch diesen Mechanismus kann eine gigantische Vielfalt an verschiedenen TCR-Spezifitäten gebildet werden, wodurch T-Zellen jeden möglichen Krankheitserreger erkennen können. Allerdings entstehen mit einer gewissen Wahrscheinlichkeit auch TCRs, die körpereigene Antigene erkennen. Diese autoreaktiven T-Zellen werden großteils durch negative Selektion entfernt, aber einige wenige gelangen vom Thymus in die Peripherie. Um diese zu kontrollieren, existieren sogenannte regulatorische T-Zellen (Treg), die durch folgende zwei Merkmale charakterisiert werden: Expression der α-Kette des Interleukin-2 Rezeptors (CD25) und des X-chromosomal kodierten Transkriptionsfaktors Foxp3 (Forkhead box P3). Letzterer ist essentiell für die Funktion von Treg. Entfernung des Foxp3-Gens führt in Mäusen 3-4 Wochen nach der Geburt zu einer tödlichen Autoimmunerkrankung, während in Menschen Foxp3 Mutationen für die Autoimmunerkrankung IPEX (immune dysregulation, polyendocrinopathy, X-linked) verantwortlich sind.

Treg können unter gewissen Umständen aus normalen T-Zellen in der Peripherie entstehen, aber die meisten Treg entwickeln sich im Thymus. Dieser Prozess kann durch Antigenerkennung auf thymischen medullären Epithelzellen (mTEC) ausgelöst werden, wobei die exakten Mechanismen noch unklar sind.

In dieser Studie sollten Einblicke in die Treg Entwicklung im Thymus gewonnen werden. Dazu wurde ein TCR-transgenes Mausmodell verwendet, in dem individuelle T-Zellen, die spezifisch für Haemagglutin sind, verfolgt werden können. In AIRE-HA × TCR-HA Mäusen, die das Protein Haemagglutinin (HA) unter der Kontrolle des Aire Promotors exprimieren, entwickelt sich ein Teil der HA-spezifischen T-Zellen zu Treg.

Durch intrathymischen Transfer von naiven TCR-HA$^+$ Thymocyten in AIRE-HA Mäuse wurde gezeigt, dass die Entwicklung von CD25$^+$ Foxp3$^+$ Treg während des CD4$^+$ positiven Stadiums der T-Zell Entwicklung möglich ist. Die Effizienz dieses Prozesses war einerseits vom Reifestadium der T-Zelle und andererseits auch vom Level der Antigenexpression abhängig. Sowohl durch intrathymischen Transfer *in vivo* als auch durch *in vitro* Experimente konnten CD25$^-$ Foxp3$^-$ GITR$^+$ und CD25$^+$ Foxp3$^-$ Thymocyten als Vorläuferzellen von Foxp3$^+$ Treg charakterisiert werden. Für deren Entwicklung war eine weitere TCR-Stimulation nicht notwendig.

Die Aufklärung von mechanistischen Details der Treg Entwicklung würde sich deutlich vereinfachen, wenn ein passendes *in vitro* System zur Verfügung stehen würde. In dieser Arbeit konnte gezeigt werden, dass thymische dendritische Zellen und mTEC *in vitro* CD4$^+$ Thymocyten zu Foxp3$^+$ Treg konvertieren, was ein einfaches experimentelles System für Modifikationen darstellen könnte.

3 INTRODUCTION

3.1 THE IMMUNE SYSTEM

The immune system is the body's defense system against pathogenic bacteria and viruses. For this purpose two distinct mechanisms have evolved, namely the adaptive and the innate immune system, the latter being evolutionarily older and thus present in all animal species.

Innate immunity mainly works by recognizing conserved structures common for pathogenic organisms, also known as PAMPs (pathogen associated molecular pattern). Thus, the innate immune system can react immediately to a wide range of pathogens, but does not offer specificity or immunological memory.

For this purpose, higher vertebrates have evolved adaptive immunity. Consisting of a variety of highly specialized cells, the adaptive immune system offers both the ability to attack every single pathogenic organism in a very specific manner, and immunological memory. To do so, a complex machinery has evolved in which immune cells randomly generate receptors by somatic rearrangement of certain gene fragments, thus giving the immune system the ability to react against every possible antigen.

The two effector cell types equipped with these skills are T cells and B cells. The latter are generated in the bone marrow and responsible for the production of antibodies, which mark antigens for attack by other immune cells and the complement system. In contrast, T cells play a vital role not only by directly attacking bacteria, but rather by controlling central processes in an antigen dependent manner.

Several subclasses of T cells can be distinguished, among them $CD4^+$ T cells, which comprise a subset of so called regulatory T cells (Treg), the latter being of special interest to this work. Furthermore, $CD8^+$ cytotoxic T cells (T_C), natural killer T cells (NKT) and γδ T cells are known. These cells fulfill different specialized functions, but all of them are generated in the thymus from common lymphoid progenitor cells. The next chapter focuses on the process of T cell development in the thymus.

3.2 T CELL DEVELOPMENT IN THE THYMUS

The thymus is a two-lobed organ situated just below the upper end of the sternum. It can be morphologically divided into a cortical and medullary region, surrounded by a capsule. Since ancient beliefs claimed the thymus to represent the seat of the soul its physiological function was a matter of debate, until it could be shown in the early 1960s that the thymus is essential for the generation of mature T lymphocytes [3].

In contrast to the bone marrow, the thymus does not contain self-renewing stem cells, but is rather dependent on the periodic import of hematopoietic progenitor cells. These common lymphoid progenitor cells (CLPs) are produced in the bone marrow, circulate

through the blood and enter the thymus at the cortico-medullary junction [4]. During a series of complex developmental steps T cell progenitor cells travel through the thymic compartments and leave the thymus as mature $CD4^+$ or $CD8^+$ T cells after roughly 20 days.

3.2.1 THE DOUBLE NEGATIVE STAGE

Initially, common lymphoid progenitors are not committed to the T cell lineage and termed double negative (DN) cells or pro-T cells. Their T cell receptor (TCR) genes are still in germline configuration, and they neither express CD3 nor the CD4/CD8 coreceptors [5]. According to expression of CD25 and CD44 DN cells can be subdivided into four stages: DN1 ($CD25^-$ $CD44^+$), DN2 ($CD25^+$ $CD44^+$), DN3 ($CD25^+$ $CD44^{low}$) and DN4 ($CD25^-$ $CD44^{low}$) [6]. Development along these stages takes place while the cells migrate outward through the cortex into direction of the capsule.

After extensive proliferation at the DN1 stage it is at the late DN2 and early DN3 stage that commitment to the T cell lineage occurs. At this point developing thymocytes start to upregulate expression of the RAG enzymes (recombination activation gene) to rearrange their T cell receptor (TCR) genes, namely the α- and β-genes for αβ-T cells. The differentiation of γδ-T cells will not be discussed in any further detail as these comprise only a minority of the mature T cell repertoire.

The TCR β gene locus is rearranged first. It consists of a number of V-, D- and J gene segments, which are rearranged randomly through the action of RAG-1 and RAG-2 to yield a continuous in-frame VDJ gene sequence expressed as the TCR β chain. This polypeptide pairs with an invariant pre-T α chain to yield the pre-T cell receptor complex, an event representing the first developmental checkpoint for αβ-T cells known as β-selection. Signaling through the pre-TCR, which seems to be cell intrinsic [7], is essential for survival of the developing thymocyte, and cells that fail to successfully rearrange their TCR β genes die by apoptosis.

As a result of pre-TCR expression a series of profound changes is initiated: suspension of VDJ gene rearrangement at the second TCR β allele, extensive proliferation and upregulation of CD4 and CD8 expression on the mRNA level [6]. Within a next step, rearrangement of the TCR α gene locus is started, which comprises only V and J segements. Functional TCR α chains replace the pre-T α chain and form together with the β chain a mature T cell receptor. Concurrent with expression of CD4 and CD8 on the cell surface this event marks the transition from the DN to the double positive (DP) stage.

3.2.2 THE DOUBLE POSITIVE STAGE AND POSITIVE SELECTION

DP thymocytes reside in the subcapsular region of the cortex and closely interact with cortical epithelial cells (cTECs) while undergoing positive selection. Basically, positive

selection refers to the process in which DP thymocytes recognizing peptide-MHC (major histocompatibility complex) molecules presented by cTECs with low affinity receive a survival signal and continue to develop, while those cells that fail to bind self-MHC molecules die by apoptosis. This developmental checkpoint ensures that mature T cells do have the ability to interact with self MHC molecules. Elegant experiments addressing this issue were done in the late 1970s showing that the developmental environment determines the MHC restriction of T cells [8]. Thymocytes of mice deficient in the TCR α chain do not develop past the DP stage demonstrating that a functional TCR is essential [9].

DP thymocytes comprise up to 80 to 90 % of thymic cellularity, but only 5-10% of these cells survive the positive selection checkpoint [10] [11] and continue to develop either to $CD4^+$ or $CD8^+$ SPs, while they migrate back through the cortex into direction of the medulla. Depending on whether the TCR recognizes MHC class I or class II molecules one of the coreceptors must be specifically downregulated, as $CD4^+$ T cells exclusively recognize antigens presented in the context of MHC class II, while $CD8^+$ T cells bind to MHC class I molecules.

There are different models explaining this process. The instructive model states that appropriate TCR-MHC interactions determine expression of the correct coreceptor, while the stochastic model implies that expression of CD4 and CD8 is regulated randomly, followed by survival of cells with the correct MHC restriction of the TCR. Recently, a third model has been suggested. According to this work, after positive selection expression of CD8 is downregulated yielding $CD4^+$ $CD8^{int}$ cells which remain uncommitted. Development to $CD4^+$ SPs occurs if TCR signalling continues, while a diminished TCR signal results in development to $CD8^+$ SPs [12].

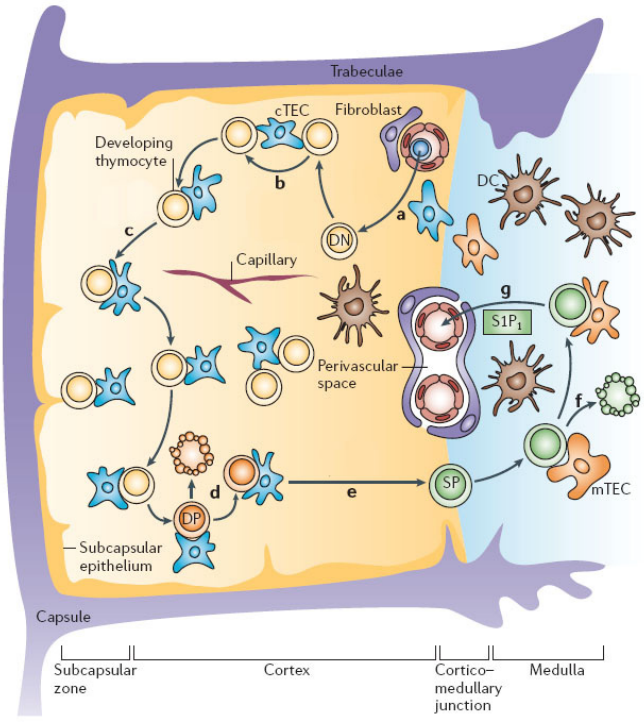

Figure 1: **Route of developing T cells in the thymus** (adapted from [2])
Common lymphoid progenitor cells enter the thymus from the blood stream at the cortico-medullary junction **(a).** During migration through the cortex outwards they mature from DN to DP cells while interacting with cortical epithelial cells **(b** and **c)**. At the DP stage only thymocytes that are positively selected survive, while other cells undergo apoptosis **(d)**. As a next step, the CD4/CD8 lineage decision is made followed by migration into the thymic medulla **(e)**.
In the medulla single positive thymocytes interact with medullary epithelial cells **(g)** and are negatively selected or continue to mature, finally leaving the thymus in an $S1P_1$ dependent fashion **(g)**.

3.3 AUTOREACTIVITY AND TOLERANCE

After the CD4/CD8 lineage decision has been made, SP thymocytes migrate into the medulla, where they are going to interact with medullary epithelial cells (mTECs) and dendritic cells (DCs). During their residence time in the medulla SPs continue to mature and undergo stringent selection processes to ensure tolerance to self antigen, which will be discussed in the next chapter. Finally, cells leave the thymus as mature T cells via chemotaxis to $S1P_1$, a chemoattractant abundant in the circulation [13]. An overview of thymocyte development from the DN stage to the SP stage, depicting stages and interacting cell types is given in Figure 1.
Positive selection ensures that only those thymocytes binding to self MHC molecules survive. Another issue of crucial importance in T cell development is negative selection. Due to the random rearrangement of TCR gene fragments there is a certain probability that receptors strongly reacting with self antigen will arise. As these cells could initiate fatal autoimmunity, three mechanisms exist to deal with this problem: clonal deletion, anergy and receptor editing [14].
During clonal deletion apoptosis is induced in thymocytes expressing receptors with high affinity for self antigen-MHC molecules. This process can occur both at the DP and early SP stage and clears a high proportion of self reactive thymocytes, thus making it one of the key mechanisms of central tolerance [15].

3.3.1 CENTRAL TOLERANCE

In general, the term tolerance covers all processes that shape the repertoire of the adaptive immune system in such a way that it remains tolerant against self components. One can distinguish between central tolerance, which involves processes during early lymphocyte development, and peripheral tolerance, which acts on mature immune cells by mechanisms like anergy or ignorance.
Since the visionary Frank Macfarlane Burnet published his thoughts regarding the clonal selection theory in 1957 [16, 17, 18], establishment of tolerance has been one of the most fundamental immunological questions. Much light has been shed on this puzzle since that, and it is now widely accepted that central tolerance is the most important mechanism, acting by recessive and dominant mechanisms. As mentioned above, recessive tolerance involves clonal deletion, anergy and receptor editing, thereby efficiently purging the T cell repertoire of self reactive cells [14].
The existence of clonal deletion as a key component of recessive tolerance could be confirmed only two decades after Burnet's postulation of this process. In two independent key experiments it was demonstrated that first, T cells specific for the MHC molecule I-E are absent in I-E expressing mice [19] and second, TCR-transgenic cells specific for the male antigen H-Y are deleted in male, but not in female mice [20].
It was known that some self-reactive lymphocytes might escape clonal deletion, but peripheral tolerance mechanisms such as anergy were thought to keep them in check.

After initial descriptions of suppressive immune cells in the 1970s [21] it was only in the late 1980s that evidence for the existence of dominant (i.e. trans-acting) tolerance mechanisms was provided by experiments in chicken, which showed that beside central tolerance being induced by thymic epithelium only a small fraction of peripheral T cells is sufficient for control of self-reactivity [22].

For the mouse system, similar landmark experiments were published in 1990 [23]. Within this work, embryonic thymi from C3H mice (haplotype H-2^k) were transplanted to nude BALB/c mice (C3H → BALB/$c^{nu/nu}$) at the age of 1-10 days, allowing T cell development in the transplanted thymus. Subsequently, either C3H, BALB/c or C57Bl/6 skin grafts were transplanted to C3H → BALB/$c^{nu/nu}$ chimeras and control BALB/$c^{nu/nu}$ mice. In the chimeras, BALB/c and C3H grafts were accepted, while C57Bl/6 grafts were all rejected, demonstrating that the transplanted thymic epithelium confers tolerance to both C3H and BALB/c. Importantly, T cells from C3H → BALB/$c^{nu/nu}$ chimeras retained the ability to react with C3H lymphocytes while accepting the skin grafts, definitely showing that clonal deletion can not be the only tolerance mechanism involved.

Due to this and other work the existence of a specific cell type which is able to suppress autoimmunity and generated in the thymus was postulated. This form of trans-acting tolerance, in other words dominant tolerance, became known as the third function of the thymus [24].

However, how the thymus could tolerize the T cell repertoire against the huge variety of self antigens present in one organism remained a puzzle for several years. After reports suggesting transport of peripheral antigens into the thymus, key experiments in the Kyewski lab proved that there is indeed a cell type in the thymus, namely medullary thymic epithelial cells (mTECs), specialized for expression of otherwise tissue restricted antigens [25]. This phenomenon was termed promiscuous gene expression, and subsequent work identified the transcription factor Aire (autoimmune regulator) as an essential regulator of this process [26]. Aire was shown to drive transcription of a large number of tissue restricted antigens specifically in mTECs, emphasizing the crucial importance of this cell type in tolerance induction. Although the detailed molecular mechanism involved remains largely unknown, epigenetic mechanisms have been suggested to be involved [15].

3.3.2 REGULATORY T CELLS

As mentioned above, first descriptions of T cells capable of suppressing immune responses date back to the 1970s [21], and work in the late 1980s strongly argued for the existence of suppressive immune cells. The absence of markers to identify such cells and the inability to define any suppressive factors misguided the scientific community to doubt the existence of regulatory immune cells [27]. Nevertheless, key experiments published by Shimon Sakaguchi in 1995 identified CD25 (the α chain of

the IL-2 receptor) as a marker for a $CD4^+$ T cell population that could control fatal autoimmune disease resulting from adoptive transfer of $CD4^+ CD25^-$ T cells into nude mice [28].

These $CD4^+$ T cells constitutively expressing $CD25^+$ were named regulatory T cells (Treg), and interest into the field was rapidly revived. Subsequent work aimed at characterizing the properties of these cells. It could be shown that Treg are able to suppress the proliferation of both $CD4^+$ $CD25^-$ and $CD8^+$ naïve effector T cells *in vitro*. Nevertheless, Treg themselves remain anergic after TCR stimulation *in vitro*. This anergy can be broken by TCR stimuli in the presence of IL-2, but after removal of IL-2 the cells return to their anergic state [29].

Contrary to these *in vitro* features, it could be shown *in vivo* that Treg proliferate extensively in an MHC class II dependent way when placed in a lymphopenic environment, but without losing their suppressive capacity [30 , 31]. More detailed studies applying cotransfer of TCR transgenic effector cells with Treg revealed that after immunization Treg indeed proliferate as much as effector T cells, but maintain their suppressive capacity *in vivo* and *in vitro* [32].

Beside that, $CD4^+$ $CD25^+$ Treg are unable to produce IL-2 or T-helper type 1 or type 2 cytokines, and can be induced *in vitro* by the presence of transforming growth factor-β (TGF-β) [33], which will be discussed in more detail later.

3.3.3 FOXP3

One of the caveats of using CD25 as a marker for Tregs is that CD25 is upregulated by activated nonregulatory T cells as well. Thus, an important breakthrough in the field was the discovery of the gene for the X chromosome encoded transcription factor Foxp3 (Forkhead box P3). Foxp3 was initially discovered by genetic mapping of the gene responsible for the phenotype of scurfy (*sf*) mice, a spontaneous mouse mutant that shows overproliferation of T lymphocytes followed by death 3-4 weeks after birth [34]. At the same time, human patients suffering from a severe autoimmune disease called IPEX (immune dysregulation, polyendocrinopathy, X-linked) were shown to have a mutation in the Foxp3 gene [35]. A remarkable feature of these diseases is that only hemizygous males, but not heterozygous females are affected, implying that in females cells carrying the wildtype Foxp3 allele keep the mutant cells in check. This observation suggested a dominant mode of action, possibly involving regulatory T cells.

Indeed, two years later both the Sakaguchi and the Rudensky lab showed that Foxp3 is specifically expressed in $CD4^+$ $CD25^+$ Tregs and essential for their development [36, 37, 38]. Foxp3 knockout mice, as *scurfy* mice, lack regulatory T cells and die at the age of 3-4 weeks due to severe lymphoproliferation and aggressive autoimmune disease involving several organs, but can be rescued by adoptive transfer of $Foxp3^+$ Tregs. Furthermore, reconstitution of lethally irradiated mice with wildtype and $Foxp3^{-/-}$ bone marrow showed that only $Foxp3^+$ stem cells could give rise to Tregs. The crucial

function of Foxp3 was further underscored by the fact that ectopic expression of Foxp3 with a retroviral vector in peripheral $CD4^+$ effector cells confers suppressive function to them [36].
Handling $Foxp3^+$ Treg was greatly simplified by the generation of mice expressing a fusion protein of Foxp3 and eGFP (enhanced green fluorescent protein), which allows the analysis of Foxp3 expression at the single cell level by flow cytometry [39]. Within this work it could be shown that T cell specific ablation of Foxp3 mimics the phenotype of Foxp3 null mice, suggesting that Foxp3 expression and function is limited to a subset of αβ-T cells. However, recent analysis by real-time PCR and immunohistochemistry showed expression of Foxp3 in non-lymphoid cells, namely in several epithelial cell types [40]. The functional importance of this findings remains to be clarified.
Collectively, these results positioned Foxp3 as a master regulator of the Treg lineage and therefore much work has been attributed to the characterization of Foxp3 function in Treg biology, which will be discussed in detail in the next chapter.

3.4 FOXP3 AND REGULATORY T CELL BIOLOGY

3.4.1 FUNCTION OF FOXP3 IN TREGS

To directly address the role of $Foxp3^+$ Treg in tolerance maintenance an elegant knock-in model was used [41, 42]. The sequence of the diphtheria toxin receptor (DTR) was inserted downstream of the Foxp3 gene ($Foxp3^{DTR}$), making all Tregs expressing the DTR on their surface. Daily treatment of both newborn and adult $Foxp3^{DTR}$ animals with diphtheria toxin completely eliminated the $Foxp3^+$ Treg population in these mice, leading to severe autoimmunity even more aggressive than observed in Foxp3 null mice, followed by rapid death within 10-24 days. This observation definitely proved that Tregs are absolutely crucial for the maintenance of tolerance throughout life while other possible mechanisms of peripheral tolerance are insufficient.
At the same time the importance of $Foxp3^+$ as a lineage specification factor was clarified. For this purpose a floxed Foxp3 allele was deleted in mature $Foxp3^+$ Tregs by Cre-Lox mediated recombination. Unexpectedly, this resulted in the loss of suppressive function followed by production of IL-2 and pro-inflammatory cytokines such as TNF (tumor necrosis factor) and IFN-γ (interferon gamma) [43]. It was concluded that Foxp3 is required to maintain Treg cell identity and does not simply induce a genetic program that sustains itself.
A recent study focused on analysis of the TCR repertoire of conventional effector cells and Treg in TCR-β chain transgenic mice, showing that there are differences between conventional TCRs and thymic Treg. Importantly, in $Foxp3^{-/-}$ mice activated $CD25^+$ T cells responsible for autoimmune reactions show considerable repertoire overlap with Treg in wildtype mice, suggesting that in the wildtype setting autoreactive thymocytes develop to $Foxp3^+$ Treg [44].

On a molecular basis, two reports showed that both interaction with NFAT (nuclear factor of activated T cells) [45] and AML1/Runx1 (acute myeloid leukaemia 1 / runt-related transcription factor 1) [46] is necessary for Foxp3 function. Interestingly, these transcription factors are involved in upregulation of IL-2, and interaction of Foxp3 with NFAT and AML1 reversed this function. According to these observations Foxp3 might mechanistically work by repressing alternative T cell fates through interaction with transcription factors that are involved. This idea is substantiated by the observation that the TGF-β dependent generation of Foxp3$^+$ Treg antagonizes TGF-β/IL-6 dependent generation of pro-inflammatory IL-17 producing T_H17 cells [47]. The reciprocal relationship between Foxp3$^+$ Treg and T_H17 cells expressing the orphan nuclear receptor RORγt has been emphasized by work published during preparation of this thesis [48].

More insight into the Foxp3 dependent regulation of Treg biology came from recent publications providing comprehensive gene profiling analysis. Two studies employed chromatin immunoprecipitation (ChIP) combined with mouse genome arrays to find direct targets of Foxp3. The Rudensky lab used *ex vivo* isolated Treg followed by ChIP, showing that Foxp3 directly binds to roughly 700 genes and acts both as a transcriptional repressor and activator [49], while the von Boehmer lab used Foxp3 transduced murine hybridoma cell lines [50]. The latter study distinguished genes occupied by Foxp3 in unstimulated and stimulated cells, revealing that differential gene expression was far more pronounced in stimulated cells. However, both studies concluded that a high proportion of Foxp3 direct target genes are involved in T cell receptor signaling, suggesting that modulation of TCR signals is an important facet of Foxp3 function.

A key aspect of Treg biology was revealed by the generation of knock-in mice expressing a nonfunctional Foxp3 allele together with eGFP (Foxp3$^{KO\text{-}GFP}$) [51]. In heterozygous females, which carry one functional Foxp3 allele and one Foxp3$^{KO\text{-}GFP}$ allele, autoimmunity is kept in check by the WT Foxp3$^+$ Treg population, thereby allowing the *in* vivo analysis of GFP marked “Treg” cells that actively transcribe Foxp3 but lack the functional protein (further on called T^{FN} cells). These T^{FN} cells were not deleted in the thymus, and most unexpectedly, showed some Treg features, namely anergy and intermediate expression levels of CD25 and GITR. However, T^{FN} cells were not suppressive and lost expression of the Foxp3$^{GFP\text{-}KO}$ after adoptive transfer. Gene expression analysis of T^{FN} and Treg cells revealed that a proportion of the characteristic Treg expression profile was already present in T^{FN} cells, meaning that Foxp3 is not necessary for initial induction of the Treg genetic program.

Collectively these results show that Foxp3 amplifies Treg features which are already induced prior to Foxp3 expression. Thus, Foxp3 is of crucial importance for the fixation and maintenance of the Treg transcriptional program, but not for its induction.

3.4.2 THYMIC DEVELOPMENT OF REGULATORY T CELLS

Since its initial discovery several aspects of Foxp3 function in Treg biology have been unraveled, but the main question of how Treg are generated remains unresolved. Early work in the 1970s has shown that thymectomy of wildtype mice 3 days after birth results in autoimmunity, which can be prevented by transfer of peripheral $CD4^+$ $CD25^+$ Treg [52]. Together with the observation of suppressive $CD4^+$ $CD25^+$ cells in the thymus [53] it is now widely accepted that mainly thymic processes are involved in Treg generation. However, peripheral conversion of T cells under certain conditions, for example in the gut [54] has been also shown to play an important role during the development of an immune response.

During the last years, models using T cell receptor transgenic mice were used to address thymic Treg development. These models offer the significant advantage of tracking single T cells of known antigen specificity *in vivo*, while one possible caveat of these systems could be that expression of the transgenic TCR at inappropriate developmental stages may distort results [55].

In a first study published in 2001 mice expressing the TCR-HA (recognizing the $peptide_{107\text{-}119}$ of influenza haemagglutinin in the context of $I\text{-}E^d$ MHC-class II molecules) were crossed to mouse strains expressing the agonist HA-peptide under control of various promotors. Developing $TCR\text{-}HA^+$ thymocytes underwent either deletion or Treg induction depending on the expression level of the ligand [56]. In a subsequent study DO11.10 mice, expressing a TCR specific for ovalbumin (OVA) were crossed to RIP-OVA mice (expressing ovalbumin under control of the rat insulin promotor), recapitulating the Treg generation seen in the TCR-HA system [31].

These results convincingly indicated that Treg can be induced in the thymus after interaction with peptide-MHC class II complexes, whereby the avidity of the interaction could play a decisive role. In this context an important aspect was provided by the subsequent observation, that costimulation via CD28 is required as well [57].

To gain a more detailed understanding of agonist induced thymic Treg development, recent work from our lab tried to elucidate the thymic cell type involved in antigen presentation [58]. For this purpose mice expressing influenza haemagglutinin (HA) under control of the Aire-promotor were generated (AIRE-HA), mimicking the physiological situation of Aire-dependent expression of self-antigens. Crossing of AIRE-HA mice to TCR-HA mice resulted in the induction of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg. By the generation of various bone marrow chimeras, it could be shown that antigen presentation by medullary thymic epithelial cells (mTECs) was indeed sufficient for Treg generation. In contrast, CD11c-driven antigen expression by dendritic cells (DCs) favoured deletion.

The notion that agonist peptide-MHC class II complexes can induce Treg *de novo* was challenged by a publication in 2004 [59]. Using a transgenic TCR recognizing moth cytochrome c (TCR-MCC) in the context of an inducible tetracycline-system to drive expression of agonist MCC-peptide it was observed that increased levels of MCC

yielded higher proportions of $CD25^+$ Treg, but only a modest increase in absolute numbers. Accordingly, the authors speculated that Treg development is not instructed by TCR-MHC class II complexes, but occurs stochastically.

3.4.3 FACTORS INFLUENCING THYMIC TREG DEVELOPMENT

The molecular basis underlying the decision whether a thymocyte interacting with self peptide-MHC class II complexes undergoes deletion or Treg development remains a mystery.

During the last years work from many labs focused on TGF-β, with the prevailing notion of its function undergoing several successive changes. Initially it was thought that TGF-β could play an important role in Treg development, as *in vitro* TCR stimulation of naive T cells in the presence of TGF-β is sufficient to induce Foxp3 [33] and TGF-β knockout mice die due to severe autoimmunity 3-4 weeks after birth [60]. Unexpectedly, analysis of young TGF-β knockout mice before the onset of any autoimmunity revealed roughly normal numbers of $Foxp3^+$ Tregs in the thymus, but not in the periphery, suggesting that TGF-β is required for peripheral maintenance, but not for thymic generation of Tregs [61]. These results were further strengthened by the generation of mice with a T cell specific deletion of the TGF-β receptor II, where thymic Treg development was unaffected, but peripheral Treg numbers were reduced as well [62].

However, a recent publication challenged these results, revealing an intricate involvement of TGF-β [63]. Mice lacking TGF-β receptor-1 specifically in T cells showed nearly no $Foxp3^+$ thymocytes at day 3 to day 5 after birth, but already 7 days after birth their number started to raise. In line with the known observations from TGF-β knockout mice, 2-3 weeks after birth normal numbers of $Foxp3^+$ thymocytes were present. The boost in $Foxp3^+$ thymocyte count was shown to depend on Interleukin-2 (IL-2), another factor critical to Treg biology as discussed below.

After IL-2 was initially discovered as a growth factor, the generation of both IL-2- and IL-2Rα- or IL-2Rβ- knockout mice suggested that IL-2 plays a crucial role in the prevention of autoimmunity [64, 65]. Afterwards, various groups tried to elucidate the importance of IL-2 signaling for Treg development, arguing for an essential function in the generation of $CD4^+$ $CD25^+$ Treg [66].

However, the unreliability of CD25 as a Treg marker challenged these results, and after the discovery of Foxp3 two studies re-addressed the significance of IL-2 for Treg biology. The Rudensky lab investigated polyclonal Treg development [67], while our lab followed agonist-driven Treg generation in $IL\text{-}2^{-/-}$ and $IL\text{-}2R\alpha^{-/-}$ mice [68]. Unexpectedly, it was found that thymic development of Treg was largely undisturbed in the absence of IL-2, with only a modest decrease observed in the polyclonal system. However, in both systems peripheral loss of Treg occurred, indicating a nonredundant role of IL-2 in

peripheral survival of Treg. Of note, IL-2 and IL-2Rα deficiency did not disturb *in vitro* suppression activity.

Beside IL-2 there are other common γ-chain cytokines such as IL-7, IL-9 or IL-15, which require the common γ-chain cytokine receptor (CD132), signal via the JAK/STAT pathway and are collectively known to modulate survival and differentiation of developing lymphocytes [69]. Initial analysis of CD132 knockout mice showed a complete absence of Foxp3$^+$ Treg [67], strongly arguing for a non-redundant role of this signaling pathway.

A subsequent study extended this findings, showing that IL-2Rβ knockout mice had reduced numbers of Foxp3$^+$ thymocytes, while in IL-2/IL-15 double knockout mice hardly any Foxp3$^+$ thymocytes could be found [70]. As both in IL-2 or IL-15 single knockouts only a slight reduction of Foxp3$^+$ thymocyte numbers was observed, this observation strongly suggests that IL-2 and IL-15 act as redundant factors during Treg development. On a molecular level, T cell specific deletion of STAT5 was shown to abrogate Treg development [70], unambiguously revealing the importance of cytokine signaling via the JAK/STAT pathway for Treg development. Furthermore, T-cell specific deletion of the MAP kinase TAK1, which is critical for integrating both TCR- and common γ-chain signals, results in nearly complete Treg deficiency [71].

On the level of TCR signaling some work focused on the role of the adaptor molecule LAT (linker of activated T cells). Mice with a specific amino acid substitution in LAT (replacing tyrosine 136 with phenylalanine, LAT^{Y136F}) exhibit a partial block in late T cell maturation but still develop autoimmune disease. This was suggested to be due to a complete block in the development of Foxp3$^+$ Treg, leading to the hypothesis that signaling via phospholipase Cγ1 is crucial for Treg development [72]. A recent publication cast doubt on this conclusions, showing that LAT^{Y136F} mice do have nonfunctional Foxp3$^+$ Treg that fail to control conventional mutant LAT^{Y136F} CD4$^+$ T helper cells [73].

3.4.4 Ontogeny of Foxp3$^+$ Treg

As thymectomy of 3-day old mice leads to autoimmunity which is attributable to a lack of Treg, one can speculate that the thymic generation of Treg is delayed in comparison to conventional T cells. After one report suggesting that this is not the case [74], detailed analysis of the kinetics of thymic Treg development in newborn Foxp3^{gfp} mice revealed that the appearance of Foxp3$^+$ Treg is indeed 3-4 days delayed in comparison to conventional nonregulatory T cells, which are already present at birth [75].

Beside that, this study showed interesting facets of Foxp3 induction which are probably applicable for adult thymic Treg development as well and therefore discussed in more detail. On day 1 and 2 after birth hardly any Foxp3$^+$ cells, but instead a significant fraction of CD25$^+$ Foxp3$^-$ cells can be detected. Although in terms of surface marker expression this population is not very similar to mature CD25$^+$ Foxp3$^+$ Treg [75], it is

tempting to speculate that these $CD25^+$ cells are direct Treg precursors. This hypothesis is substantiated by the above mentioned study of $Foxp3^{KO-GFP}$ cells which are $CD25^+$ as well [51].
Additionally, analysis of CD4/CD8 expression of the few existing $Foxp3^+$ thymocytes revealed that from day 1 on these cells mainly resided in the $CD4^+$ single positive (SP) compartment. This observation argues against the induction of $Foxp3^+$ Treg at the DP stage, which has been proposed by another study [76].

3.5 INTEGRATING CURRENT KNOWLEDGE ON TREG DEVELOPMENT

The available data discussed so far allow the following conclusions: Mechanistically, high affinity interaction with peptide-MHC class II complexes, costimulation via CD28 and signaling of common γ-chain cytokines are necessary. Both TGF-β and IL-2/IL-15 seem to dictate Treg development in a delicate manner, with some redundancy between these factors.
On a cellular level, Foxp3 expression is needed to sustain and amplify the Treg genetic signature, but is not required for induction of some Treg features such as anergy, upregulation of CD25 and low expression of IL-7Rα.
Very recent reports provided more detailed insight into the mechanistic puzzle of Treg induction. In one study, via transduction of a constitutively active allele of AKT it could be shown that both TGF-β dependent conversion of transduced $CD4^+$ T cells *in vitro* and thymic development of transduced DN thymocytes to $Foxp3^+$ thymocytes *in vivo* are reduced. Downstream targets of AKT include the protein mTOR (mammalian target of rapamycin), and rapamycin treatment of *in vitro* cultures could partially reverse the effect of constitutively active AKT [77]. Thus, signaling via AKT-mTOR seems to negatively regulate Treg development.
Another study readressed the importance of signaling via the the JAK/STAT pathway by transgenic mice expressing a constitutively active form of STAT5 [78]. In these mice, $Foxp3^+$ thymocytes could be detected at day 1 after birth, which is not the case in wildtype mice (see section 3.4.4). Furthermore, constitutively active STAT5 drives Treg development in $CD28^{-/-}$ and $CD132^{-/-}$ (common γ-chain) mice which are normally devoid of Treg, as stated above.
During preparation of this thesis another report was published, revealing that signals via the aryl hydrocarbon receptor (AHR) can define the decision whether peripheral T cells develop into $Foxp3^+$ Treg or proinflammatory T_H17 cells [79]. However, whether AHR plays a role in thymic Treg development remains to be tested.
The current state of knowledge concerning signals involved in Treg development is summarized in Figure 2, combined with an overview of defining Treg features.

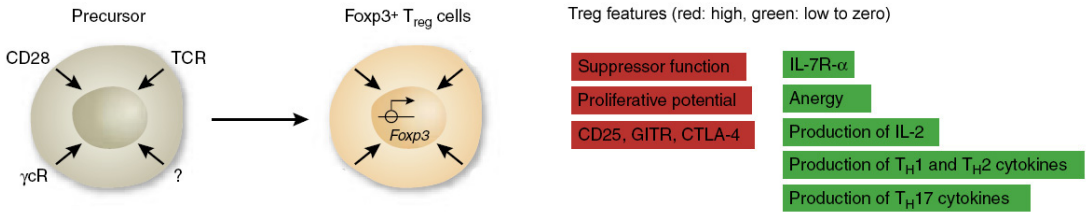

Figure 2: **Signals required to induce Foxp3$^+$ Treg cells** (adapted from [1])
Signals unambiguously shown to be involved for thymic Treg development include TCR-, CD28- and common γ-chain cytokine signals, as illustrated at the left diagram. TGF-β has recently been suggested to play a more global role in Treg development than initially thought.
Among the defining features of mature Foxp3$^+$ Treg are suppressive function and constitutive expression of CD25 and GITR. Furthermore, they express low levels of IL-7Rα and are inable to produce both IL-2 and T_H1/2 cytokines.

3.6 AIM OF THE PROJECT

As summarized above, some requirements for thymic generation of Treg have been experimentally addressed, but still the signalling pathways involved and the process per se remain enigmatic. Studying mechanistic details of thymic Treg development would be facilitated by the identification of direct precursor cells.
Thus, the aim of this work was to identify developmental precursors of mature CD4$^+$ CD25$^+$ Foxp3$^+$ Treg by using a TCR transgenic model. Additionally, by means of intrathymic transfer experiments we wanted to clarify at which stage of T cell development Treg induction takes place, thereby gaining a more detailed understanding of this process.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 BUFFERS AND MEDIA

1x PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH adjusted to 7.4
FACS buffer: 1x PBS, 2% FCS (Gibco), 1 mM EDTA
MACS buffer: 1x PBS, 0.5% BSA (Sigma Aldrich), 2 mM EDTA
Collagenase/dispase medium: IMDM (Gibco), 0.2 mg/ml dispase 1 (Roche), 0.2 mg/ml collagenase (Roche), 2% FCS (Gibco), 25 mM Hepes pH 7.2
Complete IMDM: IMDM (Gibco), 10% FCS (Gibco), glutamin, penicillin/streptamycin, β-mercaptoethanol

1x PBS (further on referred to as PBS), 0.5 M EDTA, 10x TAE, 5 M NaCl, IMDM and RPMI were all obtained from the IMP media kitchen.

4.1.2 PEPTIDES

The influenza hemagglutinin peptide $HA_{107-119}$ (SVSSFERFEIFPK, recognized by the TCR-HA in the context of I-E^d) and the ovalbumin peptide $OVA_{323-339}$ (ISQAVHAAHAEINEAGR, recognized by the DO11.10-TCR in the context of I-E^a) were synthesized in the IMP Protein Chemistry Facility.

4.1.3 ANTIBODIES

Abbreviations used for fluorescence molecules are Allophycocyanin (APC), Phycoerythrin (PE), Fluorescein isothiocyanate (FITC), Phycoerythrin-Cy7 (PE-Cy7), Allophycocyanin-Cy7 (APC-Cy7), Phycoerythrin-Cy5 (PE-Cy5, Cychrome).
The following antibodies were all purchased from BD Biosciences: APC CD45.1 (clone A20), PE-Cy7 CD25 (clone PC61), APC-Cy7 CD4 (clone L3T4), APC CD4 (clone RM4-5), PE CD4 (clone L3T4), Cychrome CD8 (clone 53-6.7), PE GITR (clone DTA-1), PE PD-1 (clone J43), Cychrome CD45 (30-F11), PE Ly51 (clone BP-1), FITC CD11c. Biotinylated antibodies against CD8, CD69 (clone H1.2F3), CD44 (clone IM7), CD24 (clone 30-F1), CD103 and Qa2 (clone 1-1-2) were from BD Biosciences as well. Streptavidin conjugated to Cychrome, PE, APC and PE-Cy7 was also obtained from BD Biosciences.
Monoclonal antibodies (mAb) to the TCR-HA (clone 6.5), TCR-OVA (clone KJ-26), against EpCAM (clone G8.8), CD4 (GK1.5) were purified from hybridoma supernatants and biotin- or fluorescence-labeled (Prozyme, following the manufacturer's protocol) in our lab. Neutralizing anti-mouse TGF-β1/2 (clone 1D11.16) was also purified from hybridoma supernatants in our lab.

All antibodies were titrated (2-fold dilution series from 1:50 down to 1:3200) and subsequently used in the optimal concentration.
Ki67 (clone B56) and Annexin-V stainings were done according to the manufacturer's instructions (BD Biosciences).

4.2 MICE AND GENOTYPING PROCEDURES

4.2.1 MICE

All mice used were on BALB/c background, unless stated otherwise. AIRE-HA × TCR-HA mice have been described previously [58] and were bred to $Foxp3^{GFP}$ reporter mice [39], and additionally for some experiments to a $CD45.1^{+/-}$ background. Both DO11.10 and TCR-HA mice were bred to a $RAG2^{-/-}$ $Foxp3^{gfp}$ $CD45.1^{+/-}$ background. All AIRE-HA mice used for experiments were heterozygous. BALB/c mice were either bred in-house or purchased from Taconic.
All animals were bred and maintained in the animal facility of the Research Institute of Molecular Pathology under pathogen-free conditions in individually ventilated cages. Animal studies were approved by local authorities (MA58) and were performed according to Austrian regulations.

4.2.2 GENOTYPING

For genotyping, mouse tails were clipped and digested in 50 µl of tail buffer for 6 hours at 55°C, followed by inactivation of enzymes at 95°C for 5 minutes. 1 µl of the digested template was used for subsequent PCR.

Tail buffer, volume 50 µl	
Reagent	Volume [µl]
10x Gitocher buffer	5 µl
10% Triton-X	2.5 µl
β-Mercaptoethanol	0.5 µl
Proteinase K [10 mg/ml]	3 µl
H_2O	39 µl
PCR reaction, volume 30 µl	
DNA template	1 µl
Primermix [2.5 µM]	3 µl
10x dNTPs [2.5 µM]	3 µl
5x PCR buffer	6 µl
Taq polymerase	1 µl
H_2O	16 µl

10x Gitocher buffer	5x PCR buffer
670 mM Tris pH 8.8 166 mM Ammonium sulfate 65 mM $MgCl_2$ 0.1% Gelatin	250 mM KCl 50 mM Tris pH 8.3 43% Glycerol 7.5 mM $MgCl_2$ 2 mM Cresol Red

The following primers were used for the respective genotypes:

TCR-HA:	5’	ACAAGGTGGCAGTAACAGGA
	3’	ACAGTCAGTCTGGTTCCTGA
DO11.10:	5’	CAGGAGGGATCCAGTGCCAGC
	3’	TGGCTCTACAGTGAGTTTGGT
RAG2:	5’	GCAACATGTTATCCAGTAGCCGGT
	3’	TTGGGAGGACACTCACTTGCCAGT
	int	GTATGCAGCCGCCGCATTGCATCA
AIRE-HA:	5’	ACAGCCACTCCTGTCTTTGC
	3’	CTCCGTCAGCCATAGCAAATTTCT
AIRE-HCO:	5’	ACAGCCACTCCTGTCTTTGC
	3’	GAATTGTTCGCATGGTAGCC
$FOXP3^{GFP}$	5’	AGACAGACCAGAGGTGTAGT
	3’	TCCTGGGGATGGGCCAAGGGCCAAGG

All genotyping PCRs were run with the following program (TD 54 × 30) and subsequently analyzed on a 1% agarose gel containing Ethidium bromide.

94°C for 3:00 min	1 cycle
94°C for 0:45 min 60°C for 0:45 min 72°C for 1:00 min	2 cycles
94°C for 0:45 min 58°C for 0:45 min 72°C for 1:00 min	2 cycles
94°C for 0:45 min 56°C for 0:45 min 72°C for 1:00 min	2 cycles
94°C for 0:45 min 54°C for 0:45 min 72°C for 1:00 min	30 cycles
72°C for 5:00 min	1 cycle

4.3 *IN VITRO* PROCEDURES

4.3.1 FACS (FLUORESCENCE ACTIVATED CELL SORTING) ANALYSIS AND STAINING PROCEDURES

Single cell suspensions of thymus, spleen or lymph nodes were prepared by gently mincing the organs between two glass slides and subsequent filtering through a cell strainer. Staining was performed according to standard procedures at a concentration of 1-4×10^6 cells in 100 µl FACS buffer (containing the appropriate antibodies) for at least 15 minutes on ice. Washing steps were performed by adding an excess of FACS buffer and centrifugation (5 minutes, 750g). Cells were filtered through a cell strainer prior to analysis. Flow cytometry was done on a FACSCanto (Becton Dickinson) followed by analysis with FlowJo (Treestar). Cell sorting was performed on a FACSAria (Becton Dickinson) into FCS-rinsed tubes.

4.3.2 MACS DEPLETION

Both for analysis of intrathymic injections and for sorting of $CD4^+$ SPs, thymi had to be depleted of all $CD8^+$ cells to yield cell numbers suitable for flow cytometric analysis.
Single cells suspensions were prepared, counted (Casy Counter, Schärfe Systems) and incubated with 1 ml of biotinylated CD8-antibody solution for 15 minutes on ice. After one washing step cells were resuspended in Strepatividin Microbeads (Milteny) which were diluted 1:10 with MACS buffer. For 100×10^6 cells 20 µl of Microbeads were used. After incubation at 4°C for at least 15 minutes cells were washed and resuspended in 1 ml of MACS buffer.
Cells suspensions were applied to pre-equilibrated MACS LS columns (Milteny), whereby thymi larger than 220×10^6 cells were split to two columns. Columns were rinsed 3 times with 3 ml of MACS Buffer, while the flowthrough was collected on ice. Cells were pelleted and stained for FACS as described in section 4.3.1.

4.3.3 PKH26 LABELING

For pkh26 labelling (Sigma Aldrich) 1-2×10^6 cells were washed two times with PBS and after complete removal of the supernatant resuspended in 100 µl of diluent C (Sigma Aldrich). 100 µl of 2x pkh26 solution were prepared (i.e. 1:500 pkh26 in diluent C to yield a final concentration of 1:1000) and added to the cell suspension. Cells were incubated for 3 minutes at room temperature and gently mixed every minute, followed by addition of 200 µl of FCS. After incubation for another minute, 400 µl of FACS buffer were added, and after centrifugation (5 min, 750 g, 4°C), the cells were resuspended either in FACS buffer or PBS and washed for an additional 2 times. All volumes were upscaled according to the used cell number (maximum 10×10^6).

4.3.4 CFSE LABELING

1×10^7 cells in 1 ml of labeling buffer (PBS with 0.1% BSA) were incubated for 10 minutes at 37°C with Carboxy-Fluorescein Diacetate Succinimidyl Ester (CFSE, final concentration 10 µM, Roche), followed by two washing steps either in FACS buffer or PBS.

4.3.5 *IN VITRO* ASSAYS

For in vitro assays, sorted T cells (either from TCR-HA RAG2$^{-/-}$ or DO11.10 RAG2$^{-/-}$ animals) were washed once with medium and then resuspended in complete IMDM. 5 x 10^4 T cells and 2 x 10^4 antigen presenting cells (sorted mTECs or DCs, see section 4.4.3) were cultured together in 96-well round-bottom plates in a final volume of 200 µl, unless stated otherwise medium was supplemented with 100 units/ml IL-2 (Preprotech). If necessary, HA- or OVA-peptide was added. For some assays, the activin receptor-like kinase inhibitor SB-431542 (conc. 3µM) [80] or neutralizing anti-TGF-β antibody (clone 1D11.16, conc 10 µg/ml) was used. Assays were stained for CD4 and CD25 and analyzed by flow cytometry 3-5 days later.

4.3.6 ANTIGEN PRESENTATION ASSAYS

For A5 antigen presentation assays, 2×10^4 sorted antigen presenting cells (either mTEC, DCs) were cultured with 2×10^4 A5 hybridoma cells in 200 µl IMDM including 1% FCS (Gibco) in 96-well round-bottom plates. If necessary, HA-peptide was added in a particular concentration. The assay was analyzed for expression of GFP by flow cytometry 17 to 20 hours later.

4.4 *IN VIVO* EXPERIMENTS

4.4.1 INTRATHYMIC TRANSFER

Intrathymic (IT) transfer has been described first in 1986 [81] and was slightly optimized for our experiments. Cells for injection were sorted to a purity > 95%, washed twice with PBS and then carefully resuspended in sterile PBS to yield a concentration of 5×10^5 cells in 3 µl (unless stated otherwise).

Recipient mice were anesthetesized with Ketamine / Xylazine intraperitoneally. After fixation of the animals with elastic straps, a small cut was made in the skin overlying the upper thoracic region, 2-4 mm above the sternum. Fat and connective tissue was carefully displaced and then the sternum was cut open for roughly 4 mm with fine scissors, thereby exposing the underlying thymus. Using a Hamilton syringe (Model 600, volume 2.5 to 5 µl) arranged in a flat angle, 3 µl of cell suspension were directly

injected into the anterior superior region of one thymic lobe. Care had to be taken not to injure the heart by passing through the thymus Afterwards the incision was closed with wound clips. Survival rate was more than 97%.
At various time points after transfer recipient animals were sacrificed, thymi were depleted of $CD8^+$ cells as described in section 4.3.2 and stained for FACS analysis.

4.4.2 BONE MARROW CHIMERAS

Tibiae and femur were collected from donor animals and carefully cleaned from surrounding muscle and connective tissue. Bones were sterilized in 70% ethanol for 2 minutes and washed with PBS. Then they were cut open at the ends and bone marrow (BM) was flushed out with PBS and the help of a syringe. Single cells suspensions were made by repeated resuspension, followed by filtering through a cell strainer. Finally, magnetic T cell depletion (using CD4 and CD8 antibodies) as described in section 4.3.2 was performed.
Recipient mice were lethally split-dose irradiated (for BALB/c animals 2 times 450 rad, for F1 (BALB/c C57Bl/6) animals 2 times 500 rad) and reconstituted by intravenous injection with $5\text{-}8\times10^6$ T cell depleted BM cells. Chimeras were used for intrathymic transfer experiments 5-8 weeks later.

4.4.3 PREPARATION OF THYMIC STROMA

Thymi were collected followed by careful removal of fat and connective tissue. Organs were cut into pieces using small scissors and then transferred to 15 ml tubes (Falcon) and incubated with collagenase/dispase medium including DNAse (added prior to use, final conc. 25 µg/ml) at 37°C for 45 minutes. For each thymus, 0.5 to 0.75 ml of collagenase/dispase medium was used.
To disrupt cell-cell interactions, the suspension was resuspended very frequently, first with a 1000 µl pipette, then using 5 ml syringes with microlance no. 5 needles (Becton Dickinson). After 45 minutes the digest was stopped by addition of EDTA to yield a final concentration of 5 mM and incubation for 5 minutes at 37°C. After EDTA incubation cells were transferred to ice immediately, washed with excess of FACS buffer and counted (Casy Counter, Schärfe Systems). After complete removal of the supernatant the pellet was carefully resuspended in high density percoll (GE Healthcare, $\rho = 1.115$). An intermediate layer of low density percoll solution (GE Healthcare, $\rho = 1.045 – 1.06$ according to titration) followed by an upper layer of IMDM was very carefully pipetted to yield an inverse discontinous density gradient.
After centrifugation at 1350 g for 30 minutes at 4°C (without brake, medium acceleration) low density cells (including mTECs and DCs) could be found within the upper interphase. Cells were removed using a 200 µl pipette, washed with FACS buffer und used for subsequent staining or sorting as described in section 4.3.

High density percoll was generated by diluting Percoll pure (GE Healthcare) with 10x PBS including 25 mM HEPES, low density percoll by diluting high density percoll with PBS. Calculation of densities was performed as follows:

$$v_2 = \frac{v_1 \times (D_1 - D_2)}{D_2 - D}$$

v_1 … volume of stock solution
v_2 … volume of diluting solution
D_1 … density of stock solution
D_2 … density to reach
D … density of PBS

4.5 RNA WORK

4.5.1 RNA ISOLATION

Sorted cells were washed two times with PBS and then resuspended in 200 µl PBS. RNA was purified using the High Pure RNA isolation Kit from Roche according to the manufacturer's protocol for RNA isolation from cultured cells. Final elution was performed in 50 µl elution buffer and RNA was either used directly for cDNA synthesis or stored at -80°C.

4.5.2 CDNA SYNTHESIS

cDNA was prepared using either SuperScript II First-Strand synthesis system (Invitrogen) or iScript cDNA synthesis kit (BioRad). For both kits the possible maximum amount of RNA template was used, following the manufacturers' protocols. cDNA was stored at 4°C until further use.

4.5.3 QUANTITATIVE REAL TIME PCR

qPCR was performed using the Bio-Rad iQ SYBR Green Supermix. 7.5 µl of SYBR Green Supermix were mixed with 0.3 µl of the appropriate primer stock (10 µM, see primer list). 7.2 µl of cDNA template (duplicates) were pipetted into 96-well plates (Bio-Rad), followed by addition of the primer-Supermix mixture and sealing of the plate with an optical qPCR film seal (Bio-Rad). qPCR was performed with a Bio-Rad iCycler using the following program:

95°C for 3:00 min	1 cycle
95°C for 0:45 min 56°C for 0:45 min 72°C for 1:00 min	45 cycles
55-95°C, 0.5 degrees change every 30 seconds	melting curve

Analysis was performed with Bio-Rad iQ5 software and Microsoft Excel using the $\Delta\Delta C_T$ method [82], while the correct size of the PCR fragment was verified on a 2% agarose gel. Primers used were as follows:

Foxp3:	5’	TTCTCACAACCAGGCCACTTG
	3’	ATCATGGAGAAGAGGCGAAGGG
IL2Rα:	5’	CGGGATACAAGGCTCTA
	3’	AAGTCTGTGGTGGTTATGG
IL2Rβ:	5’	AGAAGGGTTGGCGTAG
	3’	TCTGGGTATCAATGTGC
IL7Rα:	5’	CCCTCTGACCTGAAA
	3’	TCTGTGGGATTGTTGT
CTLA-4:	5’	TTTGTAGCCCTGCTCACT
	3’	GGAAGCCCACTGTATTCTT
SOCS-2	5’	TCGCATTCAGACTACCT
	3’	CTGTCCGTTTATCCTTG
Nrp-2	5’	TGATAGGGAAGGGACGAT
	3’	TTCTTTGCCAGATGAGGG

5 RESULTS

5.1 PHENOTYPIC CHARACTERIZATION OF THE AIRE-HA × TCR-HA SYSTEM

5.1.1 REGULATORY T CELL INDUCTION IN A DOUBLE TRANSGENIC MOUSE MODEL

As mentioned in the introduction, work from our lab [58] has shown that thymocytes expressing a transgenic class II-restricted αβ-T cell receptor specific for hemagglutinin (TCR-HA) give rise to $CD4^+$ $CD25^+$ regulatory T cells (Treg) if the agonist ligand hemagglutinin is presented by medullary thymic epithelial cells (mTECs) under control of the Aire promotor (AIRE-HA).

Here we used AIRE-HA × TCR-HA mice to further characterize the process of thymic Treg development. To facilitate identification of $Foxp3^+$ Treg cells AIRE-HA × TCR-HA mice were crossed to $Foxp3^{gfp}$ mice, which express a fusion protein of Foxp3 and eGFP (enhanced green fluorescent protein) [75]. Thus we were able to analyze and culture *ex vivo* isolated $Foxp3^+$ cells without the need for intracellular staining.

As already published [58], both TCR-HA and AIRE-HA × TCR-HA mice display normal thymic cellularity and no change in the percentage of CD4 single positive thymocytes ($CD4^+$ SPs). In TCR-HA mice about 25% of $CD4^+$ SPs stain positive for TCR-HA, and nearly all of these cells remain phenotypically naïve in terms of CD25 and Foxp3 expression. In contrast, in AIRE-HA × TCR-HA mice only 5-10% of the cells at the $CD4^+$ SP stage are $TCR\text{-}HA^+$ (Figure 3). A significant fraction of these $TCR\text{-}HA^+$ $CD4^+$ SPs express CD25 and Foxp3 and have been characterized as being functional Tregs [58].

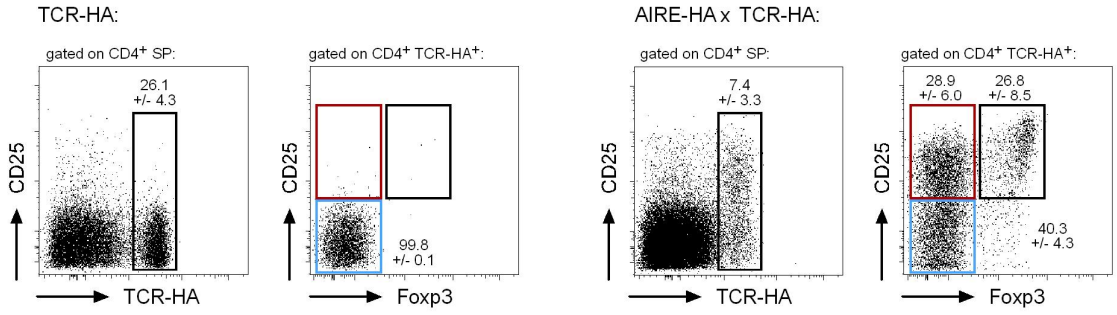

Figure 3: **$CD4^+$ SP compartment of TCR-HA and AIRE-HA × TCR-HA thymi**
$TCR\text{-}HA^+$ $CD4^+$ SPs in TCR-HA mice are phenotypically naïve in terms of CD25 and Foxp3 (left dot plots). In contrast, $TCR\text{-}HA^+$ $CD4^+$ SPs in AIRE-HA × TCR-HA mice (right dot plots) can be divided into three subsets based on CD25 and Foxp3 expression (coloured light blue, red, and black). Note the 4-fold decrease of $TCR\text{-}HA^+$ $CD4^+$ SPs in AIRE- HA × TCR-HA thymi.
Gating is denoted above dot plots, and given percentages for the indicated gates are mean values of 5 (TCR-HA) and 30 (AIRE- HA × TCR-HA) mice aged 4-6 weeks, respectively.

Interestingly, there was also a population of cells expressing only $CD25^+$ but not Foxp3. Appearance of $CD25^+$ $Foxp3^-$ thymocytes has been described during ontogeny [75] and in $Foxp3^{KO\text{-}GFP}$ cells [51], but the fate of these cells has not been experimentally addressed until recently. Two opposing possibilities could be envisioned: $CD25^+$ $Foxp3^-$ cells could represent either a "dead end" or an intermediate developmental step during Treg development. Using AIRE-HA × TCR-HA mice we wanted to address this issue and as a starting point analysed expression of various activation markers.

For this purpose, $CD4^+$ $TCR\text{-}HA^+$ thymocytes from AIRE-HA × TCR-HA mice were separated into the following three subsets (Figure 3): $CD25^-$ $Foxp3^-$ (designated subset 1, blue), $CD25^+$ $Foxp3^-$ (designated subset 2, red) and $CD25^+$ $Foxp3^+$ cells (designated subset 3, black). Results of flow cytometric analysis are discussed within the next paragraphs.

5.1.2 ACTIVATION MARKERS

Several surface molecules are known to be differentially regulated during activation of T cells. CD69 and CD44, markers indicative for T cell receptor (TCR) signalling, were found to be upregulated by $CD25^+$ $Foxp3^-$ cells (Figure 4). Diminished CD69 levels within the $CD25^+$ $Foxp3^+$ subset suggest, due to the transient nature of CD69 expression [83], a decrease in TCR triggering.

The tumor-necrosis-factor-receptor family member GITR (glucocorticoid induced tumor necrosis factor receptor) is rapidly induced after stimulation of naïve T cells, and known to be constitutively expressed by Tregs [84]. Consistent with that high expression of GITR was detected on $CD25^+$ $Foxp3^+$ cells. $CD25^+$ $Foxp3^-$ cells displayed slightly lower GITR levels, while for $CD25^-$ $Foxp3^-$ cells an unexpected biphasic expression pattern was observed, with 30-60% of cells expressing intermediate levels of GITR. This suggests that although being naïve in terms of CD25, these $GITR^+$ cells have already been activated.

Programmed death receptor 1 (PD-1) is expressed by a variety of immune cells and also upregulated on T cells within 24 hours after activation [85]. Both the $CD25^+$ $Foxp3^-$ and $CD25^+$ $Foxp3^+$ subsets expressed intermediate levels of PD-1. Interestingly, as for GITR, upregulation of PD-1 by 20-40% of $CD25^-$ $Foxp3^-$ cells signified ongoing activation. Of note, co-staining for PD-1 and GITR revealed that within the $CD25^-$ $Foxp3^-$ subset not all $GITR^+$ cells were $PD\text{-}1^+$ and vice versa (data not shown).

Inducible T cell costimulator (ICOS) belongs to the CD28 protein family and is upregulated after T cell activation as well [86]. In terms of ICOS expression no major differences could be detected between $TCR\text{-}HA^+$ subsets, with only a few percent of $CD25^+$ $Foxp3^+$ being $ICOS^+$.

The function of CD103 (α_E integrin) in Treg biology has not been clarified, although it has been reported to be involved in Treg function [87] and homing to epithelial

compartments. In agreement with these reports a fraction of CD103$^+$ cells within the CD25$^+$ Foxp3$^+$ compartment was observed.

5.1.3 MATURATION MARKERS

Since the analysis described above indicated different activation levels of TCR-HA$^+$ cells in AIRE-HA × TCR-HA mice, we aimed to characterize the maturity of these subsets. For this reason stainings for maturation markers, which are differentially regulated during development of CD4$^+$ SPs, were performed. CD62L (also known as L-selectin) and Qa-2 are known to be expressed at low to intermediate levels directly after transition from the double positive compartment, while mature CD4$^+$ SPs increase expression of these molecules. In contrast, CD24 expression is known to decline with increasing maturity of CD4$^+$ SPs [6]. For all these markers we detected an increase in maturity from CD25$^-$ Foxp3$^-$ to CD25$^+$ Foxp3$^-$ and CD25$^+$ Foxp3$^+$ cells, with the largest differences between the latter two subsets (Figure 4).

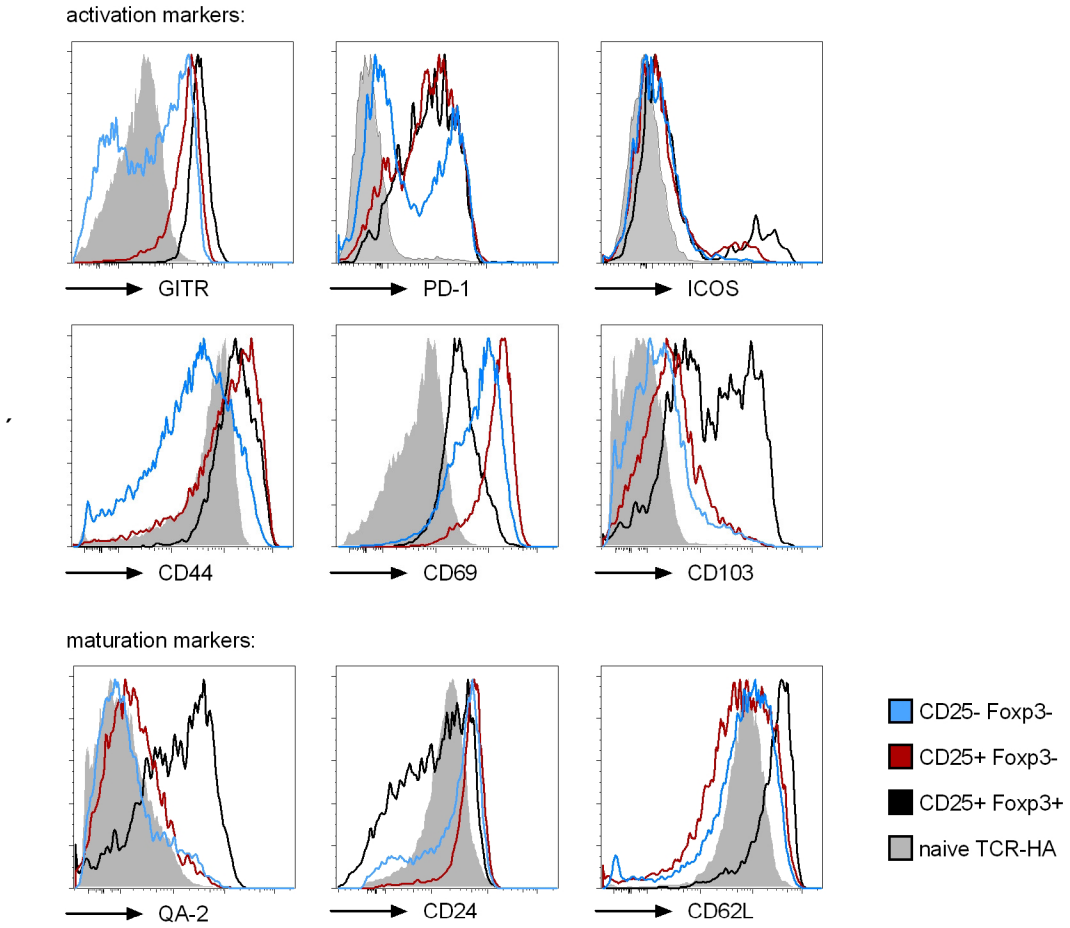

Figure 4: **Expression of activation and maturation markers by TCR-HA$^+$ CD4$^+$ subsets in AIRE-HA × TCR-HA mice.**
All plots are gated on TCR-HA$^+$ CD4$^+$ CD8$^-$ thymocytes. Color code for histograms is as shown in Figure 1 with light blue for CD25$^-$ Foxp3$^-$, red for CD25$^+$ Foxp3$^-$ and black for CD25$^+$ Foxp3$^+$ cells. Grey shaded histograms represent TCR-HA$^+$ CD4$^+$ SPs from TCR-HA mice. Stainings have been repeated three times with similar results.

In conclusion, the expression analyses of the depicted activation and maturation markers suggest a developmental precursor-progeny relationship between the three subsets of TCR-HA$^+$ CD4$^+$ SPs in AIRE-HA × TCR-HA mice. To gain additional insight into this potential relationship we wanted to assess proliferation and apoptosis within these subsets.

5.1.4 PROLIFERATION AND APOPTOSIS

To test whether CD25$^+$ Foxp3$^-$ cells are destined for negative selection an Annexin-V staining was carried out. Annexin-V binds to Phosphatidylserin molecules, which are normally found only in the inner layer of the cell membrane lipid bilayer. In cells undergoing apoptosis, one of the earliest events is flipping of Phosphatidylserine to the outer membrane leaflet, where it can be bound by Annexin-V.

In comparison to naïve TCR-HA$^+$ CD4$^+$ SPs from TCR-HA mice, a 4- to 5-fold increase in apoptosis was seen both in TCR-HA$^+$ CD25$^-$ Foxp3$^-$ and CD25$^+$ Foxp3$^-$ cells from AIRE-HA × TCR-HA mice (Figure 5b). This finding does neither establish nor exclude the possibility that one of these subsets is undergoing negative selection. Of note, the percentage of cells undergoing apoptosis *in vivo* might be underestimated by these stainings because in the thymus apoptotic cells are rapidly cleared by resident macrophages [88].

As our analysis of activation markers indicated ongoing TCR-signaling, we wanted to address the proliferative status of CD4$^+$ SPs in AIRE-HA × TCR-HA mice. For this purpose stainings for KI-67, a proliferation marker, were done. Both within the CD25$^-$ Foxp3$^-$ and CD25$^+$ Foxp3$^-$ population up to 30% of cells were proliferating, indicating that CD25$^-$ Foxp3$^-$ cells are indeed not naïve but already activated (Figure 5a).

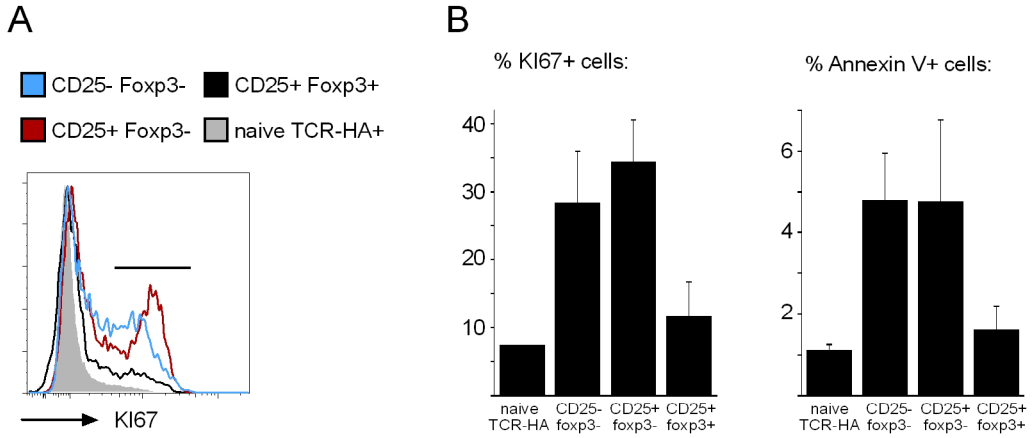

Figure 5: **Analysis of proliferation and apoptosis within TCR-HA$^+$ CD4$^+$ subsets in AIRE-HA × TCR-HA mice.**

a Intracellular staining for the proliferation marker KI-67. Histograms are gated on TCR-HA$^+$ CD4$^+$ CD8$^-$ thymocytes, color code is as in Figure 2 (light blue for CD25$^-$ Foxp3$^-$, red for CD25$^+$ Foxp3$^-$ and black for CD25$^+$ Foxp3$^+$ cells, grey shaded for TCR-HA$^+$ CD4$^+$ SPs from TCR-HA mice) **b** Percentage of cells staining positive for KI-67 (left diagram) and Annexin-V (right diagram) in TCR-HA$^+$ subsets from AIRE-HA × TCR-HA mice. Mean values from three independent experiments are shown.

5.1.5 FOXP3 MRNA EXPRESSION IN FOXP3$^-$ SUBSETS

We asked next whether the upregulation of CD25 was only the result of conventional activation due to antigen exposure or indicative of development along the Treg lineage. For this purpose, TCR-HA$^+$ CD4$^+$ SPs from AIRE-HA × TCR-HA mice were separated into the following subsets based on expression of CD25, Foxp3 and GITR: CD25$^-$ Foxp3$^-$ GITR$^-$, CD25$^-$ Foxp3$^-$ GITR$^+$, CD25$^-$ Foxp3$^+$ and CD25$^+$ Foxp3$^+$. Cells were sorted, and after RNA isolation and cDNA synthesis quantitative real-time PCR was performed.

Analysis of Foxp3 expression by qPCR revealed that in comparison to naïve CD4$^+$ SPs, upregulation of Foxp3 on mRNA level could already be detected both within CD25$^+$ Foxp3$^-$ and CD25$^-$ Foxp$^-$ GITR$^+$ cells (Figure 6). One can conclude that signaling events inducing expression of Foxp3 are taking place within these subsets which supports the hypothesis that the Foxp3$^-$ subsets seen in AIRE-HA × TCR-HA mice are precursors of mature Treg.

Beside that, changes in the expression of IL2Rα (CD25), IL2Rβ, IL7Rα, CTLA-4 and SOCS-2 were detected, all indicating ongoing TCR- and cytokine signaling events (data not shown).

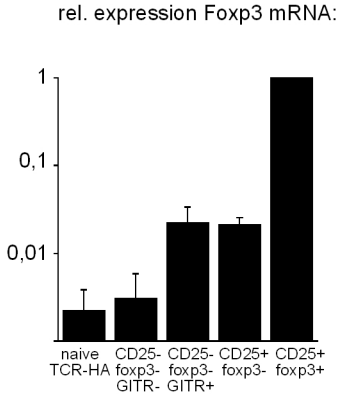

Figure 6: **qPCR analysis of Foxp3 expression within TCR-HA$^+$ CD4$^+$ SPs in AIRE-HA × TCR-HA mice.**
TCR-HA$^+$ CD4$^+$ SPs were separated based on expression of CD25, Foxp3 and GITR as denoted. Y-axis represents fold changes with expression being normalized to CD25$^+$ Foxp3$^+$ cells (arbitrary set to 1) using the $\Delta\Delta C_T$ method and β-actin as a housekeeping gene. Values represent the mean of three independent experiments.

5.2 PRECURSOR-PROGENY RELATIONSHIP

5.2.1 INTRATHYMIC TRANSFER OF SORTED TCR-HA$^+$ SUBSETS

The data presented so far argue for a precursor-progeny relationship within the subsets of TCR-HA$^+$ CD4$^+$ SPs in AIRE-HA × TCR-HA mice. However, the assumption that CD25$^+$ Foxp3$^-$ cells represent a “dead end” can not be dismissed because staining for Annexin-V did not allow unambiguous conclusions. To definitely address this issue intrathymic transfer experiments were conducted.

CD25$^-$ Foxp3$^-$ GITR$^-$, CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells were sorted from the TCR-HA$^+$ CD4$^+$ compartment of AIRE-HA × TCR-HA Foxp3^{GFP} CD45.1 mice and intrathymically injected into both AIRE-HA (CD45.2) and WT (CD45.2) recipients. Note that transfer of CD25$^+$ Foxp3$^+$ cells is not discussed here because as expected more

than 90% of cells retained their phenotype upon transfer (data not shown), which has already been reported for Foxp3$^+$ Treg earlier [1].

Analysis four days after transfer revealed that in AIRE-HA recipients all transferred subsets gave rise to CD25$^+$ Foxp3$^+$ Treg, notably with stepwise increasing efficiency from CD25$^-$ Foxp3$^-$ GITR$^-$ to CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells (Figure 7). This observation provides additional evidence for a precursor progeny relationship between these subsets, and precludes the possibility that all cells of a particular subset represent a developmental dead end.

Interestingly, after injection into WT animals both CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells gave rise to Foxp3$^+$ cells as well. Thus, at least for a fraction of cells within these subsets TCR-stimulation is no longer necessary to continue their development. In contrast, CD25$^-$ Foxp3$^-$ GITR$^-$ stayed phenotypically naïve in the absence of TCR signaling (Figure 7).

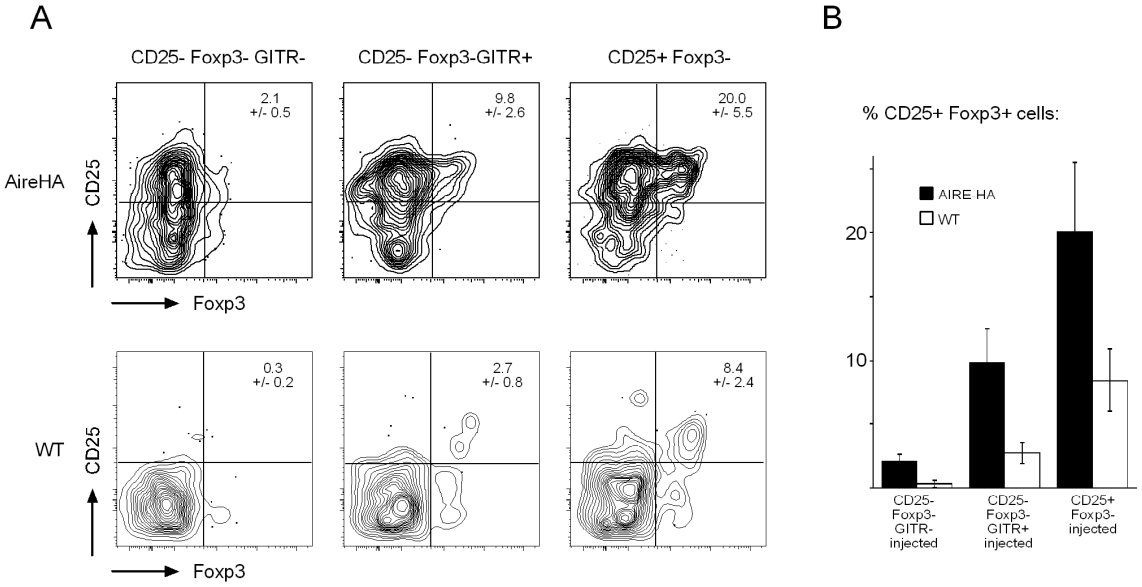

Figure 7: **All TCR-HA$^+$ CD4$^+$ subsets from AIRE-HA × TCR-HA mice can give rise to Foxp3$^+$ Treg after intrathymic transfer into AIRE-HA recipients.**

CD25$^-$ Foxp3$^-$ GITR$^-$, CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells were sorted from the TCR-HA$^+$ CD4$^+$ SP compartment of AIRE-HA × TCR-HA Foxp3^{GFP} CD45.1 mice and intrathymically transferred (3 x 10^5 cells per subset) into AIRE-HA and WT recipients. Analysis for Foxp3 and CD25 expression was performed four days after transfer.

a Contour plots are gated on transferred cells (TCR-HA$^+$ CD4$^+$ CD8$^-$ CD45.1$^+$) and representative of two independent experiments. Genotypes of recipient mice are indicated to the left, injected cell type above plots. Numbers in the plots indicate mean percentage (n=3-5) of CD25$^+$ Foxp3$^+$ cells. **b** Mean percentage of CD25$^+$ Foxp3$^+$ cells after intrathymic transfer into AIRE-HA (black bars) and WT (open bars) mice. Values are mean of 3-5 mice per group.

5.2.2 POLYCLONAL CD25$^+$ FOXP3$^-$ THYMOCYTES GIVE RISE TO FOXP3$^+$ TREG

To ensure that our observations are not the result of some predisposition of the TCR-HA system we asked whether we could reproduce our results using polyclonal thymocytes.

GITR staining of polyclonal CD4$^+$ SPs reveals that not all of them are GITR negative, but that there is a large proportion of cells expressing intermediate levels of GITR [89]. Thus, we sorted polyclonal CD25$^-$ Foxp3$^-$ GITR^{low}, CD25$^-$ Foxp3$^-$ GITR^{high} and CD25$^+$ Foxp3$^-$ cells (note that gating for GITR was set arbitrary to choose the lowest and highest GITR expressing cells within the CD25$^-$ pool) followed by intrathymic injection into WT mice.

Recipients were analyzed six days later, revealing that the results resembled the TCR-transgenic system, with both CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells giving rise to Foxp3$^+$ cells (Figure 8). Of note, in contrast to the TCR-HA system, polyclonal CD25$^-$ Foxp3$^-$ GITR^{low} developed to Foxp3$^+$ Treg as well. While HA-specific thymocytes can not encounter their cognate antigen within WT mice, polyclonal thymocytes will do so with a certain probability. The population of CD25$^+$ Foxp3$^-$ cells in these recipient mice expressed lower levels of CD25 compared to intrathymic transfer of TCR-HA$^+$ cells, which will be discussed in more detail later.

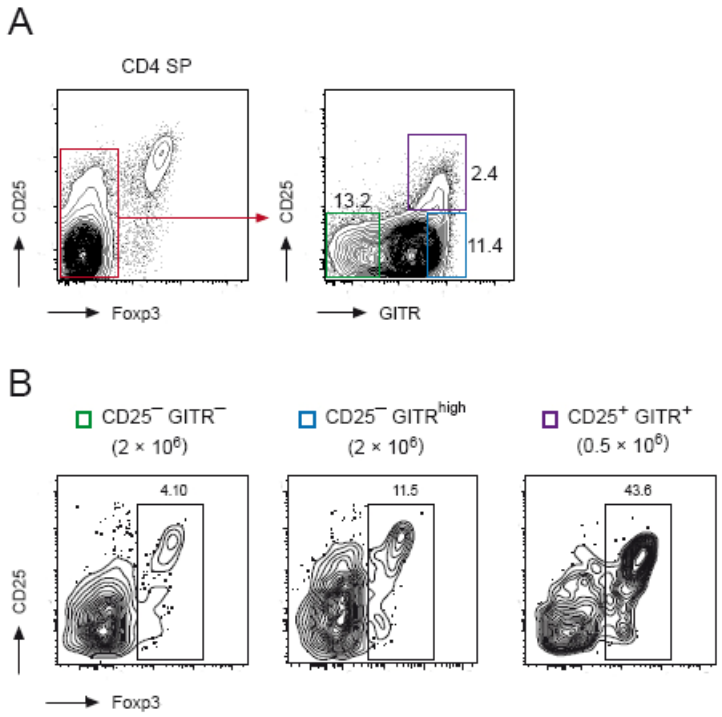

Figure 8: **Polyclonal CD25$^+$ Foxp3$^-$ and CD25$^-$ Foxp3$^-$ GITR$^+$ contain precursor cells to mature Foxp3$^+$ Treg.**
a Gating strategy for sorting of the injected subsets. Numbers indicate percentage of cells within the respective gate **b** Subsets depicted above contour plots were sorted from WT CD45.1 mice and intrathymically transferred into WT CD45.2 recipients (numbers in brackets depict number of transferred cells). Analysis was performed six days later. Numbers in the plots (gated on CD4$^+$ CD8$^-$ CD45.1$^+$) indicate percentage of Foxp3$^+$ cells. Plots shown are representative pictures of one experiment with 2-3 mice per group.

In conclusion, both TCR-transgenic and polyclonal intrathymic transfer experiments strongly suggest a precursor-progeny relationship between CD25$^-$ Foxp3$^-$ GITR$^+$, CD25$^+$ Foxp3$^-$ and mature CD25$^+$ Foxp3$^+$ Treg, and show that the latter two subsets can develop to Foxp3$^+$ Treg independent of further TCR-stimulation.

5.3 TREG INDUCTION AFTER INTRATHYMIC TRANSFER OF NAÏVE CD4$^+$ SPS

5.3.1 INTRATHYMIC TRANSFER INTO AIRE-HA MICE

Work from several groups has suggested that Tregs can be induced by thymic agonist ligands. However, it has been argued that this process may not represent *de novo* differentiation, but rather selective survival of committed precursors [59]. Beside that, it has not been clearly shown so far at which stage of thymocyte development cells commit to the Treg lineage, with one publication arguing for the double positive (DP) stage [76]. Another model suggested that already at the DN stage developing thymocytes might be trans-conditioned for development along the Treg lineage [90].
Based on our findings of a precursor-progeny relationship in TCR-HA$^+$ CD4$^+$ SPs of AIRE-HA × TCR-HA mice we wanted to elucidate whether late stage induction to the Treg lineage is possible. For this reason, intrathymic transfer of naïve CD4$^+$ SPs from TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{gfp} CD45.1 mice into either AIRE-HA (CD45.2) or WT (CD45.2) recipients was performed (Figure 9). By using this transfer method the fate of naïve, truly antigen unexperienced CD4$^+$ SPs at a defined developmental stage can be followed in their natural thymic environment, either in the presence or absence of cognate antigen. Note that usage of endogenously rearranged TCR α-chains was excluded by using mice on a RAG2$^{-/-}$ background.

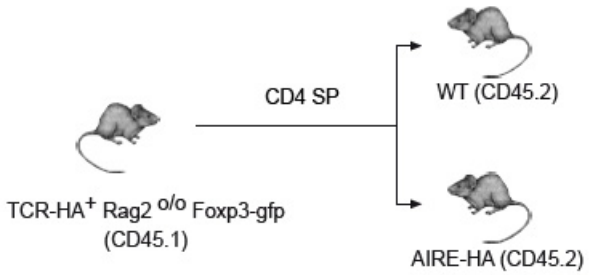

Figure 9: **Design of congenic intrathymic transfer experiments**
5x10^5 (unless stated otherwise) CD4$^+$ SPs or CD4$^+$ peripheral T cells were isolated from TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{gfp} CD45.1 donor animals and injected into the thymus of either WT or AIRE-HA recipients (CD45.2). During analysis, injected cells were identified via expression of CD45.1

Recipient mice were killed at day 1 to day 8 after intrathymic transfer and the injected cells were examined for CD25 and Foxp3 expression. Strikingly, in AIRE-HA but not in WT recipients Foxp3 induction was observed 3 to 4 days after transfer, which definitely proves that Treg induction can happen after encounter of cognate antigen at the single positive stage of thymocyte development (Figure 10a).

Comprehensive analysis of recipients for each day revealed interesting details. In WT recipients, for each time-point analyzed essentially all of the injected cells stayed naïve ($CD25^-$ $Foxp3^-$), arguing against the presence of any committed precursors in the transferred $CD4^+$ SP population.

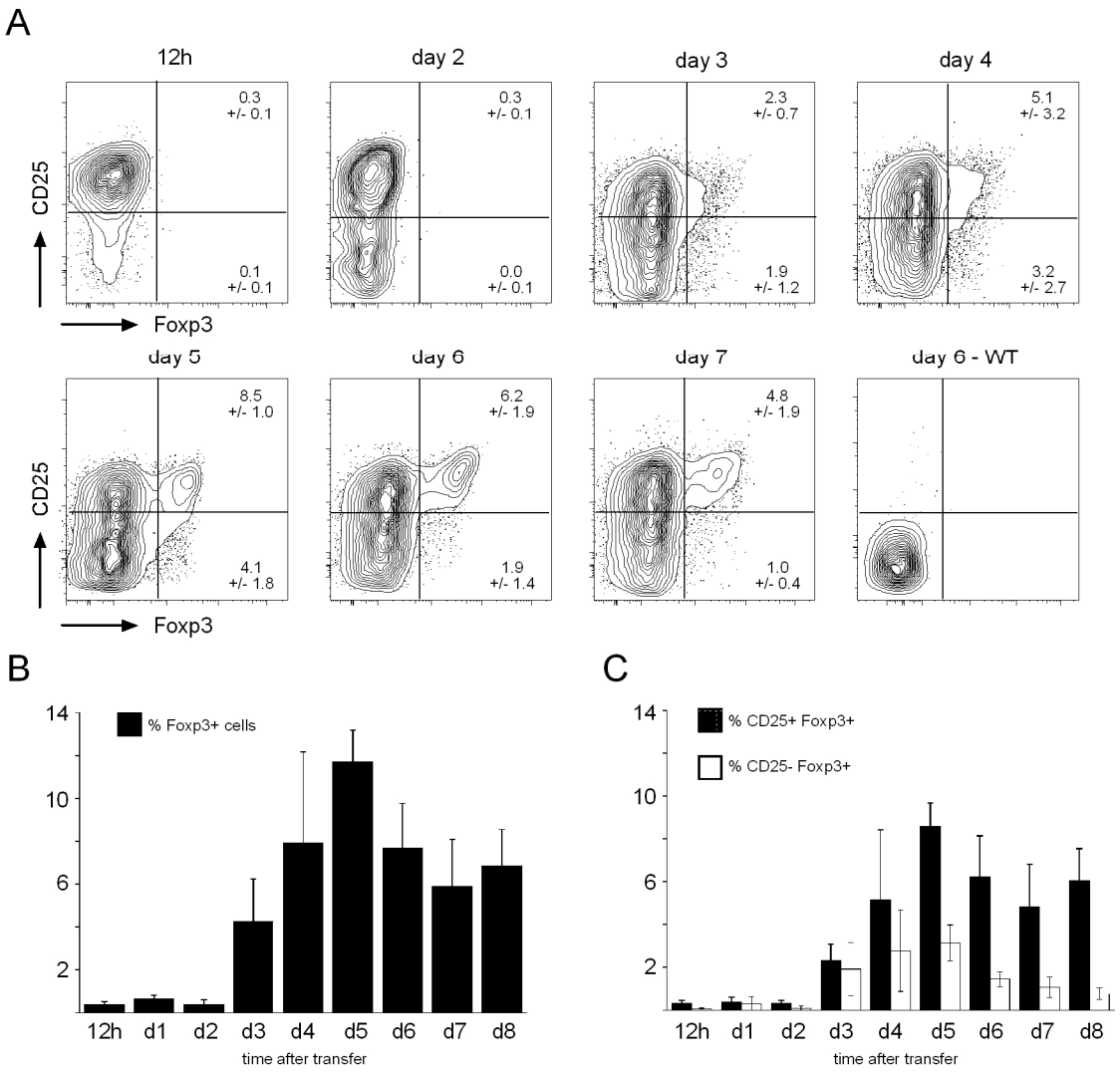

Figure 10**: Induction of $Foxp3^+$ Treg at the $CD4^+$ SP stage is possible.**
5×10^5 $TCR\text{-}HA^+$ $RAG2^{-/-}$ $CD4^+$ SPs were intrathymically transferred into AIRE-HA and WT recipients followed by analysis for CD25 and Foxp3 expression on day 1-8 after transfer. **a** Representative contour plots of transferred cells ($CD4^+$ $CD8^-$ $CD45.1^+$) at the indicated time points. Numbers indicate mean percentages (n=3-5) of cells in the respective gate. **b** Emergence of $CD25^+$ $Foxp3^+$ cells in AIRE-HA recipients at the indicated time points. **c** Percentages of $CD25^+$ $Foxp3^+$ and $CD25^-$ $Foxp3^+$ cells at the indicated time points. Note the selective increase of $CD25^-$ $Foxp3^+$ cells at day 3 to day 5.

In AIRE-HA recipients most of the cells (91.1 $^+/_-$ 1.9%) became activated in terms of CD25 upregulation as early as 12 hours after injection. Until day 3 the ratio between

CD25$^-$ Foxp3$^-$ and CD25$^+$ Foxp3$^-$ cells moved gradually to an equal distribution (55.4 $^+/_-$ 4.8% for CD25$^-$ Foxp3$^-$ and 39.6 $^+/_-$ 7.4% for CD25$^+$ Foxp3$^-$ cells), which is also seen in AIRE-HA × TCR-HA mice (compare Figure 3). Due to the obtained cell numbers at day 1 and day 3 (Figure 11) the CD25$^+$ cells are not likely to undergo apoptosis, but rather lose CD25 expression again to give rise to the CD25$^-$ population.
The percentage of Foxp3$^+$ cells in AIRE-HA recipients increased until day 5 (11.7 $^+/_-$ 1.5%), then remaining constant at a slightly lower level (Figure 10b). Of note, we observed that for day 3 to day 5 after transfer a high proportion of Foxp3$^+$ cells showed no or only intermediate CD25 expression (Figure 10c), suggesting that CD25 upregulation (confering IL-2 responsiveness) is not a prerequisite for Foxp3 induction [68].
Concerning the number of recovered cells, in WT recipients a gradual reduction down to 1000 injected cells was observed, consistent with the idea that the transferred thymocytes continue to mature and finally leave the thymus after four to five days [91]. For AIRE-HA recipients even less cells than in WT recipients were recovered at early time-points, possibly indicating negative selection of TCR-HA$^+$ cells in the antigen expressing environment. From day three on the number of recovered cells started to increase, then decreased again until the end of the analyzed time period (Figure 11a). Of note, even after 14 days 500-2000 cells could be detected (data not shown), indicating that antigen exposure prolongs the average residence time of developing CD4$^+$ SPs. Alternatively, cells could reimmigrate to the thymus.

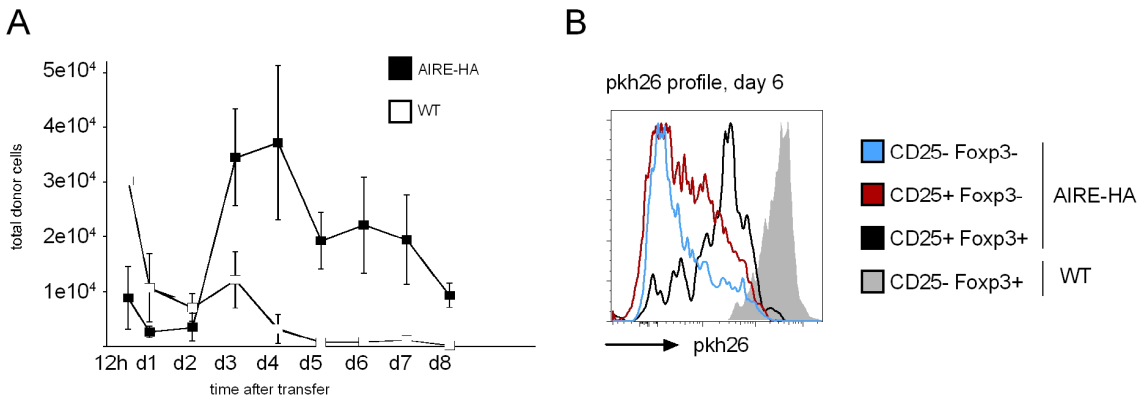

Figure 11: **Analysis of cell count and proliferation after intrathymic transfer of naïve TCR-HA$^+$ RAG2$^{-/-}$ CD4$^+$ SPs into AIRE-HA mice.**
a Absolute number of recovered cells after intrathymic transfer at the indicated time points for AIRE-HA (black squares) and WT (open squares) recipients (n=3-5). Note the steady decrease for WT recipients, while in AIRE-HA recipients cell numbers increase at day 3. **b** Induction of proliferation after intrathymic antigen encounter. Representative pkh26 profile for the indicated subsets of transferred cells (gated on CD4$^+$ CD8$^-$ CD45.1$^+$) in AIRE-HA recipients, with CD25$^+$ Foxp3$^+$ cells dividing least.

To analyze the proliferative history of the recovered cells, pkh26 labeling experiments were performed. pkh26 is a liphophilic compound which is incooperated into the cell membrane and diluted with each cell division. Although Carboxy Fluoroscein Succinimidyl Ester (CFSE) would have been superior to pkh26 in terms of signal sharpness, CFSE could not be used because its signal would have been in the same fluorescence channel as the GFP signal of Foxp^{GFP}.
After intrathymic transfer of pkh26 labelled cells no reduction of the fluorescence signal was observed at day one and two (data not shown), while for the following days the pkh26 signal steadily decreased, indicative of continuous proliferation. Analysis of subsets revealed that at all time-points CD25$^+$ Foxp3$^+$ cells had divided least (Figure 11b).
In WT recipients all of the injected cells remained pkh26 high, only losing roughly 10% of their initial fluorescence intensity which is the expected stochastic loss according to the manufacturer.

5.3.2 Effects of intraclonal competition

Having shown that induction of Treg can take place at the CD4$^+$ SP stage we asked how this process would be affected by intraclonal competition, i.e. the competition for agonist ligands. Therefore we performed intrathymic transfer of naïve TCR-HA$^+$ RAG2$^{-/-}$ CD4$^+$ SPs into AIRE-HA × TCR-HA mice. Analysis 6 days after transfer revealed the unexpected findings that in terms of percentage more Foxp3$^+$ cells developed, but according to the pkh26 signal for all subsets less proliferation than in AIRE-HA recipients was observed (Figure 12). The latter was reflected by the reduced number of recovered cells.

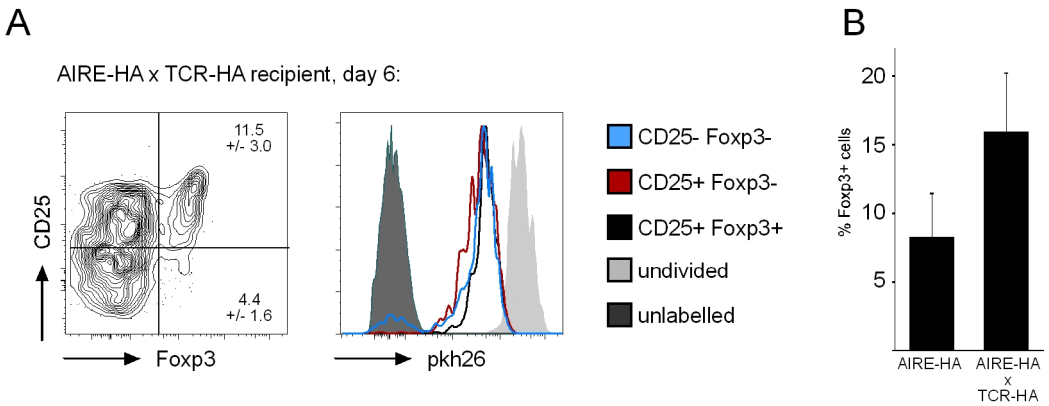

Figure 12: **Decreased proliferation after intrathymic transfer of TCR-HA$^+$ RAG2$^{-/-}$ CD4$^+$ SPs into AIRE-HA × TCR-HA recipients**
a Representative plots for transferred cells (CD4$^+$ CD8$^-$ CD45.1$^+$) at day 6 after transfer. Values indicate mean percentage (n=6-8) of cells in the respective gate. The pkh26 profile shows reduced proliferation of all subsets compared to AIRE-HA recipients (see Figure 11b). **b** Statistics for the percentage of Foxp3$^+$ cells in AIRE-HA and AIRE-HA × TCR-HA recipients at day 6 after intrathymic transfer.

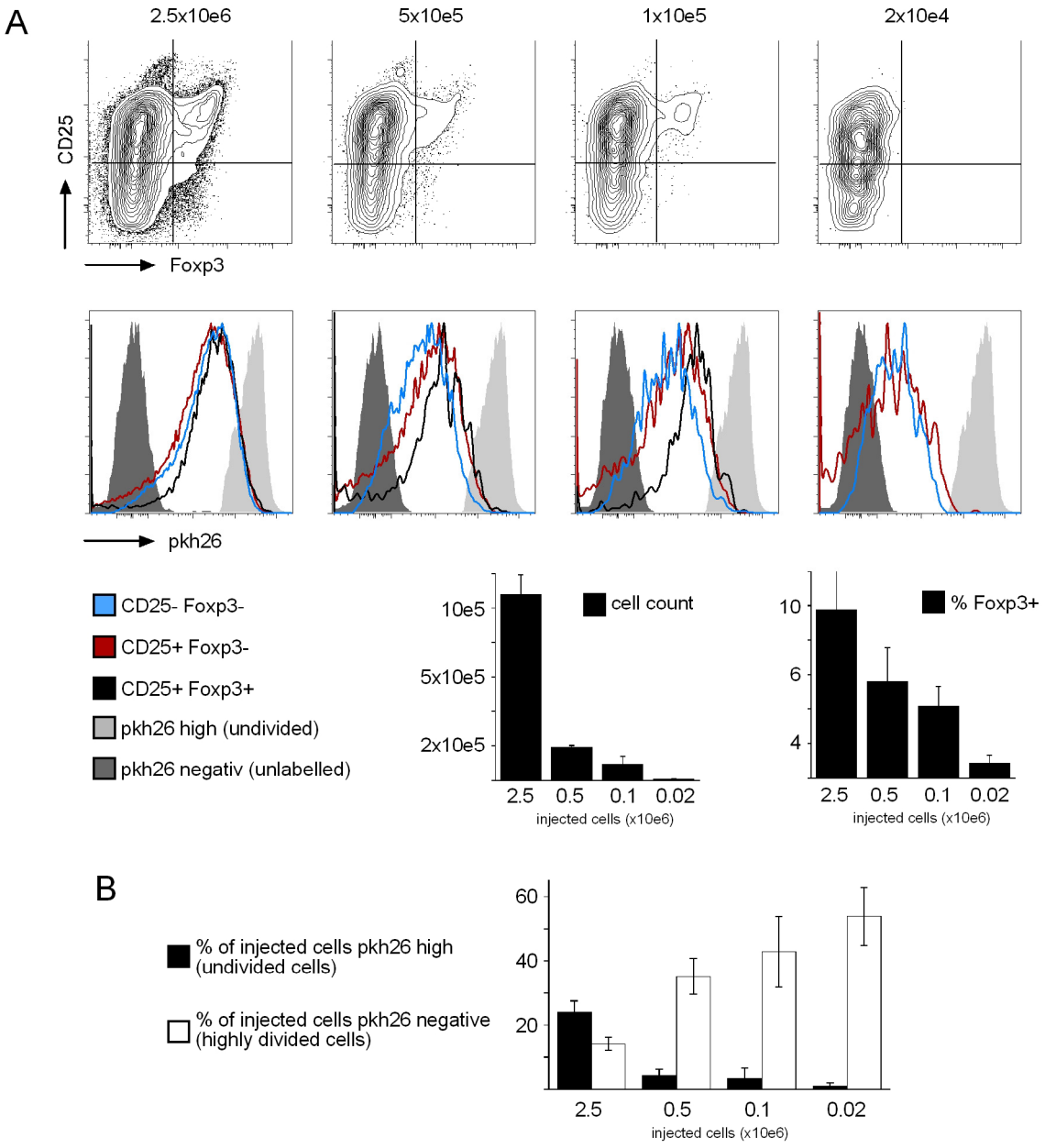

Figure 13: **Analysis of cell count and proliferation within a 5-fold dilution series of transferred naïve TCR-HA$^+$ RAG2$^{-/-}$ CD4$^+$ SPs into AIRE-HA mice.**
a Intraclonal competition affects both the proliferation and the efficiency of Treg induction. Representative plots of transferred cells (CD4$^+$ CD8$^-$ CD45.1$^+$) after five days. Injected cell numbers are indicated above, pkh26 profile of the indicated subsets below with the same color code used throughout this work. Both recovered cell numbers and percentages of Foxp3$^+$ cells decreased continuously from 2.5×10^6 down to 0.02×10^6 injected cells, as statistically shown in the bar diagrams (n=3). Note that the lower percentage of Foxp3$^+$ cells for intrathymic transfer of 0.5×10^6 cells as compared to Figure 10 might be due to slightly reduced fitness of cells because of the pkh26 labelling. **b** Statistics for the percentage of injected cells not having divided at all and having divided at least 10 times.

Due to the results described above and the observation that after intrathymic transfer the emerging Foxp3$^+$ cells have divided less than the other subsets we asked whether proliferation and differentiation are opposing processes within our system. We hypothesized that transferring a high number of cells would result in competition for both available growth factor and antigen binding sites, consequently reducing proliferation. To examine the effects of intraclonal competition in more detail, 2.5×10^6, 0.5×10^6, 1×10^5 and 0.2×10^5 pkh26 labelled cells were injected into AIRE-HA mice. Recipient mice were analyzed 5 days after intrathymic transfer. The more cells we injected the more were recovered, roughly reflecting the initial five-fold dilution series. But strikingly, there was a strong correlation between the number of injected cells and the percentage of Foxp3$^+$ cells, with hardly any Foxp3 expression following injection of 2×10^4 cells (Figure 13a).
Analysis of the pkh26 profile revealed that at a higher number of injected cells less proliferation took place (Figure 13b). Losing the pkh26 signal completely corresponds to at least 10 cell divisions according to the manufacturer, implying that these cells underwent extensive cycling. Furthermore, we observed again that CD25$^+$ Foxp3$^+$ cells were cycling less than the other subsets.
These results suggest a model in which proliferation and differentiation of Treg precursors are opposing processes and will be discussed in more detail later.

5.4 Developmental plasticity within CD4$^+$ SPs

5.4.1 Intrathymic injection of CD69$^+$ and CD69$^-$ CD4$^+$ SPs

Another issue we wanted to clarify is whether induction of natural Tregs can only happen at certain developmental stages, for example at the early CD4$^+$ SP stage. This hypothesis was based on a more detailed examination or AIRE-HA × TCR-HA mice showing that most of the TCR-HA$^+$ cells reside within the CD4$^+$ CD8^{int} compartment.
Thus we asked whether immature CD4$^+$ SPs have a higher capacity to develop into Foxp3$^+$ cells than more mature thymocytes. As expression of a transgenic TCR is known to slightly alter the thymocyte distribution in terms of maturity and percentage [55], we stained both WT and TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{gfp} animals for CD69 and CD24. In the CD4$^+$ SP compartment of TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{gfp} mice we observed biphasic expression of CD69, which was more pronounced than in the CD4$^+$ CD8$^-$ compartment of BALB/c animals. Costaining for CD24 showed that CD4$^+$ CD69$^-$ SPs expressed lower levels of CD24 than CD4$^+$ CD69$^+$ cells (Figure 14a), confirming that CD69$^+$ CD4$^+$ SPs can be regarded immature and CD69$^-$ CD4$^+$ SPs mature [6].
Intrathymic transfer of these two subsets into AIRE-HA and AIRE-HA × TCR-HA recipients revealed that a 2-3 fold higher percentage of CD69$^+$ than CD69$^-$ CD4$^+$ SPs developed into Treg (Figure 14b). In terms of recovered cell number we could not detect any significant differences.

Additionally, we injected peripheral $CD4^+$ cells, which also developed to $Foxp3^+$ cells in AIRE-HA recipients. This demonstrates that conversion to Treg is still possible even in this mature state of T cell development. Interestingly, in AIRE-HA × TCR-HA recipients we could rarely detect any of the injected peripheral cells (only 50-100 cells per mouse).

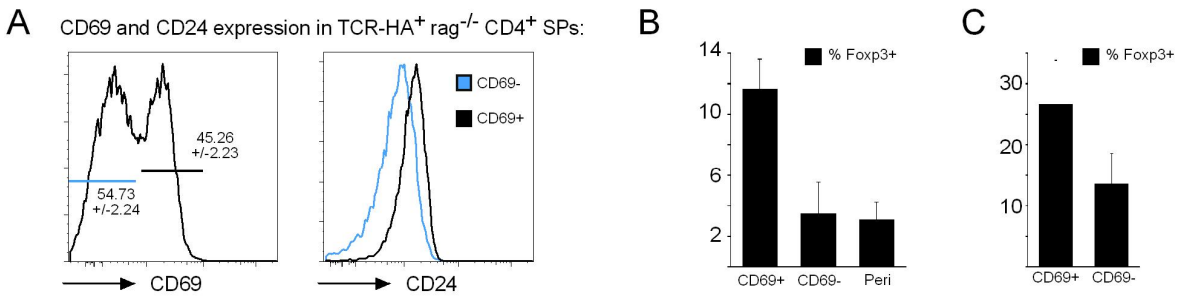

Figure 14: **Intrathymic transfer of naïve TCR-HA$^+$ RAG2$^{-/-}$ CD69$^+$ CD4$^+$ and CD69$^-$ CD4$^+$ SPs into AIRE-HA or AIRE-HA × TCR-HA recipients**
a Costaining for CD69 and CD24 of $CD4^+$ SPs in TCR-HA$^+$ RAG2$^{-/-}$ animals, showing that there are two populations in terms of CD69 expression, with lower CD24 levels in the $CD69^-$ population. **c** and **b** Intrathymic transfer of mature und immature $CD4^+$ SPs shows different efficacy of Treg induction. Percentages of $Foxp3^+$ cells after intrathymic transfer into AIRE-HA (b) and AIRE-HA × TCR-HA (c) recipients (n=3-4) is shown. Note that in AIRE-HA × TCR-HA recipients peripheral cells could not be detected after intrathymic transfer.

5.5 Role of antigen presentation by hematopoietic cells for Treg induction at the $CD4^+$ SP stage

5.5.1 Intrathymic Transfer and the influence of antigen expression levels

Although published for the steady state situation of AIRE-HA × TCR-HA mice [58] we wanted to definitely rule out that antigen presentation by hematopoietic cells is necessary for Treg induction at the $CD4^+$ SP stage. Due to the fact that on BALB/c background no MHC class II knockout mice are available and CIITA knockout mice still show some basal MHC class II expression [92], we had to use a different strategy involving bone marrow chimeras.

For this purpose AIRE-HA$^{+/-}$ F1 (BALB/c × C57Bl/6) mice were bred, which are supposed to express the MHC class II haplotypes I-E^d and I-A^b. Within these mice the amount of I-E^d molecules on the surface of antigen presenting cells is expected to decrease more than only 50% because the available MHC class II chains can pair with each other at unknown efficiency. To measure antigen presentation levels, we co-cultivated splenic HA-pulsed APCs from F1 (H-2b/d) and BALB/c (H-2d/d) animals with A5 hybridoma cells expressing the TCR-HA. TCR triggering of these hybridoma cells

leads to costimulation independent expression of a GFP reporter construct, thereby directly measuring the strength of TCR-signal. Analysis of GFP expression over a range of HA-peptide concentrations revealed a roughly 25-fold difference between BALB/c and F1 APCs (data not shown).

Subsequent intrathymic transfer experiments showed that in comparison to AIRE-HA mice on BALB/c background in AIRE-HA F1 recipients a 2-3 fold higher percentage of injected cells developed to Foxp3$^+$ Treg (Figure 15a). However, as the total number of recovered cells in AIRE-HA F1 recipients was lower, the absolute count of Foxp3$^+$ Treg was roughly equal in both AIRE-HA and AIRE-HA F1 recipients (Figure 15b). These observations indicate that the reduced amount of agonist ligand in F1 (H-2b/d) thymi affects proliferation of antigen-specific thymocytes, while differentiation to Treg as such remains intact.

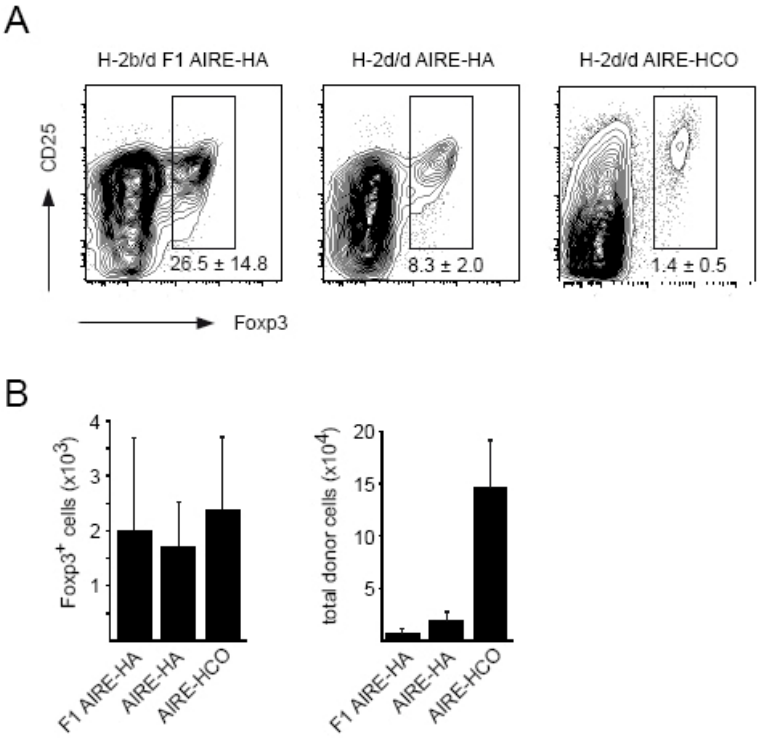

Figure 15: **Intrathymic transfer of naïve TCR-HA$^+$ RAG2$^{-/-}$ CD4$^+$ SPs into AIRE-HA F1 (BALB/c × C57Bl/6) recipients**

a Decreased levels of antigen presentation increase the relative rate of Treg induction. Representative plots of transferred cells (CD4$^+$ CD8$^-$ CD45.1$^+$) after five days are shown. Haplotype of MHC class II expression in the respective recipient animal is shown above dot plot.

b Absolute numbers of Foxp3$^+$ cells after intrathymic transfer into the indicated recipient animals (left plot), and absolute number of donor recovered cells (right plot). Note the significant increase for AIRE-HCO recipients.

To extend this hypothesis, intrathymic transfer of TCR-HA^{+} cells into AIRE-HCO recipients was performed. AIRE-HCO mice have been generated in our lab recently (see section 5.6) and express much higher levels of antigen than AIRE-HA mice, as quantified by stimulation of A5 hybridoma cells (data not shown). Analysis of injected cells revealed massive proliferation, with 1% of injected cells developing to Foxp3^{+} Treg (Figure 15a and b). These observations further emphasize the role of antigen level and will be discussed in more detail later.

5.5.2 FOXP3 INDUCTION AFTER INTRATHYMIC TRANSFER IS INDEPENDENT OF ANTIGEN PRESENTATION BY HEMATOPOIETIC CELLS

To address the initial question whether antigen presentation by hematopoietic cells plays a role for Treg induction at the CD4^{+} SP stage a series of bone marrow chimeras was generated using AIRE-HA F1 (BALB/c × C57Bl/6) animals. These mice were lethally irradiated and reconstituted with either BALB/c bone marrow (expressing I-A^{d} and I-E^{d} MHC class II molecules) or C57Bl/6 bone marrow (expressing only I-A^{b} class II molecules). As the TCR-HA recognizes hemagglutinin only in the context of I-E^{d} [93], in AIRE-HA F1 animals reconstituted with C57Bl/6 bone marrow (further on referred to as F1 AIRE-HA$^{BM\ H\text{-}2b/b}$) only radioresistant epithelial cells, but not hematopoietic cells were able to present the cognate antigen.

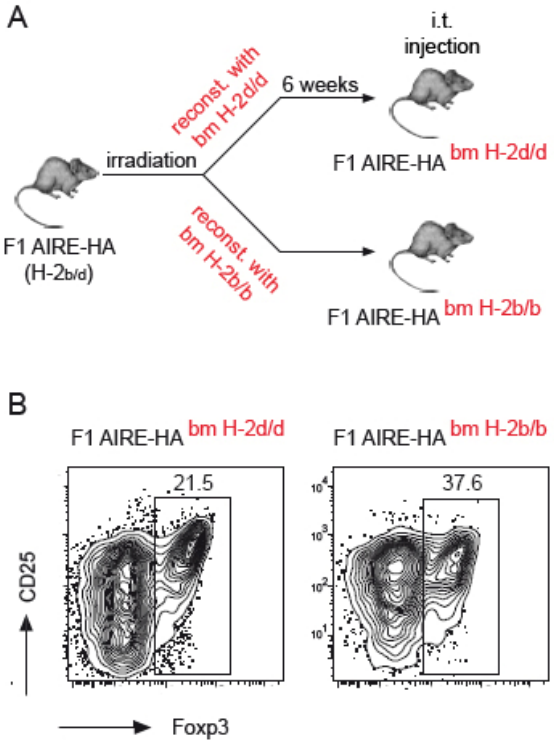

Figure 16: **Intrathymic transfer of naïve TCR-HA^{+} RAG2$^{-/-}$ CD4^{+} SPs into AIRE-HA F1 (BALB/c × C57Bl/6) bone marrow chimeras**
a Design of intratyhmic transfer experiments. Note that in F1 AIRE-HA$^{BM\ H\text{-}2b/b}$ animals the TCR-HA cannot recognize antigen on hematopoietic cells because of its restriction to I-E^{d}.
b Percentage of Foxp3^{+} cells after intrathymic transfer into the indicated bone marrow chimeras after five days. Note that absence of antigen presentation on haematopoietic cells does not disturb Treg induction. Representative plots for three recipient mice are shown.

Six weeks after reconstitution the bone marrow chimeras were intrathymically injected with 5×10^5 sorted $CD4^+$ SPs from TCR-HA^+ $RAG2^{-/-}$ $Foxp3^{gfp}$ $CD45.1^+$ mice and analyzed six days later. Development of $CD25^+$ $Foxp3^+$ cells was not disturbed, and as already noted for AIRE-HA F1 mice, both in F1 AIRE-$HA^{BM\ H\text{-}2b/b}$ and F1 AIRE-$HA^{BM\ H\text{-}2d/d}$ recipients 20-30% of injected cells developed to $Foxp3^+$ Treg (Figure 16b). Therefore, cross-presentation of antigen by hematopoietic cells is not essential for the conversion of $CD4^+$ SPs to Treg.

5.6 VALIDATION WITH ANOTHER TCR TRANSGENIC MODEL

To formally exclude any potential "bias" of the TCR-HA towards differentiation to $Foxp3^+$ Tregs we repeated some of the transfer experiments with the DO11.10 TCR transgenic system which recognizes amino acids 323-339 of Ovalbumin (OVA) in the context of $I\text{-}E^d$. As recipients we used AIRE-HCO mice recently generated in our lab, which express a fusion protein of hemagglutinin, C-reactive protein and ovalbumin under control of the Aire promotor. The phenotype of double transgenic AIRE-HCO × DO11.10 mice has been published previously [58].

Intrathymic transfer of $CD4^+$ SPs from $DO11.10^+$ $RAG2^{-/-}$ $Foxp3^{gfp}$ $CD45.1^+$ mice into AIRE-HCO recipients essentially phenocopied the results obtained for the TCR-HA. At early time-points upregulation of CD25 was observed, while after 3-4 days $Foxp3^+$ cells appeared (Figure 17), with around 10% $Foxp3^+$ cells after 8 days.

These results, in combination with the data shown so far, unequivocally show that naïve, antigen unexperienced $CD4^+$ SPs can develop into $CD25^+$ $Foxp3^+$ Tregs after encountering their cognate antigen within the thymic medulla.

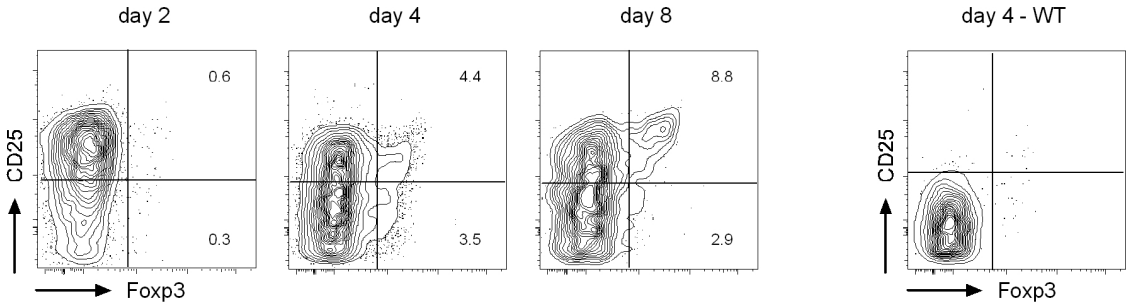

Figure 17: **Intrathymic transfer of naïve $DO11.10^+$ $CD4^+$ SPs into AIRE-HCO mice resembles the results obtained with the TCR-HA.**
Naive $CD4^+$ SPs from $DO11.10^+$ $RAG^{-/-}$ $Foxp3^{gfp}$ were transferred to AIRE-HCO recipients, contour plots are gated on transferred cells. Time-point after transfer is shown above, numbers in the plots indicate percentage of cells in the respective gate.

5.7 *IN VITRO* EXPERIMENTS

5.7.1 *IN VITRO* CULTURE OF TCR-HA$^+$ SUBSETS FROM AIRE-HA × TCR-HA MICE

The in vivo transfer experiments (section 5.2) together with the analysis of maturation and activation markers (section 5.1.2) suggest that there is a direct developmental relationship between CD25$^-$ Foxp3$^-$, CD25$^+$ Foxp3$^-$ and CD25$^+$ Foxp3$^+$ cells in double transgenic AIRE-HA × TCR-HA mice. To more precisely address the role of interacting cell types and the required stimuli at each developmental step, *in vitro* experiments were done. As a first approach, the same TCR-HA$^+$ subsets as in our *in vivo* experiments were sorted from AIRE-HA × TCR-HA mice (CD25$^-$ Foxp3$^-$ GITR$^-$, CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells). For control purposes naïve CD4$^+$ SPs from TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{GFP} mice were included.

After cultivation under various conditions, several remarkable observations were made. Most interestingly, stimulation of CD25$^+$ Foxp3$^-$ cells without any further TCR-stimulus but only with IL-2 resulted in expression of Foxp3 by 44.7% of cells after three days. This effect was seen to a lesser extent for CD25$^-$ Foxp3$^-$ GITR$^+$ cells (30.5%), but not for CD25$^-$ Foxp3$^-$ GITR$^-$ (4.3%) and naïve TCR-HA$^+$ RAG2$^{-/-}$ cells (Figure 18a).

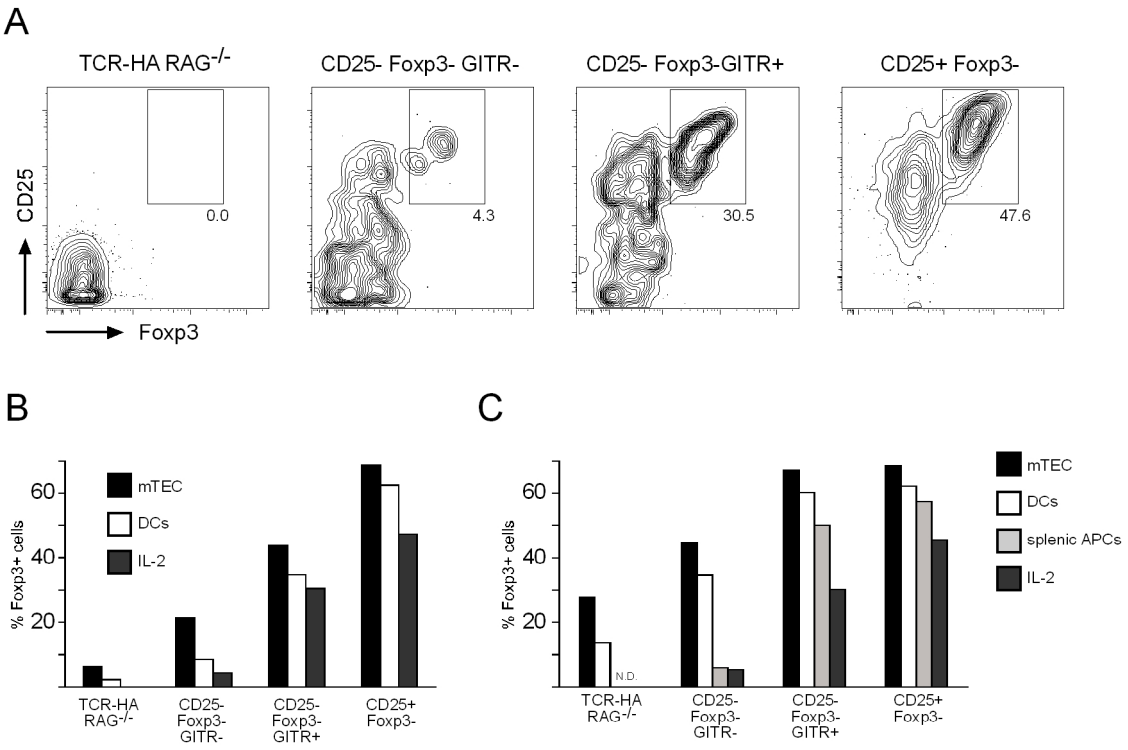

Figure 18: **TCR-independent development of CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ to Foxp3$^+$ Treg**
a Flow cytometric analysis of naïve CD4$^+$ SPs from TCR-HA RAG2$^{-/-}$ mice or sorted subsets from AIRE-HA × TCR-HA mice after three days of culture in the presence of 100 Units/ml IL-2. **b** Statistical analysis of Foxp3 expression after cultivation of the indicated thymocyte subsets for three days with AIRE-HA mTEC and IL-2 (black bars), AIRE-HA DCs and IL-2 (open bars) and IL-2 alone (dark grey bars).
c Statistical analysis of Foxp3 expression after five days. AIRE-HA mTECs and IL-2 (black bars), AIRE-HA DCs and IL-2 (open bars), splenic APCs with 5 µg/ml HA-peptide and IL-2 (light grey bars), IL-2 alone (dark grey bars). Note that for CD25$^-$ Foxp3$^-$ GITR$^-$ cells Foxp3$^+$ expression is hardly induced by splenic APCs, which is not the case for CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ cells. (N.D. not done)

Therefore, the CD25$^+$ Foxp3$^-$ subset contains a high proportion of direct precursors for mature Foxp3$^+$ Treg. Very recently, similar experiments with polyclonal cells have been done by another group, allowing the same conclusion [94] which emphasizes that our data indeed mimics the polyclonal system. However, our experiments provide additional insights into these previously uncharacterized processes preceding Foxp3 induction.

Both the CD25$^+$ Foxp3$^-$ and the CD25$^-$ Foxp3$^-$ GITR$^+$ subsets contain cells which continue to develop to Foxp3 only in the presence of IL-2, but in either case the presence of a TCR stimulus enhanced this process (Figure 18b and c) up to twofold. Strikingly, coculture with AIRE-HA mTECs or thymic AIRE-HA DCs allowed Foxp3 induction in CD25$^-$ Foxp3$^-$ GITR$^-$ cells, which was only very inefficiently the case for irradiated splenic APCs pulsed with 5 µg/ml HA-peptide. As in this experiment no titration of the agonist-peptide was performed, for the splenic APCs dose-dependent effects can not be ruled out, but still one could hypothesize that for the initial step of the Treg developmental program the interacting cell type is crucial, while after that TCR stimuli provided by any APC drive Foxp3 induction.

Of note, under the experimental conditions used there seems to be an upper limit for the conversion rate of CD25$^+$ Foxp3$^-$ cells to Foxp3$^+$ Treg, because in the presence of mTECs the percentage of Foxp3$^+$ cells on day 3 equals the result after 5 days. The same percentage of conversion, roughly 60%, was reached by CD25$^-$ Foxp3$^-$ GITR$^+$ cells after 5 days as well. However, one cannot excluded that under different conditions, for example an higher ratio of mTECs to thymocytes, all CD25$^-$ Foxp3$^+$ would have the ability to upregulate Foxp3.

As an additional interesting facet, CD25$^-$ Foxp3$^-$ GITR$^-$ cells gave rise to more Foxp3$^+$ cells than naïve CD4$^+$ SPs from TCR-HA$^+$ RAG2$^{-/-}$ mice (Figure 18c), which suggests that the former population does not represent truly naïve thymocytes, but might include already activated cells.

In conclusion, these results substantiate our *in vivo* experiments and argue for sequential developmental steps in the order CD25$^-$ Foxp3$^-$ GITR$^-$ → CD25$^-$ Foxp3$^-$ GITR$^+$ → CD25$^+$ Foxp3$^-$ → mature CD25$^+$ Foxp3$^+$ Treg, which will be elaborated in more detail in the discussion.

5.7.2 *IN VITRO* CULTURE OF NAÏVE TCR-HA$^+$ CD4$^+$ SPS WITH MTECS AND DCS

Studying the efficiency of Treg induction after intrathymic transfer is complicated by the fact that thymic egress cannot be controlled. Furthermore, elucidating mechanistic details is difficult as the thymic microenvironment after injection is unknown. Therefore, we asked whether it would be possible to mimic the *in vivo* situation of natural Treg induction by simply cultivating naïve CD4$^+$ thymocytes with either mTECs or thymic DCs *in vitro*. This would allow following the contribution of the antigen presenting cells

in a defined microenvironment, in which for example individual cytokines can be neutralized or exogenously added.

In a first approach we sorted mTECs (defined as CD45$^-$ Ly51$^-$ EpCAM$^+$) from AIRE-HA mice and cultivated them with CD4$^+$ SPs from TCR-HA$^+$ RAG2$^{-/-}$ Foxp3^{gfp} mice in the presence of IL-2 as a survival factor. Indeed, mTECs were capable to convert CD4$^+$ SPs to CD25$^+$ Foxp3$^+$ regulatory T cells after 5 days of *in vitro* culture (Figure 19a). Analysis of the kinetics of this process roughly equalled our *in vivo* results (data not shown).

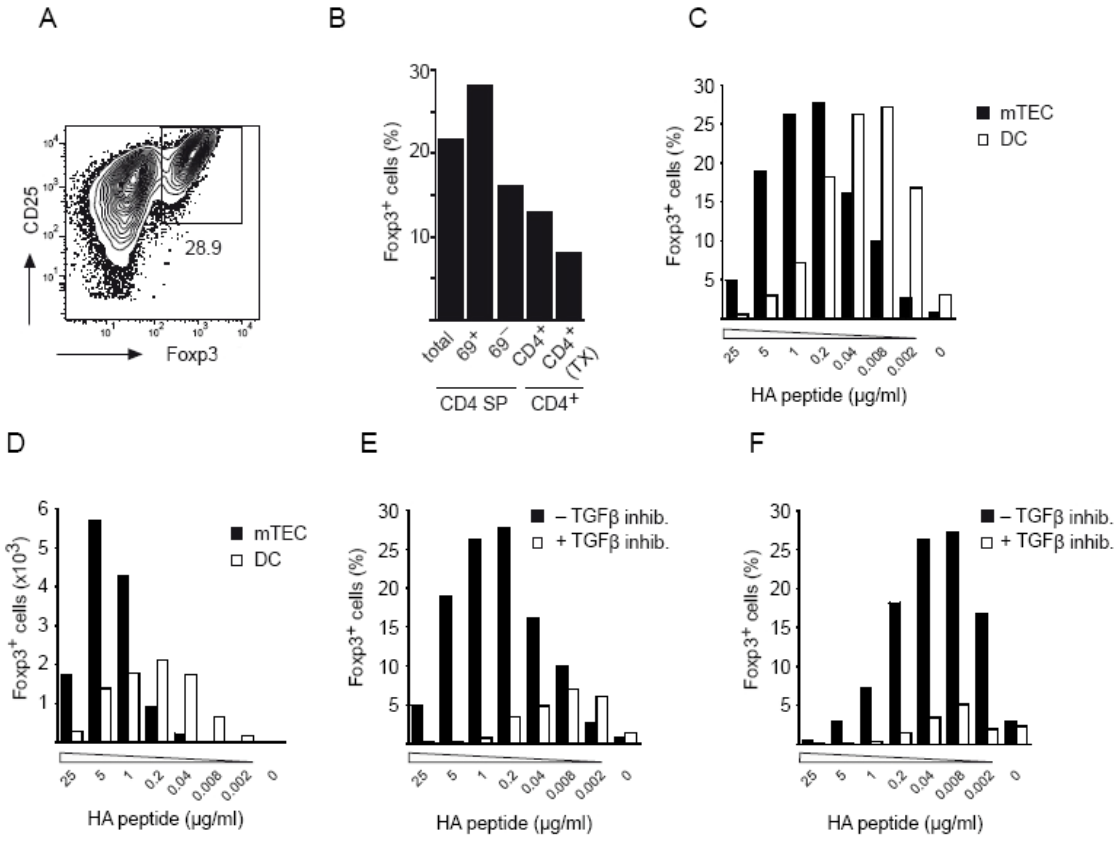

Figure 19: **mTEC and thymic DC can support differentiation of Treg *in vitro***

a CD4$^+$ SPs were cultivated with AIRE-HA mTECs for five days. One representative plot of three independent experiments is shown. Percentage of Foxp3$^+$ cells is indicated below gate. **b** Indicated T cell subsets were cultivated with AIRE-HA mTECs for five days. Note the correlation with our *in vivo* results (Figure 14) in that the most immature CD4$^+$ SPs yield the highest percentage of Foxp3$^+$ cells. CD4$^+$ T(x), peripheral CD4$^+$ T cells from a thymectomized donor mouse. **c** and **d** Percentage (c) and absolute numbers of Foxp3$^+$ cells (d) after 5 days of in vitro culture of CD4$^+$ SPs with WT mTECs and DCs at the indicated concentration of HA-peptide. **e** and **f** Percentage of Foxp3$^+$ cells with or without TGF-β inhibition (monclonal anti-TGF-β antibody and the inhibitor SB-431542) after five days of *in vitro* culture of CD4$^+$ SPs with WT mTECs (e) or WT DCs (f) and the indicated concentration of HA-peptide. Note the significant decrease in the percentage of Foxp3$^+$ cells for both APC types, but the dependence on the concentration of agonist peptide.

We asked next whether our *in vivo* results showing an inverse relationship between T cell maturity and their ability to differentiate to Treg could be reproduced *in vitro*. For this purpose, we sorted $CD69^+$ $CD4^+$ SPs, $CD69^-$ $CD4^+$ SPs and peripheral $CD4^+$ T cells from TCR-HA^+ $RAG2^{-/-}$ $Foxp3^{gfp}$ mice. After cultivation for 5 days with AIRE-HA mTECs, $CD69^+$ $CD4^+$ SPs yielded the highest percentage of $Foxp3^+$ Treg, while both $CD4^+$ $CD69^-$ thymocytes and peripheral $CD4^+$ T cells were less competent (Figure 19b). Furthermore, we used peripheral $CD4^+$ T cells from thymectomized donor mice, thereby excluding the presence of recent thymic emigrants in our starting population. In line with our hypothesis that during progressive maturation T cells lose their plasticity to develop to Treg, peripheral T cells from thymectomized mice showed even lower efficacy for Treg conversion.

To gain additional insight into the relevance of both the antigen presenting cell type and the antigen level, co-culture of WT mTECs ($CD45^-$ $Ly51^-$ $EpCAM^+$) and thymic WT DCs (defined as $CD45^+$ $CD11c^+$) with $CD4^+$ SPs in the presence of titrated amounts of HA-peptide was performed. Unexpectedly, both mTECs and DCs proved to induce Foxp3 expression efficiently, but at markedly different antigen concentrations (Figure 19c). While mTECs yielded 20-30% of $Foxp3^+$ cells at peptide concentrations higher than 0.2 µg/ml, thymic DCs did so at concentrations below 0.2 µg/ml. Interestingly, while the maximum percentage of $Foxp3^+$ cells was similar for both APC types, in terms of absolute numbers, mTECs were superior to DCs in their ability for Treg induction (Figure 19d).

5.7.3 TGF-β DEPENDENCE *IN VITRO*

As mentioned in the introduction, it was widely accepted during the last years that transforming growth factor-β (TGF-β) is only involved in the peripheral generation of Treg, but not in their thymic development. A recent publication challenged this widespread notion [63].

Therefore we sought to determine the importance of (TGF-β) in our *in vitro* system. For this purpose we used both an neutralizing TGF-β antibody and an inhibitor of TGF-β signaling, namely SB431542 [80]. The latter compound works by inhibiting Activin-receptor like kinases (ALK) 4, 5 and 7, whereby ALK-5 is the TGF-β receptor 1 which is required for the functional TGF-β receptor complex. Prior titration has shown that the neutralizing antibody was efficient at 10 µg/ml and SB-431542 at a concentration of 3 µM (data not shown).

Inhibiting TGF-β resulted in a slight increase of the total cell numbers in each well, which is due to the known antiproliferative effects of TGF-β [95]. Beside that, TGF-β inhibition had a strong effect on both mTEC- and DC-mediated Treg induction (Figure 19e and f, respectively). Of note, inhibition was not complete especially for mTECs at lower peptide concentrations, while for DCs at all concentrations the reduction in $Foxp3^+$ cells was at least 5-fold.

6 DISCUSSION

6.1 PRECURSOR-PROGENY RELATIONSHIP BETWEEN CD25$^+$FOXP3$^-$ THYMOCYTES AND CD25$^+$FOXP3$^+$ REGULATORY T CELLS

Regulatory T cells (Treg) are indispensable for prevention of autoimmune disease, but still the processes underlying their thymic development remain enigmatic. By using a T cell receptor (TCR) transgenic system which allows tracking the fate of single T cells with known TCR specificity, we identified CD25$^+$ Foxp3$^-$ thymocytes as direct precursors of mature CD25$^+$ Foxp3$^+$ Treg.

6.1.1 CD25$^+$ FOXP3$^-$ THYMOCYTES CONTINUE TO DEVELOP TO CD25$^+$ FOXP3$^+$ TREG BOTH *IN VITRO* AND *IN VIVO*

After intrathymic transfer of the sorted TCR-HA$^+$ subsets found in the CD4$^+$ SP compartment of AIRE-HA × TCR-HA mice into AIRE-HA recipients we observed that both GITR$^-$ and GITR$^+$ CD25$^-$ Foxp3$^-$ as well as CD25$^+$ Foxp3$^-$ cells gave rise to Foxp3$^+$ Treg, but the latter at the highest frequency. Thus, *in vivo* after encountering their cognate antigen all these thymocyte subsets are able to express Foxp3, but the CD25$^+$ Foxp3$^-$ subset is enriched for cells doing so. Importantly, after transfer into WT mice CD25$^+$ Foxp3$^-$ and GITR$^+$ CD25$^-$ Foxp3$^-$ cells developed to Foxp3$^+$ cells as well, albeit at a lower frequency than in AIRE-HA mice.

In vitro, we could observe that in the presence of IL-2, cultivation of TCR-HA$^+$ CD25$^-$ Foxp3$^-$ GITR$^+$ and CD25$^+$ Foxp3$^-$ subsets from AIRE-HA × TCR-HA mice led to Foxp3 expression in nearly 50% of these cells. The same tendency, albeit to a lesser extent was monitored after cultivation with IL-15 (data not shown).

Collectively, these findings prove unambiguously that CD25$^+$ Foxp3$^-$ thymocytes do not represent a developmental dead end, but instead contain direct precursors of Foxp3$^+$ Treg. Cells at the CD25$^+$ Foxp3$^-$ and CD25$^-$ Foxp3$^-$ GITR$^+$ stage do not need any further TCR stimulus, but just cytokine signalling to upregulate Foxp3 as shown by our *in vitro* experiments. This conclusion is underscored by our *in vivo* transfer experiments, where in WT recipients a fraction of injected TCR transgenic CD25$^+$ Foxp3$^-$ and CD25$^-$ Foxp3$^-$ GITR$^+$, but not GITR$^-$ cells are able to upregulate Foxp3. However, the frequency of developing Foxp3$^+$ Treg was higher *in vivo* after intrathymic transfer into AIRE-HA recipients and *in vitro* in the presence of antigen presenting cells. These observations emphasize that a microenvironment providing TCR stimulus and appropriate cytokine milieu is the most efficient differentiation environment.

Our data is partially mirrored by a very recent publication showing for the polyclonal system that CD25$^+$ Foxp3$^-$ thymocytes are Treg precursor cells [94]. The authors propose a “two-step process”, with TCR-stimuli conferring cytokine responsiveness to developing thymocytes, which then undergo cytokine dependent Foxp3 upregulation.

Our finding that the presence of antigen increases differentiation efficiency argues rather for an integration of both TCR and cytokine signals at all developmental stages.
Given the fact that *in vitro* nearly 50% of $CD25^+$ $Foxp3^-$ cells are capable of Foxp3 upregulation, while *in vivo* even in the presence of antigen only around 20% do so, a bottleneck restraining the development of these cells must exist. Several potential explanations could be envisioned, namely the limited availability of cytokines or other soluble factors, or the limited accessibility of rare antigen presenting cells such as mTECs.
Of note, *in vivo* around one third of transferred $CD25^+$ $Foxp3^-$ cells lost expression of CD25 again. As the percentage of these cells roughly equals the difference between our observed *in vitro* and *in vivo* conversion rates, it could be imagined that these $CD25^-$ cells represent the "outcompeted" population and will be cleared by negative selection.

6.2 Regulatory T cell induction at the $CD4^+$ SP stage

To follow the fate of truly naive $CD4^+$ SPs after encounter of cognate antigen in the thymus, intrathymic transfer experiments were performed. In contrast to work arguing for Treg induction at the DP stage [76, 96] we could show for two different TCR transgenic systems that in the presence of agonist ligand development to $Foxp3^+$ Treg at the $CD4^+$ SP stage is possible. Although this does not exclude that events prior to the $CD4^+$ SP stage initiate Treg development in some cells [90], our observations definitely show that Treg induction can happen completely dissociated from lineage commitment and positive selection.

6.2.1 Development of $Foxp3^+$ Treg after intrathymic transfer

Intrathymic transfer of TCR transgenic $CD4^+$ SPs yielded efficient development to Treg only in antigen expressing recipients, but not in WT mice, where all of the transferred cells stayed phenotypically naïve. This emphasizes the role of TCR interactions with peptide-MHC class II complexes, but does not formally exclude a stochastic model of Treg development as proposed by the Mathis group [59]. However, if stochastic priming of the Treg program at earlier developmental stages occurs, for example in the cortex, it is not sufficient to induce Foxp3 expression without additional agonist ligand signals.
By analyzing recipient mice each day after transfer we could delineate a series of events preceding Foxp3 induction. At early timepoints rapid upregulation of CD25 and GITR was observed, while the functional importance of these events remains unclear. CD25 expression has already been shown to be Foxp3 independent [51], and is not likely to fulfill a crucial role in terms of IL-2 signaling, as our lab [68] and others [67]

have shown that IL-2 is not essential for thymic Treg development. However, given that *in vitro* $CD25^+$ $Foxp3^-$ cells continue to develop to $Foxp3^+$ Treg in the presence of IL-2 as well as IL-15, cytokine responsiveness to common γ-chain cytokines might be an important criterion at this stage. This hypothesis is emphasized by the fact that both IL-2Rβ knockout mice [97] and IL-2/IL-15 double knockout mice [70] lack $Foxp3^+$ Treg. Since in the polyclonal system IL-2 deficiency results in a slight decrease in Treg numbers, there might be some redundancy, with a certain level of cytokine signaling being critical instead of a single cytokine. Support for this idea is provided by the fact that a $CD4^+$ T cell specific deletion of STAT5, which is required to transduce signals from the common γ-chain cytokine receptor, strongly reduces the number of mature $Foxp3^+$ Treg [70], while a constitutively active form of STAT5 is sufficient to drive Treg induction [78].

On day two and three after intrathymic transfer, a fraction of $CD25^+$ $Foxp3^-$ cells lose CD25 expression again. The first $Foxp3^+$ cells appear at day three, which tend to express only intermediate levels of CD25. Until day 5, the number of $CD25^+$ $Foxp3^+$ cells increases steadily, accompanied by upregulation of CD25 within this population, as expected because of CD25 being a direct target gene of Foxp3 [49]. Interestingly, at the same time the percentage of $CD25^{int}Foxp3^+$ cells remain roughly constant. Although one cannot exclude that these cells leave the thymus as $CD25^-$ $Foxp3^+$ cells, the kinetics of their appearance suggest that this $CD25^{int}$ $Foxp3^+$ population represents an intermediate developmental step.

6.2.2 Proliferation of TCR-HA^+ $CD4^+$ SP after intrathymic transfer

After intrathymic transfer into AIRE-HA recipients extensive proliferation both within the $CD25^+$ $Foxp3^-$ and $CD25^-$ $Foxp3^-$ subset was observed. Proliferation of $CD4^+$ SPs has already been shown earlier [98], but the expansion of autoreactive T cells appears to be counterproductive at first sight. Even more complex, titration experiments and intrathymic transfer into AIRE-HA × TCR-HA recipients suggest that within the Treg precursor pool proliferation and differentiation are counteracting processes.

However, given that not all $CD25^+$ $Foxp3^-$ are capable of developing to $Foxp3^+$ Treg both *in vivo* and *in vitro* one could envision a process in which the precursor pool expands to ensure development of a reasonable number of Treg. This idea is substantiated by the fact that in the steady state also polyclonal $CD25^+$ $Foxp3^-$ thymocytes show more proliferation than conventional $CD25^-$ cells (according to KI67 staining, data not shown), which argues against proliferation being an artifical phenomenon of TCR transgenic cells. Nevertheless, in WT mice only 1-3% of mTECs are thought to present one specific tissue restricted antigen [15], while in AIRE-HA mice HA is probably expressed by the majority of mTECs. The observed proliferative burst might be due to the abundance of HA, while for sparse antigens only modest proliferation might result.

Another reasonable explanation for the observed cell cycling of Treg precursors might be the requirement for demethylation of the Foxp3 locus. It has been suggested recently that demethylation of CpG islets in a conserved region within the Foxp3 locus is crucial for sustaining Foxp3 expression [99], but it is not clear whether this is brought about by active demethylation processes. Thus, precursor cells committing to the Treg lineage might undergo a limited number of divisions accompanied by a lack of *de novo* methylation.

An important issue is the long-term fate of the intrathymically transferred cells, as in the thymi of AIRE-HA recipients even after 14 days still 1000-3000 injected cells could be detected. However, it is known that on average $CD4^+$ SPs spend 4-7 days in the medulla [91], which equals our observations after intrathymic transfer into WT mice. The prolonged persistence of TCR-HA^+ cells in AIRE-HA mice might be due to upregulation of adhesion molecules following antigen encounter, as has been suggested earlier [100]. Since cells persisting after 14 days were mostly $CD25^-$ $Foxp3^-$ (data not shown), development along the Treg lineage does not occur for all TCR-HA^+ cells, which leaves two possibilities: either they are deleted or escape to the periphery as autoreactive T cells, where they would be kept in check by simultaneously generated Treg. Evidently, we could not address this issue by analyzing the periphery of intrathymically injected mice because during the operation *per se* a certain number of naïve injected cells will already enter the bloodstream.

However, both options might be true because in the thymus of AIRE-HA × TCR-HA a significant fraction of TCR-HA^+ cells is deleted, while in the lymph nodes of these mice still TCR-HA^+ cells exist which do not express $CD25^+$ [58]. Furthermore, it has already been shown that within the polyclonal repertoire one TCR specificity can be found both within the Treg and the conventional T cell population, suggesting that Treg induction and escape of autoreactive T cells can indeed happen side by side [44].

6.2.3 Effect of antigen presentation levels

After initial interpretations of Treg development hypothesizing that TCR affinity dictates the decision between negative selection and Treg induction it has been appreciated that this view is too simplistic [101]. However, within our intrathymic transfer system we found not the TCR affinity but the antigen level to influence both proliferation and differentiation of TCR-HA^+ $CD4^+$ SPs in a delicate way.

A 25-fold reduction in antigen presentation levels led both to enhanced conversion and reduced proliferation. In line with these observations, intrathymic transfer of TCR-HA^+ $CD4^+$ SPs into AIRE-HCO mice (which express higher antigen levels than AIRE-HA mice) led to enormous proliferation of the transferred cells, with 1% of cells giving rise to $Foxp3^+$ Treg. Importantly, in the steady state situation in AIRE-HCO × TCR-HA mice TCR-HA^+ cells are deleted (data not shown). Putting these observations together, the

antigen level might not dictate the decision without other factors, but still influences the efficiency of Treg generation in an important way.

6.2.4 DEVELOPMENTAL PLASTICITY

Does Treg development require a specialized niche within the thymus which favors Foxp3 induction, or can Treg commitment only happen at a certain stage of thymocyte development? Several observations suggested convincingly that the thymic medulla provides such a niche. The autoimmune phenotype of TRAF6 (tumor necrosis factor receptor-associated factor 6) knockout mice is due to improper organization of the thymic medulla [102], and the absence of $Foxp3^+$ Treg in neonatal mice has been ascribed to the absence of fully developed medullary islands [75].

However, our experimental data show that the developmental stage matters as well. After intrathymic transfer immature $CD69^+$ $CD4^+$ SPs gave rise to 3-4 fold more $Foxp3^+$ Treg than $CD69^-$ $CD4^+$ SPs. Since both subsets are expected to be localized in medullary regions, this implies that immature $CD4^+$ SPs are more likely to differentiate to Treg after encountering peptide-MHC class II complexes. This observation supports a model in which the thymic medulla indeed plays the major role in Treg induction, but the likelihood of Treg development is influenced by the maturation stage of the $CD4^+$ SP, i.e. cells just entering medullary regions have a higher capacity to develop along the Treg lineage.

6.2.5 RECEPTOR SPECIFICITY, AFFINITY AND TCR SIGNALING

Several studies have suggested that the Treg population contains a high frequency of self-reactive TCRs [44], which favors an agonist ligand-model of Treg induction. The notion of Treg TCRs being self-reactive was challenged by a recent publication arguing that a significant fraction of the Treg TCR repertoire recognizes non-self antigen [103]. This is not at odds with an agonist ligand model of thymic Treg development because peripheral mechanism might contribute to the Treg pool as has been demonstrated very elegantly by the von Boehmer lab [104].

However, the authors also claimed that several analyzed TCRs were found both in the Treg and conventional T cell population [103]. This would actually support a model whereby the same thymocyte expressing a certain TCR can differentiate either along the Treg lineage or escape to the periphery.

In this context it is important to discuss that the affinity of a given TCR for a certain peptide MHC-class complex is dependent on downstream signaling events and changes during development. For example, in double positive thymocytes, peptides which do not activate mature peripheral T cell are sufficient to induce negative selection [105], and antagonist peptides that normally inhibit effector T cells are sufficient to induce positive selection [106]. Although the latter study focused on

$CD8^+$ T cell positive selection, one could envision that $CD4^+$ SPs just after transition from the DP stage still have an enhanced ability to respond to low affinity antigen. This would allow agonist-ligand induced Treg induction, but the same TCR-peptide interaction would not drive peripheral proliferation after cloning into hybridomas, as done by Pacholcyzk et al. in the study mentioned above [103].
That Treg specific alterations of TCR signaling are important for their development has been suggested for a mutation in the adaptor molecule LAT (linker of activated T cells), but recent studies have cast doubt on this hypothesis [73]. However, costimulation via CD28 is known to be important for thymic Treg development and might influence TCR signaling as well [57].

6.2.6 Integrating experimental results and current knowledge into a model

Concerning the known literature and our own findings a model of agonist induced thymic Treg differentiation could be imagined as follows (Figure 20).
After transition from the double positive to the single positive compartment, immature $CD4^+$ SPs might still have an elevated sensitivity to react towards peptide-MHC class II complexes. Following antigen encounter (either on mTECs or DCs) the cells are either subject to clonal deletion, or Treg development is initiated, with the first steps being rapid upregulation of both CD25 and GITR and transcription of Foxp3 mRNA. Note that so far unknown signaling factors are likely to be involved at this step.
Depending on the number of antigen presenting cells and the antigen level proliferation is initiated, which might be strong in the case of ubiquitous thymic self antigen, but only moderate for rare tissue restricted antigens. As mentioned above, proliferation might be necessary for demethylation of CpG motifs in the Foxp3 locus, which is crucial for sustained Foxp3 expression.
After the initial activation step, some cells lose CD25 expression again, possibly depending on the contact to antigen presenting cells. Due to reduced expression of cytokine receptors for common gamma γ-chain cytokines (as shown by qPCR for IL2-Rβ and IL7-Rα) cells at the $CD25^{+/int}$ $Foxp3^-$ stage require increased amounts of cytokines for their survival and continued differentiation. Through a combination of TCR- and cytokine-derived signals, $CD25^{int}$ cells express Foxp3 at protein level. This critical step might depend on other so far unknown factors, which could be provided by a hypothetical Treg niche within the thymic medulla, possibly involving surface molecules or secreted proteins provided by mTECs. In case a certain signaling threshold is reached, cells continue to develop to mature $Foxp3^+$ Treg.
For cells that continue to interact with antigen presenting cells, but not receive additional signals, proliferation might continue, while cells that have become activated, but do not receive appropriate survival signals, undergo apoptosis. Some cells might escape as autoreactive conventional T cells, but will be kept in check by Treg, which is

in line with the above mentioned study claiming that 40% of Treg TCRs are also found on conventional T cells.

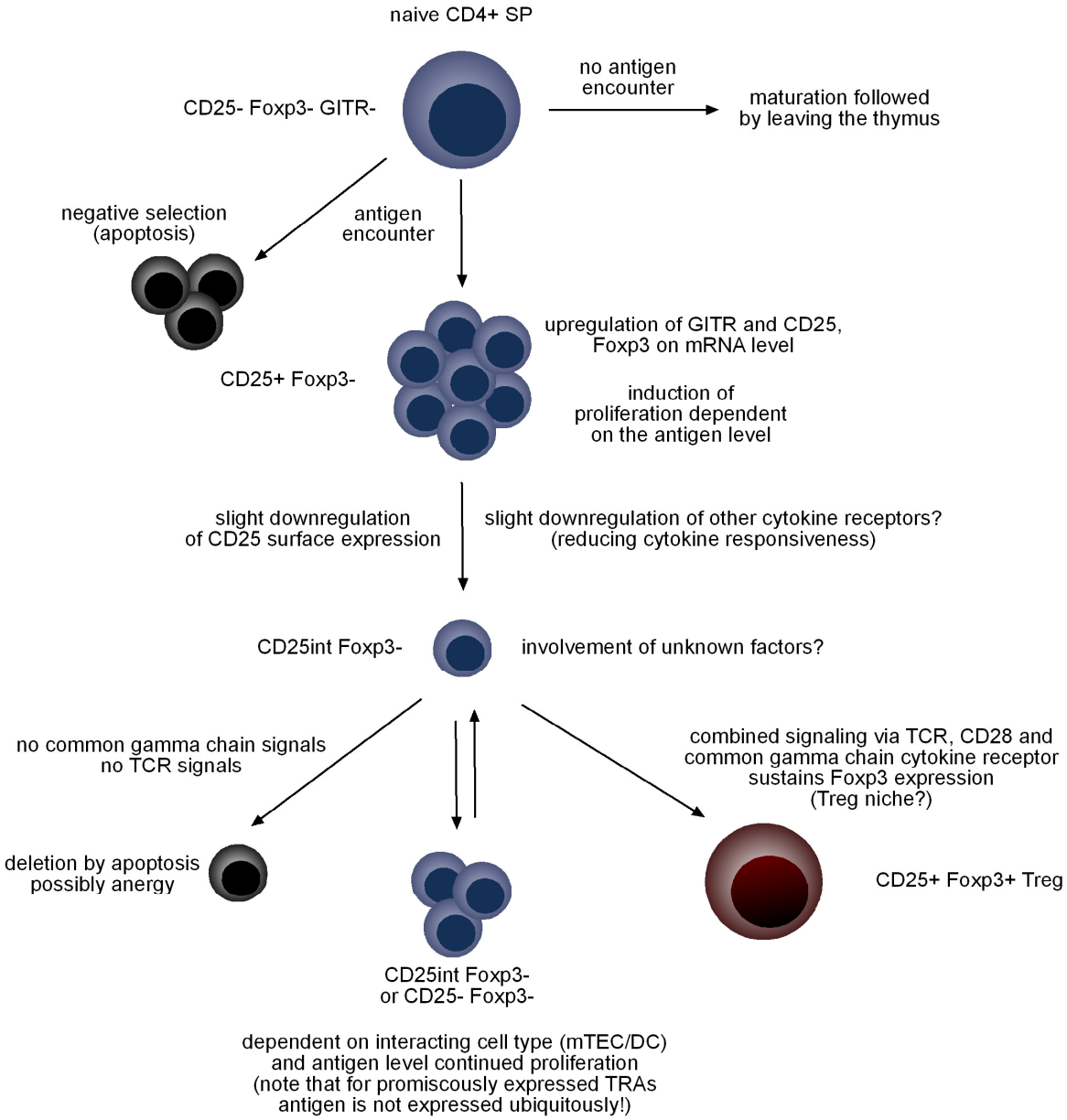

Figure 20: **Model for agonist induced Treg development**
For detailed discussion, see main text. Note that some steps have not been directly shown, but can be proposed according to our own and published findings. Further experimentation is necessary to delineate the detailed mechanistics.

6.3 *IN VITRO* CONVERSION AND TGF-β DEPENDENCE

Understanding thymic Treg development would be facilitated if the *in vivo* process could be mimicked *in vitro*. We could show by cocultivation of *ex vivo* isolated thymic APCs with naïve $CD4^+$ SPs, that both mTECs and thymic DCs are capable of $Foxp3^+$ induction *in vitro*.

Previous *in vivo* experiments in our lab have shown that antigen presentation by mTECs is sufficient for driving Treg development [58]. In contrast, other reports suggested the involvement of different stromal cell types [107, 96]. In the light of our *in vitro* observations there might be some redundancy between different thymic APCs in their principal capacity of supporting Treg induction, offering an explanation for these conflicting reports.

Importantly, the efficiency of Treg induction depended on the concentration of the agonist peptide. As we have shown earlier, mTECs and DCs do not markedly differ in the strength of delivered "signal one" [58], suggesting that additional factors such as unknown costimulatory molecules might be involved.

Concerning the involvement of TGF-β, our results support the recently published hypothesis that TGF-β serves a more elemental role in Treg development than initially thought. However, stimulation of T cells by splenic APCs or anti-CD3/CD28 antibodies has not been reported to induce Treg in the absence of TGF-β, while mTECs and to some extend thymic DCs were capable of driving Foxp3 induction even after inhibition of TGF-β signaling. Therefore, there might be considerable overlap in the mechanistic aspects of peripheral and thymic Treg development, but both thymic APC types might be equipped with particular abilities for supporting Treg development.

To elucidate the involvement of previously unknown factors such as soluble or membrane bound molecules, our *in vitro* model of Treg induction could provide a suitable defined experimental system.

6.4 CONCLUSION AND FUTURE PERSPECTIVES

Our experimental results unravelled several steps in the agonist driven development of naïve $CD4^+$ SP to $Foxp3^+$ Treg and identified $CD25^+$ $Foxp3^-$ $CD4^+$ SPs as direct precursors of mature $Foxp3^+$ Treg cells.

However, a number of questions remain to be clarified, in particular how the decision is made whether a developing T cell is deleted or deviated into the Treg lineage. According to our model a combination of TCR- and cytokine-derived signals allow Treg induction, with the developmental stage being an additional crucial factor.

Gene expression profiling of the TCR-HA^+ subsets found in AIRE-HA × TCR-HA mice could provide insight into the T cell side of this process, revealing which transcription factors are upregulated after antigen exposure, but prior to Foxp3 expression.

Additional *in vivo* transfer studies using mice deficient in factors known to be involved could show which signals are required at which developmental stage. Using this experimental approach the role of CD28 costimulation is currently investigated in our lab (M. Hinterberger, personal communication).
The most promising approach for studying the importance of single factors are *in vitro* studies. Using for example neutralizing antibodies to both soluble factors and surface molecules one could study the required cytokine milieu within a defined system.

7 ABBREVIATIONS

A summary of used abbreviations (in alphabetical order) is given below.

AIRE	autoimmune regulator
ALK	activin-receptor like kinases
APC	antigen presenting cell
BM	bone marrow
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	carboxy-fluorescein diacetate succinimidyl ester
DC	dendritic cell
DN	double negative
DP	double positive
FACS	flourescence activated cell sorter
FCS	fetal calf serum
Foxp3	forkhead box P3
GITR	glucocorticoid induced tumor necrosis factor receptor
HA	hemagglutinin
ICOS	inducible T cell costimulator
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, X-linked
IT	intrathymic
JAK	janus kinase
LN	lymph node
MHC	major histocompatibility complex
mTEC	medullary thymic epithelial cell
NFAT	nuclear factor of activated T cells
NFkB	nuclear factor kappa b
OVA	ovalbumin
PCR	polymerase chain reaction
PD1	programmed death 1
RAG	recombination activating gene
RTE	recent thymic emigrants
SOCS	suppressor of cytokine signaling
SP	single positive
STAT	signal transducer of activated T cells
TCR	T cell receptor
TGF	transforming growth factor
Treg	regulatory T cell

8 LITERATURE

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10 CURRICULUM VITAE

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