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DISSERTATION

Titel der Dissertation

The role of thymic antigen presenting cells and T cell intrinsic factors in the development of Foxp3^{+} regulatory T cells

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1. Summary

The majority of regulatory T cells (T_{regs}) are believed to be of thymic origin. It has been hypothesized that this may result from unique intrathymic environmental cues, possibly requiring a dedicated antigen presenting cell (APC). However, T cell intrinsic developmental regulation of the susceptibility to T_{reg} differentiation remains a mutually non-exclusive scenario.

We found that upon exposure of monoclonal T cells of sequential developmental stages to a thymic microenvironment expressing cognate antigen, the efficiency of T_{reg} induction inversely correlated with progressive maturation. This inclination of immature thymocytes towards T_{reg} differentiation was even seen in an APC-free *in vitro* system providing only TCR stimulation and IL-2. In support of quantitative but not qualitative features of external cues being critical, thymic epithelial cells as well as different thymic dendritic cell (DC) -subtypes efficiently induced T_{reg} development of immature thymocytes, albeit at strikingly different optimal doses of cognate antigen. We propose that the intrinsically high predisposition of immature thymocytes to T_{reg} development may contribute to the predominantly thymic origin of the T_{reg} repertoire. The underlying instructive stimulus, however, does not require unique features of a dedicated APC and can be delivered by hematopoietic as well as epithelial thymic stromal cells.

2. Zusammenfassung

Ein Großteil der regulatorischen T Zellen (T_{reg}) der Maus ist thymischen Ursprungs. Erklärungsmodelle dafür beinhalten unter anderem Thymus spezifische Zell-extrinsische Signale, möglicherweise in einer durch spezialisierte Antigen präsentierende Zellen (APC) geschaffenen Nische. Zusätzlich oder alternativ dazu könnten T-Zell-intrinsische Faktoren die Entwicklung von regulatorischen T Zellen im Thymus beeinflussen.

Im Rahmen dieser Arbeit wurden monoklonale T Zellen unterschiedlicher Entwicklungsstadien in Thymi transferiert, die das spezifische Antigen für diese T Zellen exprimieren. Die Effizienz, mit der Zellen sich in T_{reg} entwickelten, korrelierte dabei invers mit dem Reifegrad der transferierten Zellen. Diese verstärkte Neigung unreifer T Zellen in regulatorische T Zellen zu differenzieren konnte in einem APC-freien System, das nur einen T-Rezeptor-Stimulus und Interleukin-2 beinhaltet, bestätigt werden. Weiters konnte gezeigt werden, dass sowohl Thymus Epithelzellen, als auch unterschiedliche Typen von thymischen Dendritischen Zellen die Entwicklung von T_{reg} aus unreifen Thymozyten induzieren, sofern optimale Antigen Konzentrationen vorherrschten. Dies deutet darauf hin, dass die Quantität und nicht die Qualität T-Zell-extrinsischer Signale entscheidend für die intrathymische Entwicklung von T_{reg} ist.

Zusammenfassend deuten die in dieser Studie vorgestellten Resultate darauf hin, dass der intrathymische Ursprung eines Großteils der T_{reg} das Resultat einer verstärkten Prädisposition unreifer Thymozyten ist, in T_{reg} zu differenzieren. Die dafür notwendigen extrinsischen Signale können dabei sowohl von hämatopoietischen als auch epithelialen APC geliefert werden.

3. Introduction

3.1. Thymus organogenesis

The thymus is the primary lymphoid organ providing the microenvironment for the development of the T cell arm of the adaptive immune system. In evolutionary terms the appearance of a thymus is closely linked to the development of antigen receptors based on random recombination events of variable (V), diversity (D), and joining (J) DNA segments, resulting in the generation of an enormously diverse T cell receptor (TCR) repertoire. Thymic structures probably co-evolved with the enzymatic machinery that allowed T lymphocytes to acquire a somatically rearranged VDJ-TCR. Consequently thymic structures are only found in species that apply VDJ-recombination to diversify their antigen receptors, that is jawed vertebrates (1, 2). The number and anatomical location of these thymic structures varies considerably among different species with mice and humans carrying a thoracic thymus consisting of bilobular or multilobular lobes, respectively. Moreover, cervical thymi have been described in both mice (3, 4) and humans (5, 6) but their incidence in humans is somewhat unclear. Cervical thymi are frequently found in mice, albeit at markedly different frequencies in variable inbred mouse strains analyzed (3). To what extend these cervical thymi contribute to the development of the T cell pool is currently unclear.

The thymus is characterized by an enormous cellular turnover as a result of the constant development and emigration or death of T cells. Due to the highly migratorial nature of developing thymocytes and the large contribution of these cells to the overall thymic cellularity the thymus is not a solid organ. Nevertheless, it contains a scaffold of stromal cells providing the microenvironment for T

lymphopoiesis. The combination of these framework and thymocytes of various developmental stages allows for the morphological separation of the thymus into cortical and medullary regions that serve distinct functions during early and late thymocytes development, respectively.

The adult thymus is comprised of cells of various developmental origins (see Figure 1 for major thymic cell types and their developmental origin). About 98% of these cells are of hematopoietic origin and are characterized by the expression of the cell surface molecule CD45. The vast majority of these cells are thymocytes of different developmental stages. Additionally, hematopoietic antigen presenting cell types like macrophages, B cells and various dendritic cell (DC) subtypes can be found. Non-hematopoietic cells of the thymus segregate into epithelial and mesenchymal cells. Although constituting a minor fraction of thymic cellularity, thymic epithelial cells (TEC) are indispensable for the establishment of thymic microenvironments that are essential for developing thymocytes. Interaction of thymocytes with these highly specialized TEC is required at multiple stages of their development and is of fundamental importance in the acquisition of a functional and self-tolerant T cell compartment. Thymic mesenchymal cells constitute the third major cell type that can be found in the thymus. Mesenchymal cells include fibroblasts, various endothelial cells and cells forming the thymic capsule and septae.

Thymic structures originate from the third pharyngeal pouch region of the foregut at around day 10.5 (E10.5) of mouse embryonic development (2). Given the importance of epithelial cells in the formation of thymic compartments, the developmental origin of epithelial cell precursors that can be found in the “thymic anlage” is of fundamental interest. In early models it was proposed that cells of ectodermal and endodermal origin both give rise to TEC progenitors that would subsequently differentiate into

cortical or medullary epithelia cells (cTEC and mTEC), respectively. To address the germ layer origin of thymic epithelial cells, grafting experiments in birds were conducted. In these elegant experiments quail embryonic endoderm grafts were transplanted into chicken embryos and were found to give rise to both cTEC and mTEC and suggested a single germ layer origin (endodermal) of the thymic epithelium (7). More recently the question regarding the germ layer origin of TEC was readdressed in the mouse. To this end a combination of elegant experiments involving dye labeling, embryo culture techniques and grafting of endoderm-only third pouch tissue was applied. Transplantation of endoderm-only third pouch tissue under the kidney capsule of athymic recipient mice led to the ectopic development of a fully functional thymus. These results provided further experimental proof for the single endodermal origin of the thymic epithelium (8, 9).

The developmental origin of thymic mesenchymal cells is somewhat unclear. The Neural Crest (NC) was thought to provide a potential source of these cells during development. To study the contribution of NC-derived cells to the thymic mesenchyme NC-specific expression of Cre-recombinase combined with Cre-activity dependent reporters was applied to detect NC-derived cells in the developing or adult thymus (10, 11). While NC-derived cells were shown to significantly contribute to the thymic mesenchyme at early stages of thymus development (E13), these cells were confined to a cell population sandwiched between endothelial and epithelial cells lining the thymic vasculature in adult mice. Thus NC-derived thymic fibroblasts and cells forming the thymic capsule and thymic septae might be replenished with cells of as yet unknown origin during development (10).

Irrespective of its cellular contribution to the thymus, the NC was shown to serve an additional, non-redundant function during thymus development. A lack of interaction

between mesenchymal cells and TEC (progenitors) was shown to result in severe thymic hypoplasia (12, 13). This conclusions were drawn based upon studies in birds were absence of interactions between the (cephalic) NC during embryonic development led to a severe size reduction of the thymus (12). Several signaling pathways were proposed to possibly contribute to the "nurturing" function of mesenchymal cells in this context (2) and especially signals involving fibroblast growth factor 10 (FGF-10) and its specific receptor FGFR2-IIIb on epithelial cells seemed to be required for the development of a mature functional thymus since ablation of either of these molecules leads to the development of severe thymic hypoplasia, possibly due to a lack of proliferative stimulation provided by mesenchymal cells (13).

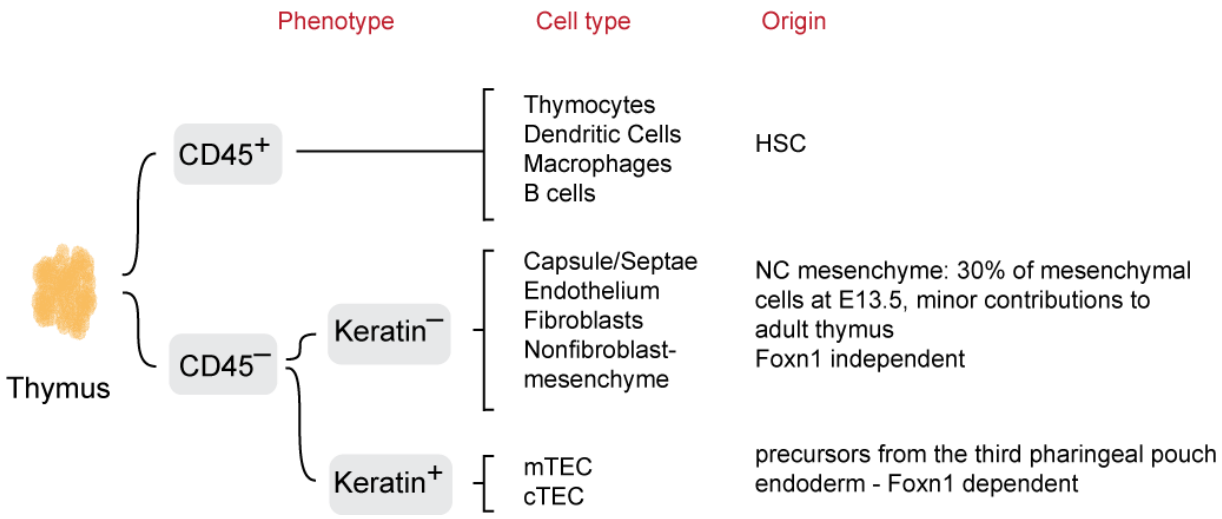

Figure 1 – Developmental origin of major cell types in the thymus. The thymus can be divided into hematopoietic cells ($CD45^+$) and non-hematopoietic stromal cells ($CD45^-$) that can be further subdivided into epithelial ($Keratin^+$) and mesenchymal cells ($Keratin^-$). The origin of mesenchymal cells appears to be heterogeneous and the cellular contribution of NC-derived cells changes considerably during ontogeny [figure structure adapted from (2)].

Prior to hematopoietic colonization of the thymus at around E12 the thymus is not compartmentalized into medullary and cortical structures. At these developmental stages TEC are thought to exhibit a precursor characteristics that eventually allow them to differentiate into mTEC and cTEC seen at later stages of thymus development. Distinct stages of TEC development are characterized by the expression of certain keratin family members. While mature TEC comprise keratin-8^+/keratin-5^- cTEC and keratin-8^-/keratin-5^+ mTEC, the majority of TEC express both of these keratins at the precursor stage (14, 15). On a population level these epithelial precursors give rise to both cTEC and mTEC. Whether, on a single cell level, these cells could give rise to both cTEC and/or mTEC and thereby constitute a pool of bi-potent precursors was recently addressed in two studies either applying single cell transfer of E12 thymic epithelial cells or a lineage tracing approach (16, 17). Both studies came to the conclusion that both cTEC and mTEC can develop from a population of bi-potent precursor cells. Although these data brought unequivocal proof for the existence of such bi-potent precursors, purification of these cells based upon the expression of cell surface markers is still not feasible.

Morphological separation of medullary and cortical thymic compartments is characterized by a gradual reduction of the keratin-8^+/keratin-5^+ pool of epithelial precursors and the differentiation of these cells into cTEC and mTEC. This developmental progression was proposed to be dependent upon hematopoietic colonization of the thymus and to require “crosstalk” between epithelial (precursor) cells and thymocytes. Evidence in favor of the importance of such interactions came from mouse models that exhibited a developmental block at early thymocyte stages that severely impaired the formation of a properly compartmentalized thymus (18, 19). Apart from these (as yet unidentified) signals affecting the developmental

progression of epithelial precursors, members of the tumor necrosis factor (TNF) receptor family have been shown to impinge on the development of TEC and the thymic architecture, mainly affecting the formation of a functional mTEC compartment. mTEC can be separated into $mTEC^{low}$ ($MHCII^{-}CD80^{-}$) and $mTEC^{high}$ ($MHCII^{+}CD80^{+}$), whereby the latter population is of special importance due to its ability to both promiscuously express otherwise tissue restricted antigens and to directly present them to developing thymocytes. Ablation of the Lymphotoxin-β receptor, receptor activator of NF-κB (RANK), and CD40 caused the development of "disorganized" medullary regions or affected the cellularity of these mTEC subpopulations (20-24). The importance of these receptors was substantiated by the analysis of animals deficient or impaired in their downstream signaling components TNF-receptor associated factor 6 (TRAF6), NF-κB-inducing kinase (NIK), IκB kinase α (IKKα), and the NF-κB subunit RelB.

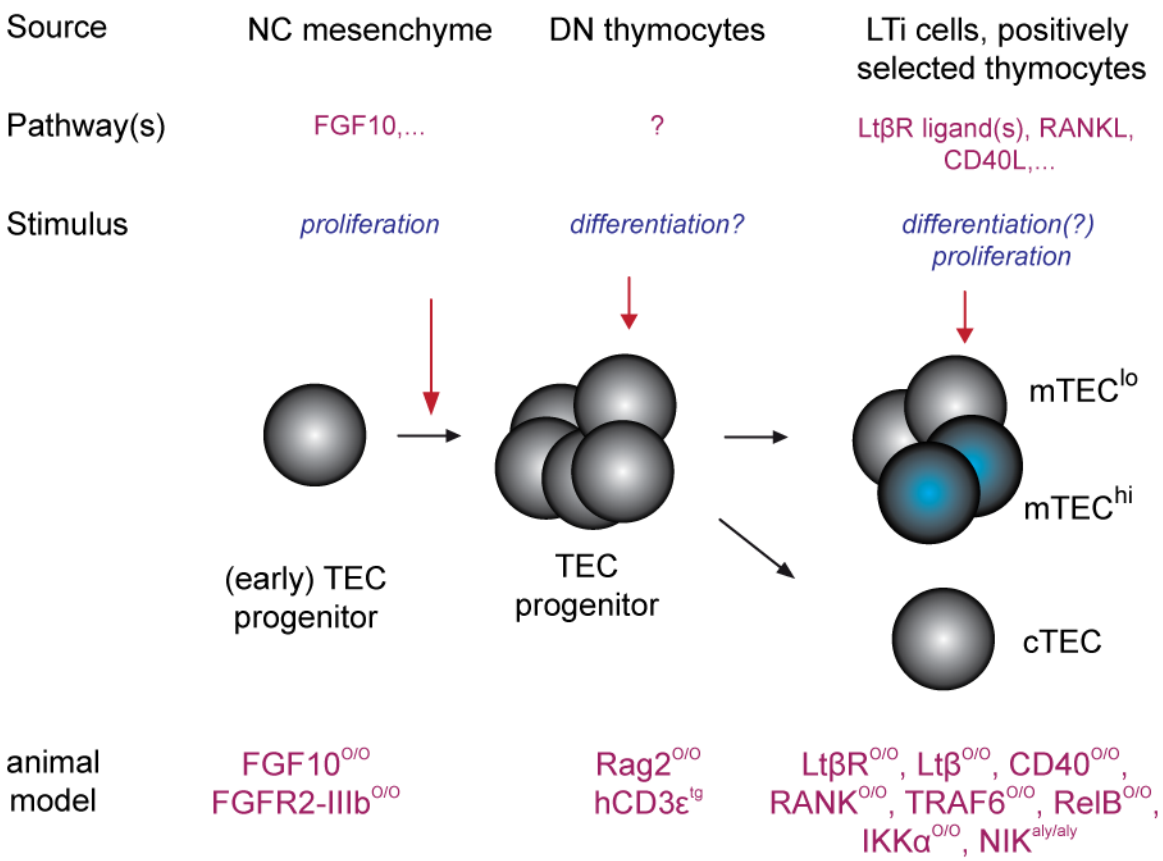

Figure 2 – Signaling pathways involved in the formation of TEC. Various sources provide proliferative and/or differentiation stimuli to TEC during thymus ontogeny. The figure depicts pathways known to affect the development of the indicated TEC stages. Question marks indicate a lack of knowledge either about pathways involved or the precise function of known stimuli in TEC development. Additionally, knockout or transgenic models used to study the role of the respective pathways are depicted (see text for references). LTi , Lymphoid Tissue inducer cell.

Defective signaling led to even more severe phenotypes concerning mTEC cellularity and the medullary architecture than observed in single-receptor deficient mice, indicating redundancies amongst members of the TNF receptor family and possibly other pathways activating these downstream signaling molecules (25-29). The general importance of these signaling pathways is also highlighted by defective tolerance induction leading to the development of autoimmunity manifestations,

probably as a result of the impaired formation of medullary structures [see Figure 2 for a schematic overview of (crosstalk) pathways involved in TEC development].

3.2. T cell progenitors and T cell lineage commitment

T cell development and maturation is, as opposed to the development of other blood cell types, strictly dependent upon the thymus. Since self-renewing potential is limited or absent in thymus resident progenitor populations (30) sustained T cell development requires continuous input of blood borne progenitor cells (31). Entry of T cell progenitors into the thymus was shown to be a gated phenomenon (32). It was proposed that the thymic microenvironment provides a limited number of niches for incoming progenitors and that mobilization of progenitor cells from the bone marrow and importation of this rare population of blood borne cells might be co-regulated (33). Additionally, the number of bone marrow resident T cell progenitors is thought to be subject to periodic fluctuations (33). Whether the periodic development of T cell progenitors in the bone marrow and their subsequent release into the blood is affected by the depletion of progenitor cells in the thymus and regulated by feedback mechanisms is unclear to date (32-34).

While studies on the developmental potential of different populations of bone marrow resident progenitor cell types have narrowed down the search for physiologically relevant precursors they did not allow for an assessment of the contribution of each of these populations to the development of the T cell compartment under physiological conditions. Most *in vivo* studies either used immune-deficient or irradiated recipient mice creating an artificial situation in terms of available niches in the thymus, homing capabilities of precursors and competition among these cells. A

prerequisite for a physiologically relevant T cell progenitor population would be its efficient and timed mobilization from the bone marrow combined with efficient homing of these cells to available intrathymic niches. Studies on the development of such progenitors and their mobilization are complicated by their low abundance, however.

Extravasation of progenitors into the thymic microenvironment has been predicted to involve rolling along the vascular endothelium as a result of low affinity interactions, probably involving selectins and their interaction partners on the vascular endothelium, followed by integrin/chemokine mediated arrest and transmigration into the thymus. Thymic vascular structures rendering the extravasation of progenitors into the thymus possible according to the paradigm illustrated above have been indentified (11). To address the thymus tropism and mode of extravasation of different bone marrow derived progenitor populations immune-deficient animals were reconstituted with these populations and numbers of cells that homed to the thymus were assessed. In these studies a subset of common lymphoid progenitor cells termed CLP-2 was seen to show robust thymus homing capabilities. Moreover, P-selectin/PSGL-1, CCR9/CCL25, and α4 or β2 integrins and their counterreceptors ICAM-1 or VCAM-1 were suggested to be mechanistically involved in this process (35). Whether this represents a general mechanism applicable to all progenitors that show thymus tropism remains to be established.

Thymus entry of T cell progenitors is thought to occur at the corticomedullary junction (CMJ) (36) and the earliest intrathymic progenitors that can be found in the thymus have been termed early T cell progenitors (ETP) and reside within the $CD4^{-/low}CD3^{-}CD8^{-}CD25^{-}CD44^{+}$ (DN1) population of cells (37). ETP are characterized by the expression of CD117 and have been found to be heterogeneous concerning the

expression of CCR9, Flt3 and CD24. This heterogeneity has been suggested to represent developmental transitions within this precursor population (38).

Thymocyte progenitors and the least mature thymocytes reside in an area termed perimedullary cortex (PMC) for up to 10 days. During this period extensive cellular expansion (~1000 fold) accompanied by the asynchronous utilization of cells to replenish the T cell pool takes place. During the stages preceding the recombination of T cell receptor (TCR) genes developing thymocytes commit to the T cell lineage in a manner that is strictly dependent upon Notch1 signaling. Inducible inactivation of either Notch1 or the downstream transcription factor recombination signal binding protein-J (RBP-J) led to a developmental block in intrathymic T cell development at or before the DN1 stage of development (39, 40). This phenotype was recapitulated in mice lacking Delta-like 4 (Dll4) on thymic epithelial cells. Hence Notch1 and Dll4 interactions play a crucial and non-redundant role in T cell development (41-43). Furthermore, Notch signaling strength affects lineage commitment, whereby abrogation of B cell development required only low levels of Notch signals, whereas intermediate levels are necessary to shift the balance towards T cells at the expense of NK lineage potential (44). The precise role of Notch1-Dll4 interactions and downstream events that eventually trigger the initiation of the T cell lineage developmental program are not well established but Notch signaling has been shown to both affect lineage commitment and maintenance of the metabolic state of T cells, possibly serving different roles at distinct developmental stages of T cell development (44, 45).

3.3. Thymic stromal microenvironments and thymocyte development

Thymocyte development proceeds along well defined stages characterized by the expression of cell surface molecules (see Figure 3) during their migration through different stromal compartments of the thymus. These stromal compartments are not separated by rigid structures but are defined by the localization of thymocytes at distinct developmental stages. Due to their complex migratorial pattern thymocytes of different developmental stages can co-localize, leading to an extensive overlap of thymic compartments as defined above. However, the combination of thymocyte extrinsic signals and cellular interaction partners combined with stage specific changes in the integration of the very same environmental cues allows for the definition of distinct signaling microenvironments (46).

Several zones have been described with the first one, the PMC being situated immediately adjacent to the CMJ. This region provides the appropriate microenvironment for DN1 cells. As stated above cells can reside in this area for up to 10 days (47) and undergo massive proliferative expansion during this period. DN1 cells do not synchronously leave the PMC but rather segregate into cells that reside in the PMC, possibly providing a (limited) source of progenitors, and cells that progress to the next developmental stage (47). This might account for the observation that T cell development, as opposed to homing of T cell progenitors, is a continuous process. A detailed mechanistic understanding of factors that mediate retention and proliferation of DN1 cells or trigger their release from the PMC is still missing.

Migration of cells out of the PMC coincides with their transition into the DN2 ($CD44^{+}CD25^{-}$) developmental stage. DN2 cells are found in the inner half of the outer

cortex and their average residence time in this area is only about 2 days (47). Area specific events include proliferative expansion and (further) commitment to the T cell lineage (46). Migration to these cortical regions is thought to involve interactions of DN2 cells with thymic stromal cells via integrins and vascular cell adhesion molecule-1 (VCAM-1). VCAM-1$^+$ cells constitute a subset of thymic stromal cells in the cortex (48) and are thought to form a lattice along which DN2 cells migrate into the outer cortical regions. DN2 cells have been found to remain in close contact with VCAM-1$^+$ cells during this migration, probably via α4β1 integrin interactions (49), and it has indeed been suggested that this reticular lattice might provide a limited number of niches for developing thymocytes to continue their development and subsequently enter the outer cortical regions (46). Additionally CXCR4 expressed on DN2 cells and its ligand CXCL12 expressed on cortical stromal cells were shown to be essential for the migration of DN2 cells from the PMC into the outer cortical regions (50-52).

Further maturation of cells coincides with migration into the outer cortical regions and developmental progression to the DN3 (CD44$^-$CD25$^+$) stage. Migration of cells to the outer cortical regions/capsule was shown to critically depend upon CCR9 and its ligand CCL25 since absence or premature expression of CCR9 affects migration of DN3 cells into the subcapsular zone of the cortex or exit from this region, respectively (53, 54). Cells reside for about two days in the subcapsular zone, undergoing proliferative expansion and irreversible commitment to the T cell lineage (55). A key process at this stage is the induction of TCRβ-locus recombination. Recombination events critically depend upon the expression of RAG genes and IL-7 signaling has been shown to induce expression of RAG1 and RAG2 at stages preceding TCRβ-locus recombination. Another function of IL-7 signaling in T cell receptor locus recombination might be the induction of locus accessibility by as yet ill defined means

(56, 57). During their residence in the subcapsular zone of the thymus, DN3 cells complete recombination of the TCRβ locus. This stage marks the first checkpoint during T cell development termed β-selection. Only successful recombination of one of the TCRβ-loci allows for the progression of cells to the CD4/8 double positive (DP) stage. Successful rearrangement is marked by the expression of the pre-TCR, a dimer of the TCRβ-chain and a pre-TCRα chain. Pre-TCR signaling is thought to occur in a ligand independent way (58) and successful rearrangement of one TCRβ-chain and signaling by the pre-TCR excludes recombination of the other locus (59).

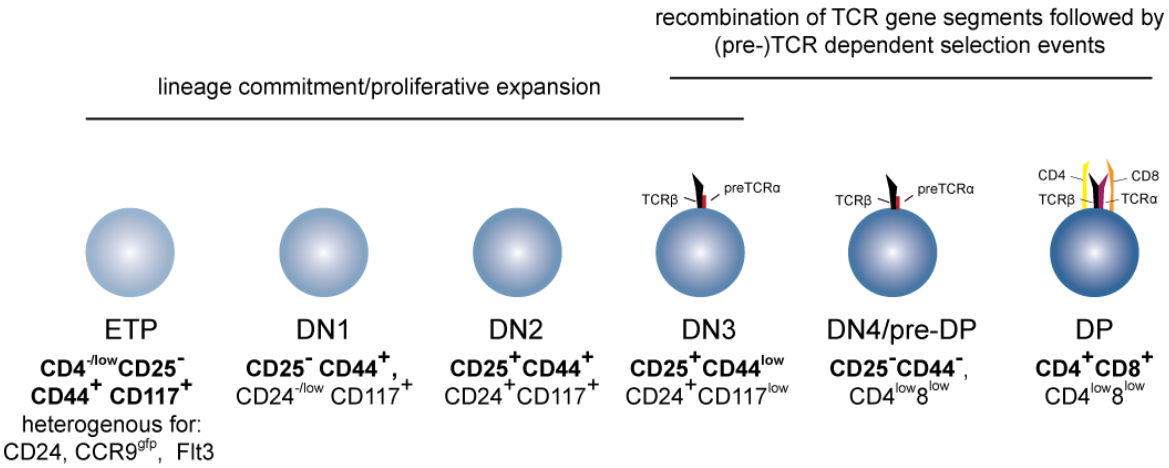

Figure 3 – Stages of thymocyte development preceding maturation in the thymic medulla. Early stages of thymocyte development can be defined by the expression of the indicated cell surface molecules. Early T cell progenitors (ETP) are a heterogeneous population of cells that eventually give rise to DN1 cells. Thymocyte development preceding the expression of a pre-TCR is characterized by strong proliferative expansion and Notch1/Dll4 dependent T cell lineage commitment. Subsequent selection events or proliferative expansion are critically dependent upon the expression of a (pre-)TCR.

Another consequence of successful rearrangement is the acquisition of a blast like morphology and a dramatic increase in proliferative activity in the subcapsular region that generates the bulk cellularity of the thymus (60). Once DN3 cells pass β-selection they rapidly upregulate CD4 and CD8 expression. Cells expressing low levels of CD4 and CD8 have been referred to as DN4 ($CD44^{-}CD25^{-}$) cells or pre-DP cells. The most critical events during the pre-DP stage are the initiation of TCRα locus recombination and the reversion of their migration polarity. The extrinsic cues responsible for the inward migration of pre-DP and DP cells are only poorly understood.

Interaction with cortical epithelial cells is essential after recombination of the TCRα locus for the next checkpoint/maturation step to occur. This checkpoint is termed positive selection and is characterized by interactions between the TCR and peptide-major histocompatibility complex (pMHC) molecules that allow for the selection of cells bearing successfully rearranged TCRs that recognize self ligands with low affinity (61). Cells that do not express T cell receptors allowing for positive selection undergo apoptosis in a process termed “death by neglect”.

Another essential process happening at the DP stage of development is the commitment to either the CD4 or CD8 T cell lineage. Although significant progress has been made in terms of understanding the molecular basis of CD4/CD8 lineage commitment (62-65) the initial steps leading to the induction of lineage specification factors remain unclear. Several models have been proposed either relying on stochastic events terminating the expression of one or the other co-receptor or instructive models suggesting mutual inhibition of co-receptor expression, depending upon either MHC class I or MHC class II molecules driving positive selection. In both cases lineage commitment only requires TCR-pMHC interaction and does not

necessarily involve additional stromal cues. Another model proposed a role for IL-7 signaling in CD8 lineage commitment. In this “co-receptor reversal” model TCR-triggering initially terminates expression of the CD8 co-receptor. If loss of CD8 expression abolishes TCR-signaling, IL-7 signaling initiates re-expression of CD8 and commitment to the cytotoxic T cell lineage. Conversely, sustained signaling in the absence of CD8 expression will inhibit IL-7 signal transduction and deviate cells into the CD4 lineage (66).

Once cells underwent positive selection and possibly lineage commitment their migration becomes polarized and is directed towards the thymic medulla. This directed migration is thought to occur in a CCR7 specific manner. Abrogation of CCR7 guided migration leads to a failure of positively selected thymocytes to enter the thymic medulla and consequently to an accumulation of mature thymocytes in the cortex (67). Full maturation of thymocytes does not depend upon migration of positively selected thymocytes into the medulla. The acquisition of a fully self-tolerant T cell repertoire is, however, tightly linked to the interaction of developing thymocytes with medullary antigen presenting cells. Consequently, “ectopic” maturation of thymocytes in the cortex leads to a failure to establish a self tolerant T cell compartment (67). Selection events in the thymic medulla are driven by cognate TCR-pMHC interactions that lead to either the elimination of auto reactive cells (termed negative selection/deletion) or the induction of regulatory T cells (T_{reg}) with both mechanisms being crucial for the maintenance of a self-tolerant immune state. As opposed to the thymic cortex the medulla contains significant numbers of dendritic cells (DC) and furthermore a population of medullary thymic epithelial cells (mTEC) that is specialized in expressing a wide range of otherwise tissue restricted antigens, a phenomenon termed “promiscuous gene expression”. Presentation of this wide

range of antigens by mTEC themselves or “distribution” of these antigens to DC-subpopulations allows medullary antigen presenting cells (APC) to mirror the peripheral self in order to tolerize the developing T cell compartment (68).

3.4. Immunological Tolerance – historical overview

With the acquisition of somatic recombination as a means to diversify the scope of antigens that cells of the adaptive immune system can recognize, a fundamental problem arose in terms of preventing the immune system from attacking self-determinants (69). Already in 1957 Burnet suggested, in his seminal paper on “clonal selection” that each lymphocyte only expresses one antigen-receptor specificity and that cells recognizing self-determinants could be subject to tolerance induction by functional impairment or clonal deletion (70). At that time it was proposed that all immune-competent cells were produced during embryonic development. Antigen encounter during an early developmental period (embryonic) would then lead to the deletion of cells, since all antigens available at this time would be self derived. This elegant model centered on the ontogeny of the individual as the key factor in the acquisition of immunological tolerance. Experimental proof for this theoretical framework was provided by transplantation experiments done by Medawar and co-workers. Transplantation of skin grafts from semi-allogenic donors onto newborn mice, i.e. mice that still lacked a fully immune-competent immunological state, was shown to result in tolerance to these grafts if it was accompanied by embryonic/neonatal transfer of hematopoietic cells of donor haplotype. A basic corollary of Burnet’s and Medawar’s theory on “actively acquired tolerance” is that grafts transplanted “early enough” should be tolerated by the recipient’s immune

system due to the ability to recognize the graft as self later on. Yet transplants were only tolerated with high incidence if embryos/neonates were pre-inoculated with hematopoietic cells. This tolerization protocol did not work if the inoculum was of non-hematopoietic origin. Results leading to similar conclusions were also derived from transplantation experiments in chicken. “Learning” self obviously required hematopoietic cells of donor origin in this setup. Back then these transplantation experiments were nevertheless interpreted as unequivocal proof for the existence of a window of time during ontogeny during which tolerance could be imposed onto or learned by developing immune cells (71).

The concept of a “developmental window” accounting for the observations seen in chicken and mouse transplantation experiments was at odds with observations suggesting that lymphocytes do not only arise during embryonic development but throughout adult life. Even if the embryonic and perinatal period represented a specialized environment for the acquisition of a tolerant state, tolerance induction would, due to the constant production of lymphocytes, have to be efficient later on to allow for the maintenance of a tolerant state. Already in 1959 Lederberg suggested that it might be the ontogeny of the lymphocyte, not the ontogeny of the whole organism, that represents the critical determinant in tolerance induction. He proposed that the outcome of antigen encounter would critically depend upon the maturity of the lymphocyte; the defining characteristics of “maturity” were unclear, however (72).

Apart from the “timing” problem another fundamental issue was the availability of self-determinants in the process of tolerance induction. Developing lymphocytes would have to encounter all possible self determinants to be rendered anergic or to be deleted to sustain immunological tolerance. Thus, crucial questions remained open at the time.

About three decades after the postulation of “developmental tolerance”, the question of self-non-self-discrimination was re-addressed using quail/chicken interspecies transplantation experiments. In these elegant experiments embryonic quail tissue was transplanted onto chicken embryos to test for the induction of tolerance. According to Burnets postulates the transplanted tissues should have been tolerated due to the immune system recognizing the graft as self during this early stage of development. Surprisingly enough the opposite was the case. As soon as the transplanted chickens acquired full immunological competence, donor derived tissues were acutely rejected (73). Thus a key postulate of the “developmental tolerance” hypothesis was proven wrong.

Another key experiment, published in 1987 showed that co-transplantation of a donor derived thymic anlage conferred tolerance to transplanted tissues. However, tolerance induction was not the result of the deletion of “autoreactive” cells as alloreactivity of T cells in transplanted animals was still evident (74). Accordingly both a reinterpretation of previous results and the incorporation of a phenomenon termed “dominant tolerance” into the concept of immunological tolerance seemed to be required.

Transplantation experiments in the quail/chicken model suffered from the lack of available tools to narrow down the population of cells responsible for this “dominant tolerance”. However, in 1990 similar conclusions were also drawn from analogous experiments in nude mice and it was additionally shown, that “dominant tolerance” was mediated by “regulatory” T cells (75, 76). The concept of regulatory T cells, or “suppressor T cells”, was already proposed in the seventies (77), but the failure to exactly define a population of cells that exert regulatory or immune modulatory

functions within the T cell compartment combined with a lack of mechanisms responsible for these effects hampered progression in this field.

The availability of appropriate tools and a series of seminal publications led to the identification of a subset of T cells that was potent in suppressing various manifestations of autoimmunity. The presumable loss of a regulatory T cell population and the induction of massive autoimmunity seen by early thymectomy combined with the restoration of a tolerant state in various models of autoimmunity by the transfer of $CD45RC^{low}$ or $CD45RA^{low}$ T cell subsets allowed for the identification of T cell populations strongly enriched for regulatory activity (78-80). This work culminated in the identification of $CD4^+$ T cells expressing high constitutive levels of CD25, the high affinity chain of the IL-2 receptor complex, as being the population of cells responsible for these regulatory effects (81). Subsequently significant progress has been made in establishing the crucial importance of these cells and thereby “dominant tolerance” by the identification of the lineage specific transcription factor Forkhead box p3 (Foxp3) that unequivocally identifies cells that have been imprinted with the transcriptional program of regulatory T cells (82-88).

3.5. Experimental proof and mechanistic basis of negative selection

Seminal studies during the last decades have proven clonal deletion as being a crucial mechanism of immunological tolerance. Although this was suggested relatively early in the field, experimental proof appeared to be difficult to obtain. It was suggested that T cells educated in an allogenic thymus were depleted of alloreactive MHC-specific cytotoxic T lymphocyte precursors [reviewed in (89)]. However, these studies suffered from a lack of experimental tools to allow for the detection of T cells

that would be lost upon antigen encounter during the process of tolerance induction. A definitive experimental proof for clonal deletion would necessitate following individual TCR-specificities during their intrathymic development, i.e. finding specificities/individual TCRs that initially arose but were deleted later on. Two experimental approaches were highly instrumental in solving this issue. First, a monoclonal antibody that recognized Vβ17a$^+$ TCRs was generated (90). This antibody allowed for the detection of a fraction of T cells and to follow this population during its intrathymic development. T cells expressing a Vβ17a$^+$ TCR were shown to be deleted in a mouse strain specific manner (91). Initially, strain specific expression of I-E MHCII molecules was thought to lead to this deletion. Later on, the “ligands” for this TCRs were shown to be mouse mammary tumor superantigens-8,-9 (92, 93). Importantly, early developmental stages of Vβ17a$^+$ cells (DP) were unaffected whereas cell numbers of mature thymocytes and peripheral T cells were markedly reduced. Second, TCR-transgenic mice were created that allowed for following a single TCR-specificity along its intrathymic development. Co-expression of the cognate ligand of the respective specificity led to a strong reduction in cell numbers of thymocytes bearing the transgenic TCR [reviewed in (94)]. Thus decades after the initial proposal of deletion as being an essential means to establish tolerance to self-constituents experimental proof was available corroborating this central paradigm of immunological tolerance. A detailed characterization of spatial and temporal requirements allowing for the efficient deletion of autoreactive cells remained challenging, however.

Negative selection requires cognate TCR-pMHC interactions. Thus the earliest stage of thymocyte development that can be negatively selected is the (late) DP stage. In addition to TCR-pMHC interactions co-stimulatory molecules have been

demonstrated to impinge on the efficiency of negative selection. Both CD28/B7 interactions and CD40/CD40L interactions have been shown to affect clonal deletion (95, 96). Whether different co-stimulatory molecules trigger downstream pathways specifically involved in negative selection or whether they act to amplify TCR signaling pathways and thereby increase negative selection is somewhat unclear. The purported redundancy of co-stimulatory pathways increasing negative selection probably favors the latter interpretation (97).

TCR-proximal signaling events are similar in positive and negative selection, nevertheless some molecules seem to be uniquely involved in negative selection (94). TCR signaling involves a large number of proteins that form an immunological synapse and a signalosome integrating stimulatory and co-stimulatory cues that result from (cognate) TCR-pMCH interactions (98). Key molecules downstream of TCR signaling involve amongst others members of the mitogen activated protein kinase (MAPK) family. It has been suggested that the activation and compartmentalization of different members of this family of kinases defines the outcome of TCR signaling in thymocyte selection processes. Negative selection has been shown to involve the activation of jun N-terminal kinases (JNK), p38, and extracellular signal-regulated kinase (ERK) whereas (weak) positive selecting signals involve only activation of ERK, albeit with different kinetics and sub cellular localization (94, 99) of ERK. Accordingly, interference with upstream components of these pathways, like the adaptor protein Grb2 that is involved in activation of the Ras pathway, ultimately leading to the activation of the JNK/p38 kinases, reduces negative selection in TCR-transgenic models and in CD3-crosslinking-mediated induction of apoptosis (100, 101). Additional evidence for the importance of JNK/p38 signaling in negative selection came from the identification and characterization of

misshapen NIKs-related kinase (MINK). MINK is highly expressed in DP thymocytes and its knockdown was shown to result in an inhibition of TCR induced JNK activation and impaired negative selection in superantigen and TCR-transgenic models of deletion (102). Although this provides another striking piece of evidence for the importance of these MAPK pathway(s) in negative selection, the precise nature of molecular interactions/signaling events linking TCR signaling with JNK activation is currently unknown. It has been suggested that MINK is linked to the TCR complex via the adaptor protein Nck and provides a scaffold for MAPKKKs and MAPKKs subsequently activating JNK. Consistent with this model the kinase activity of MINK is not required for its function in activating the JNK pathway (102, 103).

The ultimate outcome of signaling events leading to the deletion of autoreactive T cells is the induction of programmed cell death and several molecules were revealed to play a non- redundant role in this process. In principle apoptosis can be triggered via cell surface receptors of the tumor necrosis factor receptor (TNFR) family and Fas and via activation of pro-apoptotic members of the Bcl-2 family of proteins. Induction of apoptosis in thymocytes was shown to depend upon Fas ligation when high antigen doses were applied (104). Likewise, inhibition of multiple TNFR pathways by virtue of a dominant negative construct blocking apoptosis inducing signaling did not impair negative selection (105). The second pathway, involving members of the Bcl-2 family, induces apoptosis by triggering the release of mitochondrial cytochrome-C that binds the apoptosis protease activation factor-1 (APAF-1) which subsequently leads to the initiation of cleavage of caspases ultimately leading to cell death. Deletion of Apaf-1 does not significantly impair thymocyte negative selection, however, implying alternative pathways inducing the activation of the caspase cascade in the absence of Apaf-1 (106). Prominent members of the Bcl-2 family

involved in inducing apoptosis are Bim, Bax, and Bak, whereby Bax and Bak are thought to act downstream of Bim. Deletion of Bim or combined deletion of Bax and Bak results in defective negative selection in multiple model systems studied (107, 108). The combined action of Bim, Bax, and Bak is thought to lead to mitochondrial instability and subsequently to apoptosis (109). Another important molecule in negative selection is the nuclear receptor 77 (Nur77). Activation of T cells via the TCR leads to strong transcriptional activation of the Nur77 gene that can by itself trigger apoptosis. Likewise impairment of Nur77 function by virtue of overexpression of a dominant negative form of Nur77 leads to defective negative selection (110). The precise mechanisms whereby TCR signaling affects transcription or posttranslational modification of these molecules and thereby ultimately triggers apoptosis is unknown (109).

As stated above negative selection can already be initiated at the DP stage and experimental evidence indicates that developmental progression gradually reduces the susceptibility of thymocytes to undergo apoptosis as a result of antigenic stimulation (104). How (a combination of) the above mentioned pathways or alternatively a lack of survival signals co-operate to establish the “window of sensitivity” to negative selection during the ontogeny of lymphocytes that has been proposed decades ago remains to be shown in detail.

3.6. Thymic development of Foxp3$^+$ regulatory T cells

The experiments of Nicole Le Dourain and colleagues have firmly established a role of dominant tolerance in immune homeostasis. The identification of regulatory T cells several years later and the establishment of Foxp3 as the lineage defining

transcription factor of regulatory T cells have allowed for the examination of fundamental aspects of regulatory T cell induction and development.

Loss of regulatory activity within the T cell compartment by ablation of Foxp3 lead to the development of massive autoimmunity and reconstitution of these mice with T_{reg} was shown to be sufficient to abrogate this pathologic manifestations (111). Since these autoimmune manifestations were arguably driven by the recognition of self-constituents the fundamental question arose as to how the regulatory T cell repertoire inhibiting autoimmunity relates to the partly autoreactive repertoire of naïve T cells. Regulatory T cells bear multiple hallmarks of activated conventional T cells, such as expression of the cell surface molecules CD25, PD-1, GITR, and CTLA-4 indicative of (constant?) antigen encounter (112). This surface marker expression profile is already intrathymically imprinted on T_{reg}, implying a possible role for TCR-pMHC interactions during the development of T_{reg}. Evidence for the importance of TCR derived signals during the development of T_{reg} came from observations made in TCR transgenic systems. While polyclonal T cells segregate into conventional T cells and T_{reg} at a fairly constant ratio, TCR transgenic T cells that lack expression of endogenously rearranged TCR chains do not give rise to T_{reg} (113-115). This picture changes if the cognate ligand for the respective transgenic TCR is expressed intrathymically. Antigen encounter by developing thymocytes can lead to the appearance of a robust fraction of $Foxp3^+$ cells in these models (115-117). To study the potential autoreactivity of the T_{reg} repertoire in a “physiological” setting, TCR repertoires of conventional and T_{reg} cells of the thymus and the periphery of TCRβ transgenic mice were compared, allowing for studies on a diverse, yet limited repertoire of TCR specificities. These studies suggested that conventional and T_{reg} TCRs showed only a limited overlap in repertoire. Retroviral transduction of T cells

with T_{reg} TCRs and subsequent transfer of these cells into lymphopenic and nonlymphopenic hosts conferred the ability to these cells to rapidly expand, indicative of cognate antigen encounter (118, 119). These data bolstered the hypothesis that autoreactivity is an essential hallmark of these cells and that it might also be involved in the induction of these cells in the thymus.

A critical event during the development of regulatory T cells is the induction of Foxp3 expression. Although Foxp3 was shown to amplify already established features of intrathymically arising T_{reg} and to confer them with regulatory activity it appeared to be downstream of as yet ill defined events initially driving the commitment of these cells to the T_{reg} lineage. Nevertheless Foxp3 is essential for the establishment and maintenance of the T_{reg} transcriptional signature (120-123). Genetic ablation of components implicated in T_{reg} biology combined with biochemical studies on the mechanistic basis of the regulation of Foxp3 gene expression allowed for the delineation of minimal requirements for T_{reg} development *in vitro*. TCR stimulation combined with TGFβ and IL-2 signalling seemed to be sufficient to induce T_{reg} from naïve precursors (124). Studies on the suppressive potency and most importantly stability of these so called adaptive T_{reg} were contradictory, however (124-128). Additionally, genetic evidence indicated that intrathymic T_{reg} induction followed fundamentally different rules since both ablation of IL-2/CD25 or TGFβRII in T cells did not lead to a drastic reduction in intrathymic numbers of $Foxp3^+$ cells (129-131). Additionally, the need for CD28/B7 co-stimulation could be bypassed by the provision of IL-2 *in vitro* but was suggested to be required for the induction of Foxp3 expression *in vivo* (132). The interpretation of these results is complicated by the involvement of IL-2 induced and CD28/B7 co-stimulatory pathways in both cellular survival and differentiation, however (133-136).

Recent studies have readdressed the importance of TGFβ signalling in natural T_{reg} induction and came to different conclusions. Loss of TGFβ induced signals did indeed severely reduce the first wave of $Foxp3^+$ cells arising at around day three after birth. This lack of $Foxp3^+$ cells was then compensated for by massive proliferative expansion that critically depended upon IL-2 signalling (137).

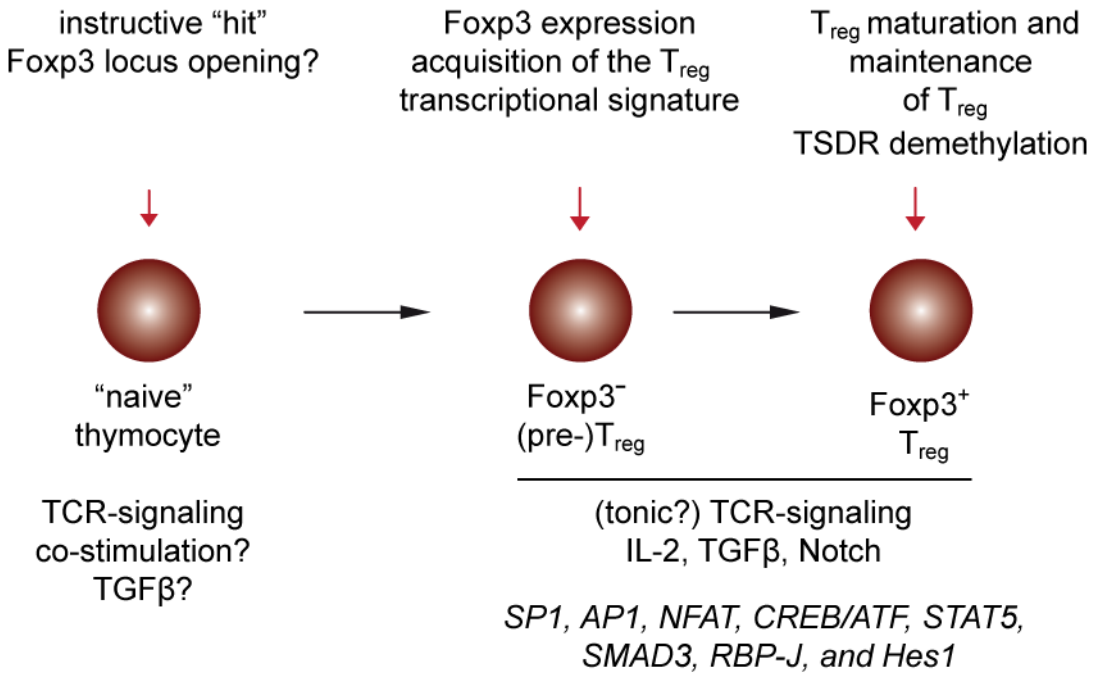

Figure 4 – Induction, differentiation and maintenance of T_{reg}. Intrathymic development of T_{reg} can be subdivided in an instructive phase and a phase of differentiation that is characterized by the expression of Foxp3 protein, demethylation of regulatory regions in the first intron of the Foxp3 gene (TSDR) and the acquisition of the Foxp3 dependent transcriptional program. Maintenance of a stable T_{reg} phenotype requires continued expression of Foxp3, consequently factors that are involved in transcriptional regulation of Foxp3 expression have been shown to be involved in the maintenance of these cells. The figure depicts signaling pathways that have been proposed to be implicated in T_{reg} development, whereby the earliest events remain ill defined. Based upon genetic evidence both cognate antigen encounter and intact TGFβ-signaling were proposed to play a prominent role.

Hence TGFβ appears to play a non-redundant role in intra- and extrathymic T_{reg} induction whereas IL-2 signalling might be involved in differentiation and maintenance of these cells but is possibly not involved in Foxp3 “locus opening” or the earliest events of T_{reg} lineage commitment. The precise molecular events allowing for initial transcription of Foxp3 are not yet clear.

Differentiation of T cell lineages and expression of lineage specific cytokines was shown to coincide with massive epigenetic changes that allowed for stable lineage commitment (138, 139). While this aspect is still somewhat underexplored in T_{reg}, stable expression of Foxp3 was shown to be accompanied by demethylation of an intronic region of the Foxp3 gene. This region was termed T cell specific demethylated region (TSDR) and did not show demethylation upon *in vitro* induction of T_{reg} differentiation in mature naive T cells (126, 140, 141). Hence it was speculated, that the TSDR methylation status would be a feature distinguishing natural from adaptive T_{reg} cells. Recent evidence indicates that demethylation can also occur in peripheral naïve cells *in vivo,* however (142). The mechanistic basis for demethylation, i.e. active demethylation versus “passive demethylation” as a consequence of lacking *de novo* methylation during cell cycling, is currently unclear.

While essential aspects of the earliest events driving cells into the regulatory T cell lineage remain poorly defined, genetic and biochemical evidence allowed for the characterisation of a transcription factor network involved in the differentiation of T_{reg} and maintenance of Foxp3 expression. Genetic studies have implicated TCR-, IL-2-, TGFβ- and Notch-derived signals as essential in this respect. Accordingly, biochemical characterisation of the Foxp3 promoter and intronic regulatory regions revealed binding sites for the TCR-signalling activated transcription factors SP1, AP1, NFAT and CREB/ATF. Additionally IL-2 induced STAT5, TGFβ induced SMAD3

and Notch induced RBP-J and Hes1 were shown to transcriptionally regulate Foxp3 expression (140, 143). Thus significant progress has been made in unravelling essential aspects of T_{reg} biology in terms of their differentiation and maintenance. A detailed picture of T cell intrinsic and T cell extrinsic factors and their effects during the "inductive" phase of T_{reg} development is still missing.

3.7. Thymic APC in tolerance induction

As mentioned above thymocyte development can be separated into developmental stages preceding the expression of a TCR and late developmental stages that involve selection events that are based upon TCR-pMHC interactions and are critical for the induction of central tolerance. Multiple APC types are involved in this process and both qualitative/quantitative as well as spatio-temporal aspects of antigen presentation by these APC impinge on the outcome of cognate TCR-pMHC interactions.

APC in the thymic cortex are mainly comprised of cTEC and a low number of DC. While providing essential cues for the positive selection of thymocytes cTEC were proposed to be inefficient in inducing negative selection (144, 145). Induction of T_{reg} in the thymic cortex was also reported (146, 147), but in terms of total numbers of $Foxp3^+$ cells that can be found in the thymus the contribution of these DP T_{reg} to the total pool of $Foxp3^+$ cells seems to be relatively low (147). Thus a separation of the thymus into cortical regions that are essential for positive selection and medullary regions that are essential for negative selection and the induction of dominant tolerance is, although obviously not absolute, an interesting scenario. This hypothesis on a division of labor between the major thymic compartments, that is

cortex and medulla, is not only bolstered by experiments addressing the principal potential of cTEC to mediate negative selection or dominant tolerance but also by the fact that “full” tolerance induction requires interaction of developing thymocytes with a broad diversity of self-ligands presented by medullary APC. This requirement is met by medullary APC in two ways. First, the promiscuous expression of otherwise tissue restricted antigens by a subset of mTEC and their presentation by both mTEC and thymic DC allows for a considerable broadening of the scope of “self” that is presented to developing thymocytes (68) and second, medullary DC consist of both autochthonous DC and DC subpopulations that arise extrathymically and migrate into the thymus later on. These migratory populations of DC have been proposed to extend the pMHC diversity presented in the thymic medulla by importing peripheral antigens (148, 149).

Negative selection has been primarily attributed to DC due to the strongly increased frequency of mature thymocytes arising in thymi that lack MHCII expression on hematopoietic cells (150). As a consequence of a lack of experimental tools, the quantitative contribution of antigen presentation by mTEC to negative selection remains difficult to be addressed. Induction of T_{reg} has been attributed to both mTEC (151) and DC (152), rendering the probability of a single APC type mediating induction of dominant tolerance low. Additionally, thymic DC show a considerable heterogeneity (153) and the contribution of different DC subtypes only begins to be unraveled.

Deciphering the role of mTEC and different medullary DC in tolerance induction needs to take into account both the principal ability of these stromal APC to contribute to recessive and dominant tolerance induction and the contribution of each of these cell types to the highly diverse peptide ligandome that is presented within

the thymic medulla. Both of these aspects are technically challenging to address *in vivo* and certainly await further refinement.

4. Aim of the present study

As stated above it is commonly accepted that the majority of natural T_{reg} originate from intrathymic selection processes. Evidence that the polyclonal T_{reg} population is enriched in autoreactive T cells (119, 154) and observations in TCR transgenic models have indicated that intrathymic self-antigen encounter molds the T_{reg} repertoire. When and how this dedicated T cell lineage branches of from "mainstream" thymocyte development remains controversial. We and others have suggested that T_{reg} arise at the CD4 single-positive (SP) stage through what may be called "altered negative selection" in the thymic medulla (116, 155). Other studies have proposed that T_{reg} differentiation is the consequence of "altered positive selection" of $CD4^+CD8^+$ double-positive (DP) thymocytes located in the cortex (146, 147, 156-158). Yet another model recently suggested that unknown factors trans-condition the propensity of thymocytes to give rise to T_{reg} even prior to positive selection (159). Importantly, any of these models would have to account for why developing T_{reg} are not subject to clonal deletion. Thus, some investigators have suggested a stochastic/selective mode of T_{reg} development, whereby thymocytes may randomly, i.e. in an at least initially antigen independent manner, commit to a developmental program that selectively protects developing T_{reg} from clonal deletion (160, 161). Alternatively, largely unknown signals provided by dedicated niches, e.g. particular stromal cell types and/or cytokine and co-stimulatory milieus, may favor T_{reg} development over clonal deletion (155, 162, 163). A variation of an instructive mode of Treg induction assumes a pivotal role of the avidity of self-antigen encounter (116, 164), thus bearing resemblance to classical models of positive selection.

Some of this controversy certainly arises from the fact that prospective identification of T_{reg} precursors remains a significant experimental challenge. Foxp3-reporter mice unable to express the functional Foxp3 protein have been instrumental in delineating the role of Foxp3 in the control of late stage T_{reg} differentiation and acquisition of full functional competence (120-122). However, because these studies position Foxp3 function relatively far downstream in T_{reg} development, they did not reveal the external cues or the molecular and phenotypic changes that coordinate T_{reg} differentiation upstream of Foxp3. Significant progress in this regard was very recently achieved by the demonstration that the $Foxp3^{-}CD25^{+}$ subset of polyclonal CD4 SP thymocytes is highly enriched in T_{reg} precursors (165). These cells appeared to represent the penultimate stage prior to Foxp3 expression, and acquisition of the "mature" $Foxp3^{+}$ Treg phenotype required only IL-2 or IL-15, but was largely independent of TCR engagement. These findings support a two-step model, whereby Treg development segregates into a TCR-driven "instructive" and a cytokine-driven "consolidation" phase. It remained open, however, at which stage of thymocyte development and by which stromal cell type the "instructive" TCR signal can be delivered. Similarly, these studies did not reveal the temporal relation between TCR signal and the acquisition of a $Foxp3^{+}$ T_{reg} phenotype.

For obvious reasons, these questions can essentially not be answered when studying the polyclonal T cell repertoire. Here, we used a previously described TCR transgenic model to circumvent these experimental limitations. In the TCR-HA × AIRE-HA model, thymocytes specific for the transgenic neo-self antigen influenza hemagglutinin (HA) efficiently differentiate into T_{reg} cells due to intrathymic expression of cognate antigen under control of the Autoimmune Regulator (aire) gene locus (155). We first verified that the precursor/progeny relationships that have

been outlined in the two-step model of polyclonal T_{reg} development also apply to the TCR-HA × AIRE-HA model. Using intrathymic transfer of post-positive selection thymocytes, we then asked whether T_{reg} conversion by agonist encounter can be temporally dissociated from the $CD4^+CD8^+$ double-positive stage of thymocyte development, and defined the temporal relation between “instructive” lineage priming and the acquisition of a mature T_{reg} phenotype. Furthermore, we aimed to reveal an eventual interrelation of a CD4 T cell’s maturation stage with its receptiveness for T_{reg} lineage commitment. Finally, we used the TCR transgenic model to address whether T_{reg} conversion of CD4 SP cells requires a dedicated antigen presenting cell.

5. Results

5.1. Heterogeneity of TCR-HA$^+$ CD4 SP cells in TCR-HA × AIRE-HA mice

Polyclonal CD25$^+$Foxp3$^-$ thymocytes have recently been shown to contain precursors of CD25$^+$Foxp3$^+$ cells (165). We first sought to ascertain that the critical hallmarks of this "two-step model of T_{reg} differentiation" also apply to the TCR-HA × AIRE-HA model.

Besides phenotypically and functionally mature CD25$^+$Foxp3$^+$ T_{reg} cells, TCR-HA$^+$ CD4 SP thymocytes from TCR-HA × AIRE-HA mice also contained Foxp3$^-$ cells. These could be subdivided into a CD25$^-$ and a CD25$^+$ subpopulation, in the following referred to as pre-T_{reg} 1 (PTR1) and PTR2, respectively (Figure 5A). Neither PTR1 nor PTR2 displayed suppressive potential *in vitro* (data not shown).

Compared to mature T_{reg}, PTR2 cells expressed similarly elevated levels of the activation markers GITR, PD-1 and CD44, whereas CD69 was expressed at substantially higher levels on PTR2 cells (Figure 5). PTR1 cells could be further subdivided into GITR$^+$ and GITR$^-$ cells. All PTR1 cells were highly positive for the early activation marker CD69, distinguishing them from truly "naïve" TCR-HA$^+$ CD4 SP thymocytes cells in TCR-HA single transgenic controls.

Analysis of maturation markers revealed that CD25$^+$Foxp3$^+$TCR-HA$^+$ cells were homogenously CD62L^{hi}. These cells also segregated into CD24^{hi} and CD24^{lo} as well as Qa-2^{hi} and Qa-2^{int} cells, respectively (Figure 6 A). PTR2 cells displayed a more immature phenotype (CD62L^{int}, CD24^{hi}, Qa-2^{lo}), and concerning these markers the surface phenotype of PTR1 cells closely resembled that of PTR2 cells.

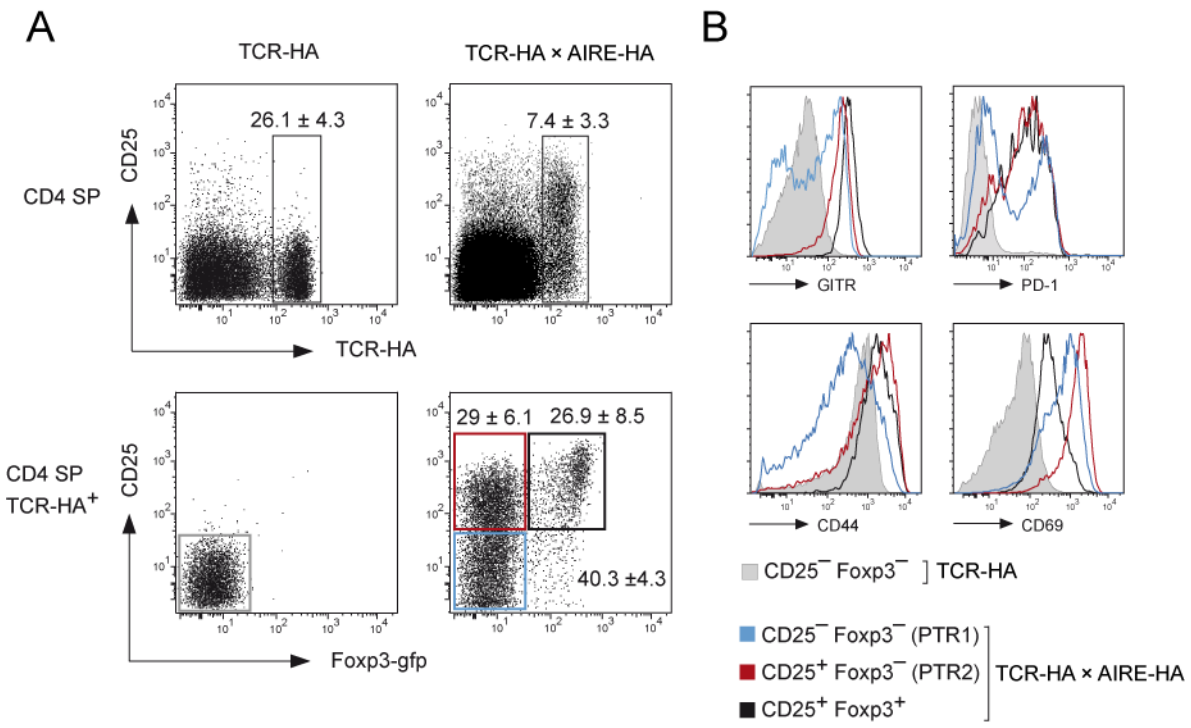

Figure 5 - Characterization of CD4 SP thymocyte subpopulations of TCR-HA × AIRE-HA. (A) Selection of T_{reg} in TCR-HA × AIRE-HA mice. The plots depict stainings of CD4 SP of 5-week-old TCR-HA or TCR-HA × AIRE-HA mice for TCR-HA and CD25 expression (upper panel) or Foxp3-gfp and CD25 expression on gated TCR-HA$^+$ CD4 SP cells (lower panel). Numbers indicate the average frequency (± s.d.) of cells within these areas. (n = 5 for TCR-HA mice, n = 35 for TCR-HA × AIRE-HA mice). (B) Activation marker expression of TCR-HA$^+$ CD4 SP cells. The histograms show overlays of naïve TCR-HA$^+$ cells and subpopulations of TCR-HA × AIRE-HA mice as outlined in the lower right panel of (A). Data are representative of 5 TCR-HA and 3 TCR-HA × AIRE-HA mice.

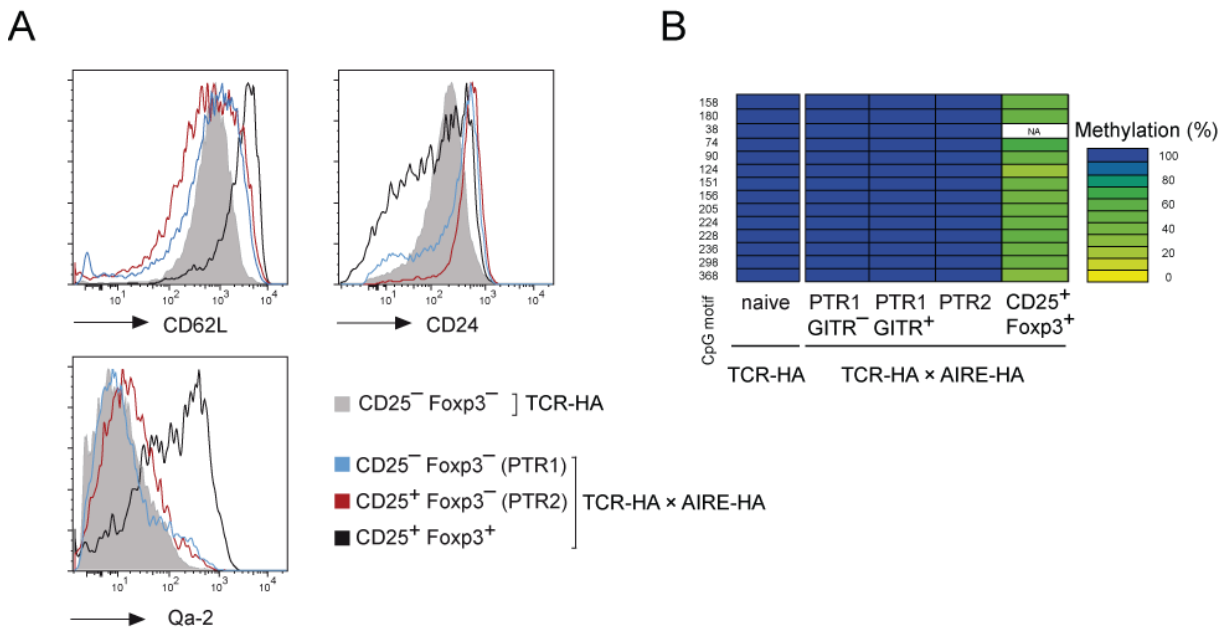

Figure 6 - Maturation marker expression and methylation state of CD4 SP subpopulations of TCR-HA × AIRE-HA mice. *(A)* The histogram shows overlays of naïve TCR-HA$^+$ cells and subpopulations of TCR-HA × AIRE-HA mice (as outlined in Figure 1 A) stained for the indicated maturation markers. (B) Methylation state of the TSDR within the Foxp3 gene. The DNA-methylation pattern of CpG motifs within an evolutionary conserved intronic enhancer region of the Foxp3 gene in indicated subpopulations of TCR-HA$^+$ CD4 SP of TCR-HA × AIRE-HA and TCR-HA mice is shown. Each box depicts the methylation-state of a single CpG motif according to the color code shown to the right.

Demethylation of CpG motifs within a conserved region of the foxp3 locus upstream of exon-1 is a hallmark of natural T_{reg}, and this T cell-specific demethylated region (TSDR) was found already to be mostly demethylated in developing Foxp3$^+$ thymocytes (126). Reminiscent of these observations in polyclonal T_{reg}, we found demethylation of about 60% of the CpG motifs in the TSDR of CD25$^+$Foxp3$^+$TCR-HA$^+$ cells (Figure 6 B). By contrast, neither PTR2 nor PTR1 cells showed evidence for DNA demethylation at these sites.

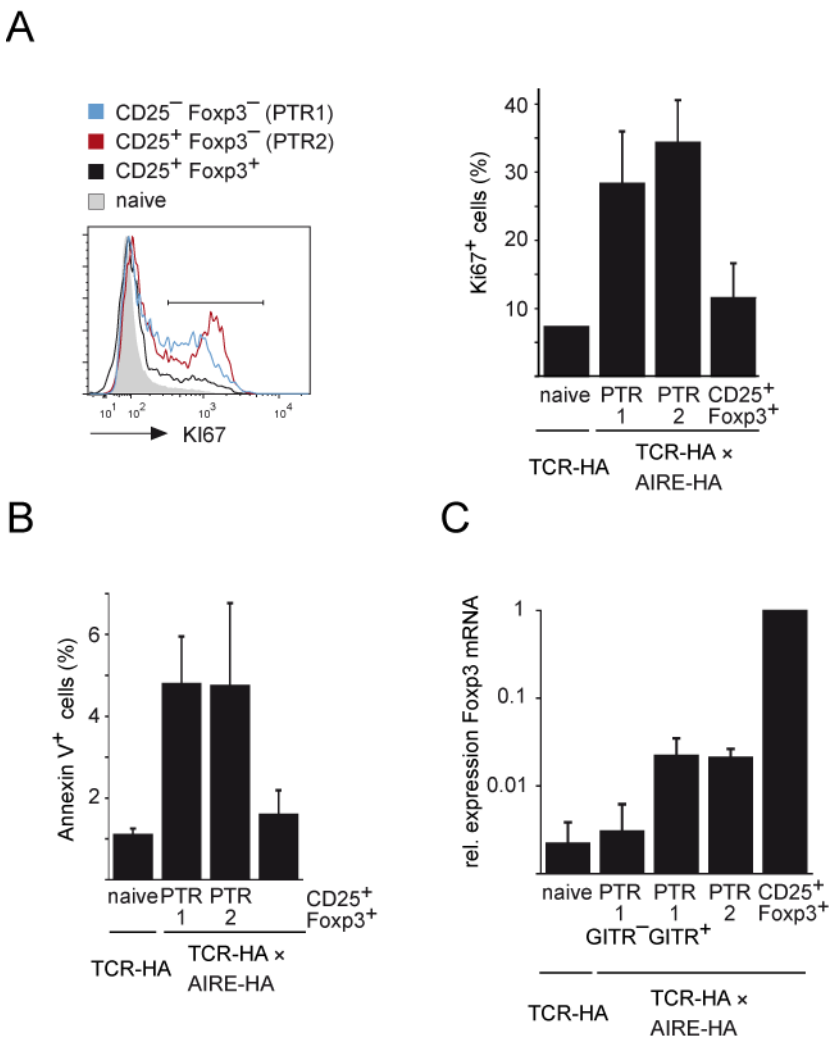

Figure 7 - TCR-HA$^+$ CD4 SP cells of TCR-HA × AIRE-HA mice show increased rates of proliferation and apoptosis and express varying levels of Foxp3 mRNA (A) Intracellular staining for the proliferation marker Ki67. The histogram (left) shows an overlay of representative stainings of subpopulations as defined in Figure 1 A. The bar-diagram (right) shows the average percentages (± s.d., n = 3) of Ki67$^+$ cells within subpopulations (gating as indicated in the histogram overlay). (B) The diagram depicts the average percentage (± s.d., n = 3) of apoptotic cells within subpopulations as determined by staining for Annexin-V. (C) Foxp3 mRNA expression of sorted subpopulations was quantified by real time PCR and normalized to the expression level of CD25$^+$Foxp3$^+$ Treg. The average normalized expression (± s.d., n = 3) is shown.

In contrast to CD25$^+$Foxp3$^+$TCR-HA$^+$ cells, PTR1 and PTR2 cells contained a substantial fraction of cells staining positive for the proliferation marker Ki67 (Figure 7 A). At the same time, the fraction of cells staining positive for the early apoptotic

marker Annexin-V was also elevated within PTR1 and PTR2 cells when compared to "mature" T_{reg} of TCR-HA × AIRE-HA mice (Figure 7 B).

The results so far indicated that essentially all $Foxp3^-$ subsets of TCR-HA × AIRE-HA mice contained antigen experienced cells. However, it remained open whether PTR1 and PTR2 may represent or at least contain precursors of $CD25^+Foxp3^+$ cells, or whether these cells had committed to a cell fate other than T_{reg} differentiation, e.g. abortive proliferation / negative selection. In favor of the former scenario, we detected elevated Foxp3 mRNA levels in $GITR^+$ PTR1 cells and in PTR2 cells as compared to $GITR^-$ PTR1 or naïve CD4 SP cells of TCR-HA single transgenic animals (Figure 7 C). Taken together, the phenotypic heterogeneity of TCR-HA^+ CD4 SP cells in the TCR-HA × AIRE-HA model recapitulated the essential phenotypic transitions that have recently been established for polyclonal T_{reg} development, consistent with the idea that at least a fraction of PTR1 and PTR2 cells in TCR-HA × AIRE-HA mice were T_{reg} precursors.

5.2. $Foxp3^-$ CD4 SP cells from TCR-HA × AIRE-HA mice can differentiate into $CD25^+Foxp3^+$ cells *in vivo*

In order to test eventual precursor/progeny relationships between the various subsets of TCR-HA^+ thymocytes from TCR-HA × AIRE-HA mice, we isolated $GITR^-$ PTR1, $GITR^+$ PTR1 and PTR2 cells (Figure 8 A) and transferred them intra-thymically (i.t.) into AIRE-HA recipients. On day four after transfer, most of the progeny of $GITR^-$ or $GITR^+$ PTR1 cells had up-regulated CD25, i.e. resembled PTR2 cells, and a substantial fraction of the progeny of $GITR^+$ PTR1 cells had indeed acquired a

$CD25^+Foxp3^+$ T_{reg} phenotype (Figure 8 B, upper panel). Transition into the T_{reg} compartment was most efficient for the progeny of PTR2 cells.

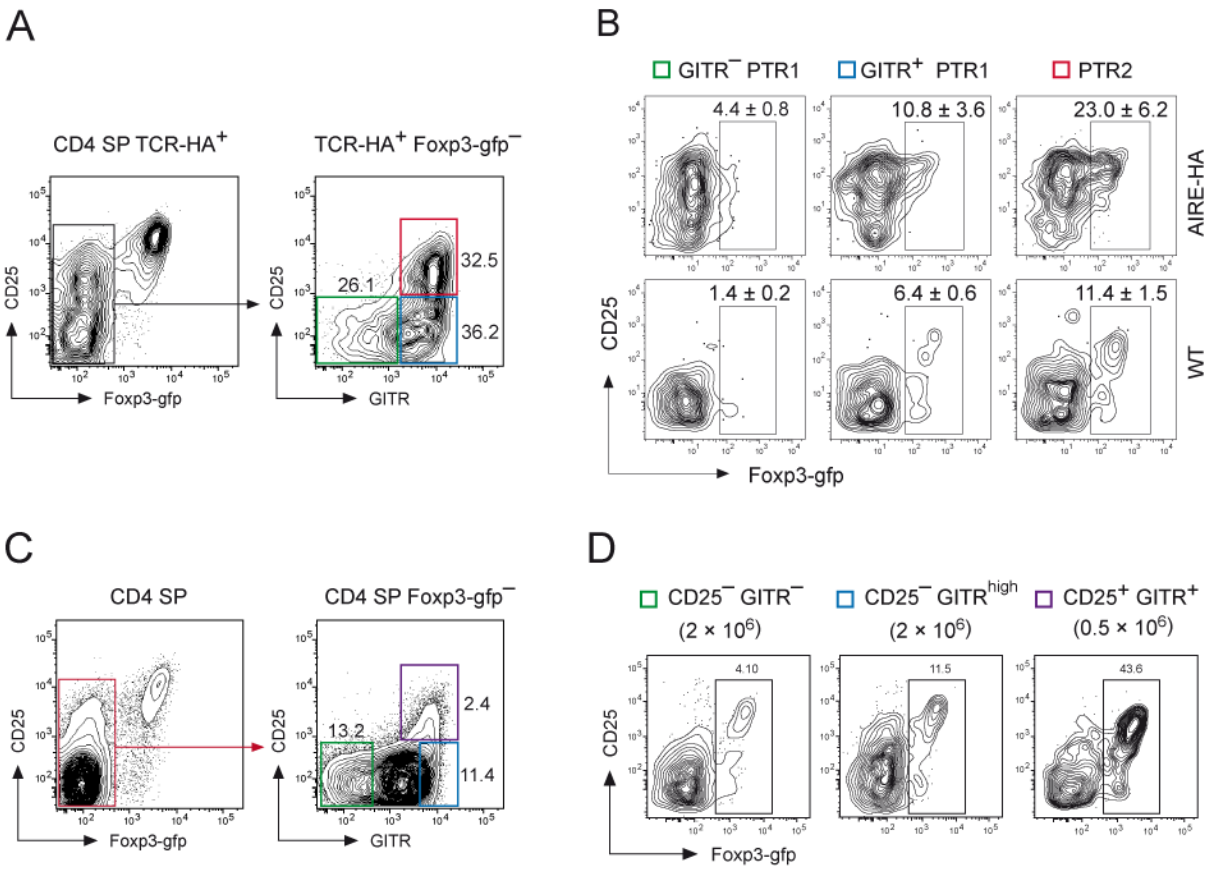

Figure 8 - $Foxp3^-$ subpopulations of CD4 SP thymocytes contain T_{reg} precursors Subsets of TCR-HA^+ Foxp3-gfp^- cells of TCR-HA × AIRE-HA CD45.1 mice were sorted based upon expression of GITR and CD25 (A). Numbers indicate the frequency of cells within sorting gates. 3 × 10^5 cells of each subpopulation were then transferred i.t. into AIRE-HA or WT recipient mice. Mice were sacrificed after four days and thymocytes were analyzed for Foxp3-gfp and CD25 expression on $CD45.1^+$ donor-type cells. Representative plots for each subset injected into either AIRE-HA or WT thymi are shown in (B). Numbers in plots indicate the average frequency (± s.d., n = 3) of Foxp3-gfp^+ cells recovered. Data are representative of four independent experiments. (C, D) Polyclonal CD4 SP subsets of CD45.1 mice were sorted as depicted in (C) and indicated numbers were transferred into WT recipient mice. After four days $CD45.1^+$ donor type cells were analyzed for Foxp3-gfp and CD25 expression. Representative plots for each subset are shown. Data are representative of two independent experiments.

These observations established that GITR$^+$ PTR1 and PTR2 cells contained cells with T_{reg} precursor potential that faithfully recapitulated the developmental potential of polyclonal thymocytes of the respective phenotype (Figure 8 C and D, and (165)).

It remained unclear whether GITR$^+$ PTR1 or PTR2 cells required continuous contact with agonist ligand in the host environment to realize their T_{reg} potential. We therefore performed i.t. transfers into WT recipients (Figure 8 B, lower panel). Under these circumstances, the majority of GITR$^-$ PTR1 cells remained CD25$^-$Foxp3$^-$. By contrast, among the progeny of GITR$^+$ PTR1 cells there was a considerable fraction of cells that had up-regulated Foxp3, indicative of antigen independent developmental progression towards a T_{reg} identity. However, in contrast to the observations in antigen-expressing recipients, these Foxp3$^+$ cells segregated into CD25$^+$ and CD25$^-$ subpopulations, suggesting inefficient induction or loss of CD25 in the absence of continuous antigen encounter. Similarly, although the capacity of PTR2 cells to progress towards a "mature" T_{reg} phenotype when deprived of continual stimulation by cognate antigen exceeded that of GITR$^+$ PTR1 cells, this process was less efficient than in AIRE-HA recipients.

Together, these data suggest a developmental progression in the order CD25$^-$Foxp3$^-$GITR$^-$ → CD25$^-$Foxp3$^-$GITR$^+$ → CD25$^+$Foxp3$^-$GITR$^+$, ultimately leading to the mature CD25$^+$Foxp3$^+$GITR$^+$ Treg phenotype. Moreover, as early as at the CD25$^-$Foxp3$^-$GITR$^+$ stage, some cells have undergone full commitment towards the T_{reg} lineage, such that subsequent to this developmental stage, further TCR stimulation appears to be beneficial, but not absolutely required for differentiation towards a CD25$^+$Foxp3$^+$ phenotype.

5.3. Antigen driven commitment to the T_{reg} lineage by "post-positive selection" CD4 SP thymocytes *in vivo*

One critical feature distinguishing the "altered positive selection" and the "altered negative selection" model of T_{reg} differentiation, i.e. the initiation of T_{reg} induction in the cortex or the medulla, respectively, is the postulation of discrete developmental windows of opportunity at which T_{reg} differentiation can be initiated. So far, our data indicated that the progression of CD4 SP thymocytes towards a "mature" T_{reg} phenotype in the TCR-HA × AIRE-HA model partitioned into an antigen-dependent and an antigen–independent phase, very much in analogy to what has recently been described for polyclonal T_{reg} development (165). However, these observations did not reveal whether the instructive TCR stimulus itself can occur at the CD4 SP stage, or whether prior contact with cognate antigen concomitant to positive selection at the $CD4^+CD8^+$ stage might be an essential prerequisite.

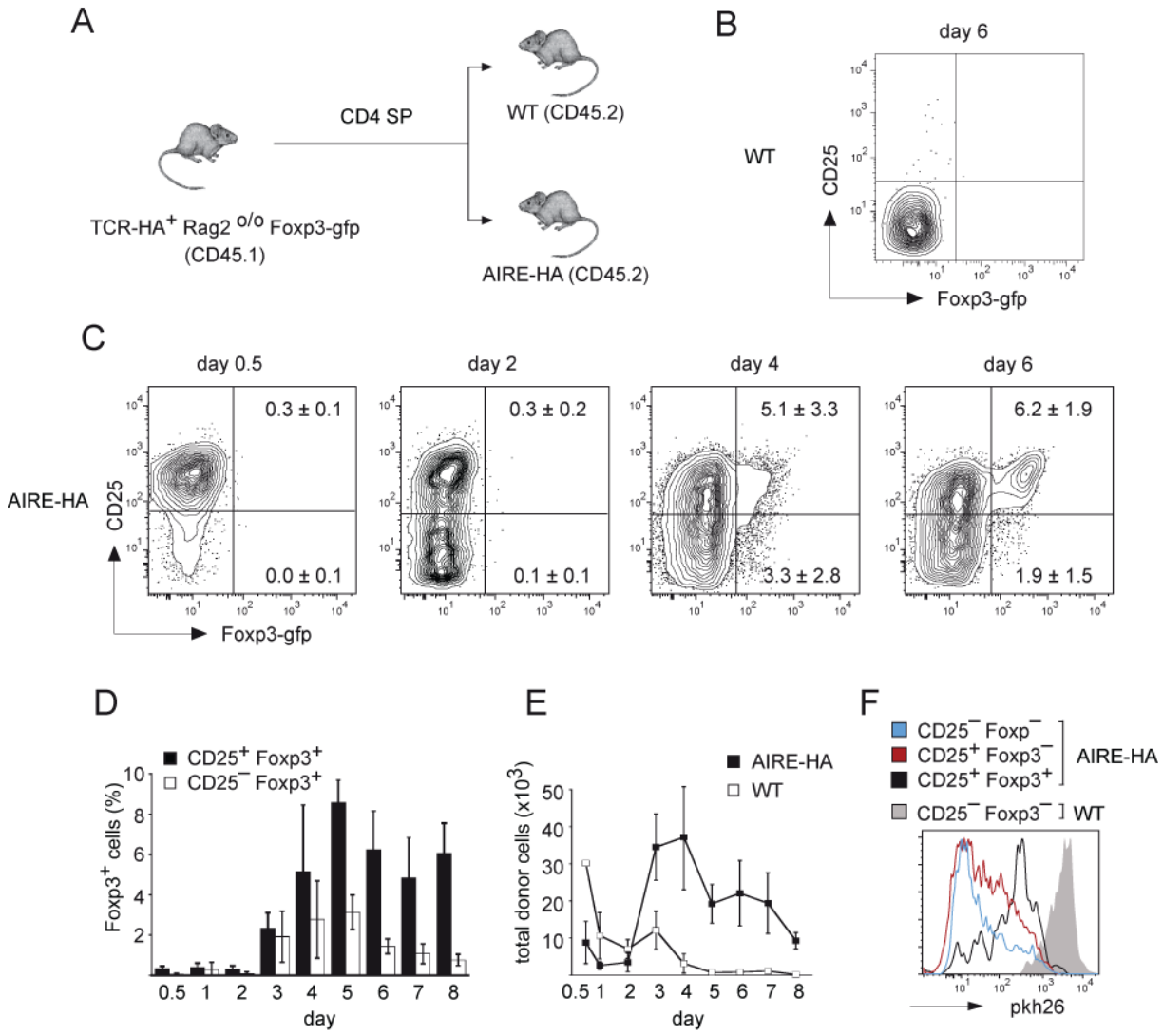

Figure 9 - Naïve TCR-HA$^+$ cells give rise to Treg after intrathymic injection into AIRE-HA recipient mice (A) Experimental design. 5 × 10^5 CD4 SP cells from CD45.1 TCR-HA$^+$ *Rag$^{o/o}$ Foxp3-gfp* mice were intrathymically transferred into either WT or AIRE-HA recipient mice (CD45.2). (B) In WT recipients, transferred cells exhibited a stable Foxp3$^-$CD25$^-$ phenotype. (C) Kinetics of intrathymic Treg development in AIRE-HA thymi. Injected cells were analyzed for Foxp3-gfp and CD25 expression at different time points as indicated above the plots. Numbers in quadrants indicate the percentage (± s.d., n = 3) of cells within the respective quadrant. (D) Emergence of CD25$^+$Foxp3$^+$ and CD25$^-$Foxp3$^+$ cells. The diagram depicts the average percentage (± s.d., n = 3) of CD25$^+$Foxp3$^+$ or CD25$^-$Foxp3$^+$ recovered from AIRE-HA recipient mice at the indicated time points. (E) Recovery of injected cells. The diagram shows the average absolute number (± s.d., n = 3) of cells recovered from intrathymically injected AIRE-HA or WT mice at the indicated time points. (F) Proliferation upon intrathymic antigen encounter. Pkh26 labeled cells were i.t. injected into either WT or

AIRE-HA recipient mice. The histogram shows an overlay of subpopulations recovered from AIRE-HA recipients and of $CD25^{-}Foxp3^{-}$ cells recovered from WT recipient mice five days after i.t. injection. Data are representative of three independent experiments.

In order to ask whether the initiation of T_{reg} differentiation by cognate antigen can be developmentally dissociated from positive selection and CD4 lineage commitment, we i.t. transferred CD4 SP cells obtained from TCR-HA *Foxp3gfp* *Rag2*$^{o/o}$ mice (Figure 9 A). These cells were free of any $Foxp3^{+}$ cells, and expectedly maintained a stable $CD25^{-}Foxp3^{-}$ phenotype following injection into control WT recipients (Figure 9 B). By contrast, intrathymic transfer into AIRE-HA recipients resulted in a swift and homogenous up-regulation of CD25 as early as twelve hours after injection (Figure 9 C). On day two, about half of the cells had down-regulated CD25 again. Between days three and four, $Foxp3^{+}$ cells appeared that segregated into $CD25^{-}$ and $CD25^{+}$ cells. $Foxp3^{+}CD25^{-}$ cells had essentially disappeared by day six, whereas the frequency of "mature" $Foxp3^{+}CD25^{+}$ cells reached a maximum between days four and five and remained relatively stable thereafter (Figure 9 D).

A second TCR transgenic system was used to verify that these findings can be generalized. Indeed, i.t. transfer of OVA-specific CD4 SP cells from DO11.10 *Rag2*$^{o/o}$ *Foxp3gfp* mice into recipients expressing the DO11.10 epitope fused to HA driven by an mTEC-specific bacterial artificial chromosome (BAC) transgene (designated AIRE-HCO) (155) yielded results that closely resembled the findings in the TCR-HA system, both in terms of percentages of $Foxp3^{+}$ cells recovered and the kinetics of their differentiation (Figure 10 A and B). Together, these findings clearly established that intrathymic T_{reg} differentiation can efficiently be initiated in a manner that is fully dissociated from positive selection.

In the steady state thymus of TCR-HA × AIRE-HA mice, differentiation into the T_{reg} lineage coincides with a substantial loss of TCR-HA$^+$ cells (Figure 1 and (155)), indicating that T_{reg} differentiation and negative selection are “competing” mechanisms of tolerance in this system. In order to address whether this scenario of concomitant T_{reg} induction and clonal deletion also applies to the “acute” TCR-HA → AIRE-HA i.t. transfer setting, we determined the absolute number of donor type cells at various time points after i.t. injection. Up to two days after injection, the recovery of donor cells in AIRE-HA thymi was considerably lower as compared to WT controls, most likely indicating that indeed a substantial fraction of the injected cells are subject to clonal elimination as a consequence of antigen recognition (Figure 9 E). Whereas in control WT recipients the number of donor cells continually decreased over time, most likely as a result of thymic egress (166), in AIRE-HA recipients a sharp increase in cell numbers set in on day two. The absolute number of donor cells in AIRE-HA recipients reached a maximum around day 4 and was then followed by a gradual decline. These dynamic changes suggested proliferative expansion of at least a fraction of the injected cohort of cells. To address this issue, donor cells were labeled with the fluorescent dye pkh26. Five days after transfer, essentially all progeny of cells injected into AIRE-HA hosts displayed a reduced pkh26 fluorescence, whereas cells injected into control WT hosts expectedly remained pkh26 bright, i.e. did not cycle to any measurable extent (Figure 9 F).

Of note, cells that had differentiated into Foxp3$^+$CD25$^+$ T_{reg} in AIRE-HA thymi retained substantially more pkh26 dye than Foxp3$^-$CD25$^+$ or Foxp3$^-$CD25$^-$ cells, indicative of a history of only limited proliferation. Consequently, the actual fraction of the starting population committing to a T_{reg} fate is considerably underestimated when

assessed solely on the basis of the relative abundance of T_{reg} cells among donor cells several days after transfer.

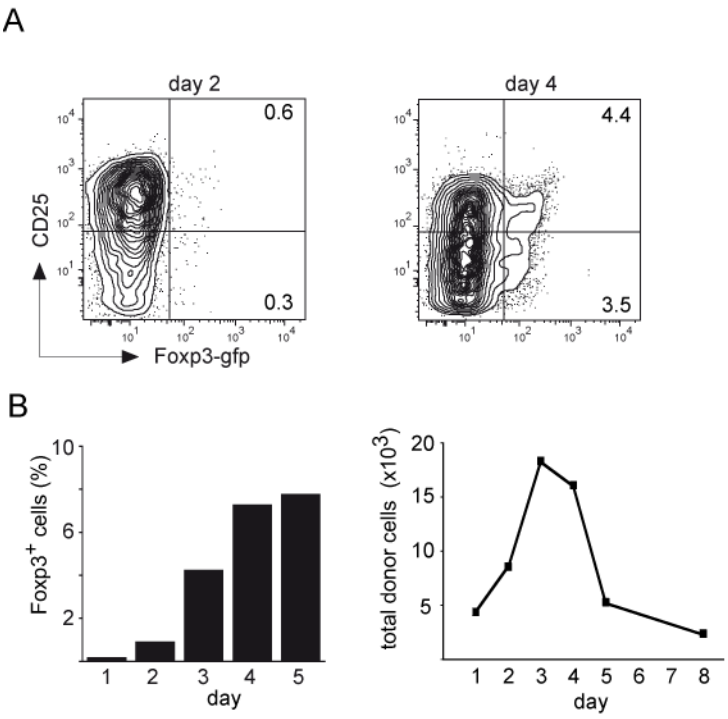

Figure 10 - DO11.10$^+$ CD4 SP cells give rise to Treg after intrathymic injection into AIRE-HCO recipient mice (A) CD4 SP thymocytes cells from CD45.1 DO11.10 *Rag$^{o/o}$ Foxp3-gfp* mice were i.t. transferred into AIRE-HCO mice and analyzed for Foxp3-gfp and CD25 expression at the indicated time points after injection. Numbers indicate the frequency of cells within the respective quadrants. (B) Kinetics of intrathymic T_{reg} development. The diagram depicts the percentage of Foxp3$^+$ cells (left) or absolute numbers of total donor cells (right) that were recovered at the indicated time points after i.t. injection.

The phenotypic heterogeneity of the progeny of CD4 SP donor cells injected into AIRE-HA recipients was strikingly reminiscent of the subsets of TCR-HA$^+$ CD4 SP cells in the steady state TCR-HA × AIRE-HA thymus (Compare Fig 5 A). Moreover, the emerging CD25$^-$Foxp3$^-$, CD25$^+$Foxp3$^-$ and CD25$^+$Foxp3$^+$ cells also seemed to

recapitulate the proliferative behavior of steady state PTR1, PTR2 and $CD25^+Foxp3^+$ subsets as revealed earlier using Ki67 staining (compare Figure 7 A).

Taken together, these data demonstrate that upon "acute" exposure to the AIRE-HA intrathymic environment, antigen inexperienced TCR-HA^+ CD4 SP cells can assume three different fates: Deletion, T_{reg} conversion or proliferative expansion.

5.4. Progressive maturation of CD4 SP thymocytes decreases their competence for T_{reg} differentiation *in vivo*

It is possible that the heterogeneous fate of a monoclonal cohort of SP thymocytes when injected into an antigen expressing host-thymus was at least in part caused by cell extrinsic factors such as cognate antigen recognition in distinct microenvironments. However, intrinsic heterogeneity within the starting population may also modulate the outcome of intrathymic antigen encounter. For example, it is well established that immature and mature CD4 SP cells display a differential susceptibility to negative selection (104), and we reasoned that the maturation stage of CD4 SP cells may similarly influence the efficacy with which these cells enter the T_{reg} lineage.

Progressive maturation of CD4 SP cells is characterized by a loss of CD69 expression and upregulation of CD62L expression. Accordingly, CD4 SP cells of TCR-HA *Rag2$^{o/o}$ Foxp3gfp* mice segregated into $CD69^+(CD62L^-)$ and $CD69^-(CD62L^+)$ subsets, and the levels of CD24 correlated with those of CD69 in the expected manner (Figure 11 A and data not shown).

When CD4 SP cells were fractionated on the basis of CD69 and CD62L expression and injected i.t. into AIRE-HA recipients, T_{reg} cells emerged at a higher frequency

among the progeny of CD69$^+$ “immature” cells (Figure 11 B). Along the same line, recovery of donor cells upon i.t. injection of CD4$^+$CD8$^+$ DP cells from TCR-HA *Rag2$^{o/o}$ Foxp3gfp* mice into AIRE-HA thymi was negligible, indicating efficient deletion in this setting, whereas upon i.t. injection of peripheral TCR-HA$^+$ CD4 T cells only a low number of cells was deviated into the T_{reg} lineage.

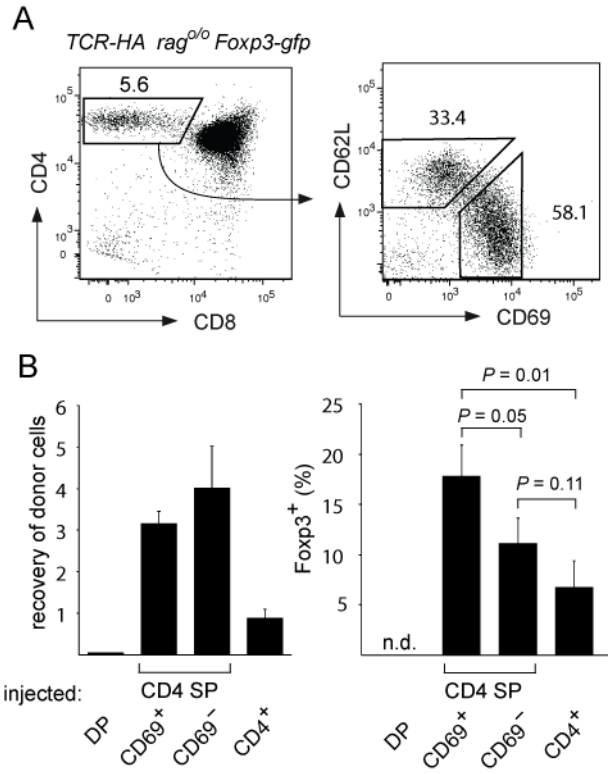

Figure 11 - The maturation stage of CD4 SP cells determines the efficiency of T_{reg} conversion *in vivo*. (A) Gated *TCR-HA Rag2$^{o/o}$* CD4 SP thymocytes (left) can be subdivided into immature (CD69$^+$CD62L$^-$) and mature (CD69$^-$CD62L$^+$) cells (right). Numbers indicate the percentage of cells in the respective gates. (B) Intrathymic transfer of thymocyte subpopulations and peripheral T cells. DP, CD69$^+$CD62L$^-$ CD4 SP, CD69$^-$CD62L$^+$ CD4 SP, and peripheral CD4$^+$ cells from *TCR-HA Rag$^{o/o}$ Foxp3-gfp* mice (CD45.1) were sorted and mixed with a PKH26 labeled reference population of total CD4 SP cells (CD45.1) at a ratio of 1 : 2.6 prior to intrathymic transfer into *AIRE-HA* recipients. After five days, the recovery of the different tester populations was determined as the ratio of the respective cells to

reference cells among donor cells ($CD45.1^+$) (left). The right diagram shows the percentage of Foxp3-gfp^+ cells among tester cells (n.d. = not detectable). Data are representative of two independent experiments with n = 3.

Taken together, these findings suggest a sliding scale of responsiveness in the CD4 T cell lineage with regard to the interpretation of cognate antigen encounter, with a predisposition of DP and presumably very early CD4 SP cells to undergo apoptosis, merging into a phase of susceptibility to T_{reg} conversion that ultimately gives way to a mature phenotype characterized by a strong propensity to proliferate.

5.5. The role of mTEC in T_{reg} differentiation *in vivo*

We had shown earlier that in the TCR-HA × AIRE-HA system efficient induction of T_{reg} was independent of antigen transfer and cross-presentation by DC, indicating that mTEC served a dual function as antigen-expressing and antigen-presenting cells (155). To further explore mechanistic commonalities between the steady state induction of T_{reg} in the TCR-HA × AIRE-HA model with the adoptive transfer system used here, we next addressed the contribution of hematopoietic versus thymic epithelial cells as APC. To this end, CD4 SP cells from TCR-HA *Rag2*$^{o/o}$ *Foxp3gfp* mice were adoptively transferred into the thymus of mice that carried the matching restriction element (I-E^d) on TEC, but were either sufficient or deficient in their ability to present the cognate antigen on hematopoietic cells. Recipient animals were generated by lethal irradiation of F1 H-$2^{b/d}$ AIRE-HA mice followed by reconstitution with H-$2^{d/d}$ or H-$2^{b/b}$ bone marrow cells (Figure 12 A).

In F1 AIRE-HA$^{bm\ H\text{-}2b/b}$ recipient animals, i.e. in the absence of the restriction element on hematopoietic cells, the induction of T_{reg} cells was similarly, if not more, efficient

as compared to hosts that expressed I-E^{d} also on hematopoietic APC (F1 AIRE-HA$^{bm\ H\text{-}2d/d}$) (Figure 12 B). Thus, cross-presentation of mTEC derived antigen by DC was dispensable, suggesting mTEC as the APC-type that co-ordinates the conversion of CD4 SP cells into T_{reg} cells in these settings.

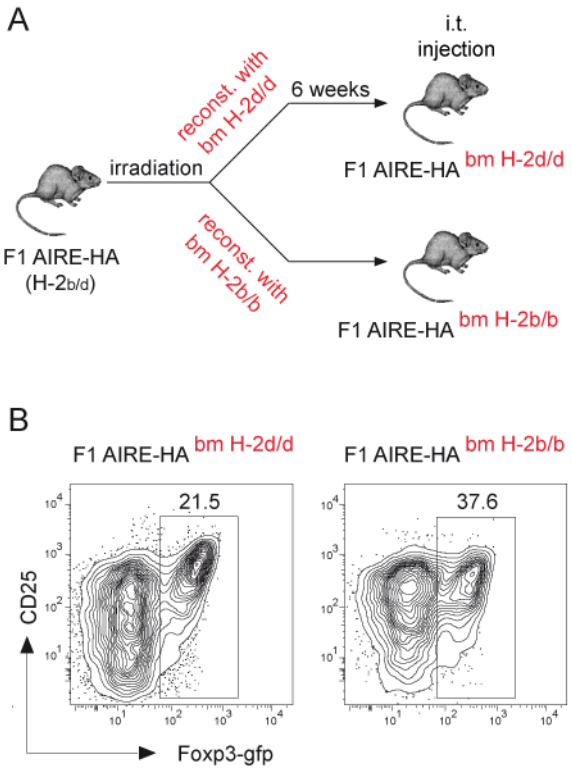

Figure 12 - Antigen presentation by hematopoietic cells is dispensable for T_{reg} induction upon i.t. injection (A) Generation of bone marrow chimaeric recipient mice. F1 H-2$^{b/d}$ AIRE-HA mice were lethally irradiated and reconstituted with either H-2$^{d/d}$ or H-2$^{b/b}$ bone marrow to generate F1 AIRE-HA$^{bm\ H\text{-}2d/d}$ or F1 AIRE-HA$^{bm\ H\text{-}2b/b}$ recipients, respectively. Six weeks later mice were injected with TCR-HA^{+} CD4 SP thymocytes (CD45.1). (B) Injected cells were analyzed for expression of Foxp3-gfp and CD25 five days after transfer. Numbers indicate the percentage of Foxp3-gfp^{+} cells. Two to three mice per group were injected and one representative plot per group is shown.

5.6. Deviation of CD4 SP cells into the T_{reg} lineage *in vitro* does not require a dedicated thymic stromal APC

Assessing the efficiency of T_{reg} induction *in vivo* upon i.t. transfer is complicated by the fact that eventually asynchronous thymic egress of cells either committing into the T_{reg} lineage or not can only insufficiently be controlled for. Thus, preferential retention or egress of a particular subset of cells may possibly complicate the correct interpretation of the actual efficacy or timing of T_{reg} induction. We considered the use of FTY720 as an inhibitor of thymic emigration, however, there is accumulating evidence that FTY720 itself may modulate the function or even promote the *de novo* induction of Treg cells (167-169). We therefore sought to establish an analogous *in vitro* system that on the one hand would eliminate these migration-related caveats, and on the other hand would reduce the complexity of cellular interaction partners.

We first sought to confirm that the recognition of endogenously expressed cognate antigen on mTEC was sufficient for T_{reg} conversion of CD4 SP cells in a minimal *in vitro* system. To this end, purified mTEC from AIRE-HA mice were co-cultured with naïve CD4 SP cells from TCR-HA *Rag2*$^{o/o}$ *Foxp3gfp* mice. After five days, cells were harvested and analyzed, revealing efficient induction of T_{reg} cells (ranging from 20% to 25% between experimental replicates, data not shown). Kinetic analyses indicated that the sequential emergence of Foxp3^{-}CD25^{+} and Foxp3^{+}CD25^{+} cells by and large reproduced the *in vivo* results (data not shown). Similar results were obtained using mTEC from AIRE-HCO mice and DO11.10 CD4 SP cells (data not shown).

In the Th1/Th2 paradigm, it is well established that multiple factors such as cytokines, APC-type and antigen dose to some extent influence the fate of mature helper T cells. We hypothesized that the avidity of cognate interactions in conjunction with

qualitative features of particular thymic stromal cells may in a similar way impinge on decision making by developing thymocytes. So far, our *in vivo* studies neither allowed delineating the relationship between antigen dose and efficiency of T_{reg} induction, nor did they exclude that stromal cells other than mTEC, e.g. thymic DC, may principally be capable of converting CD4 SP cells.

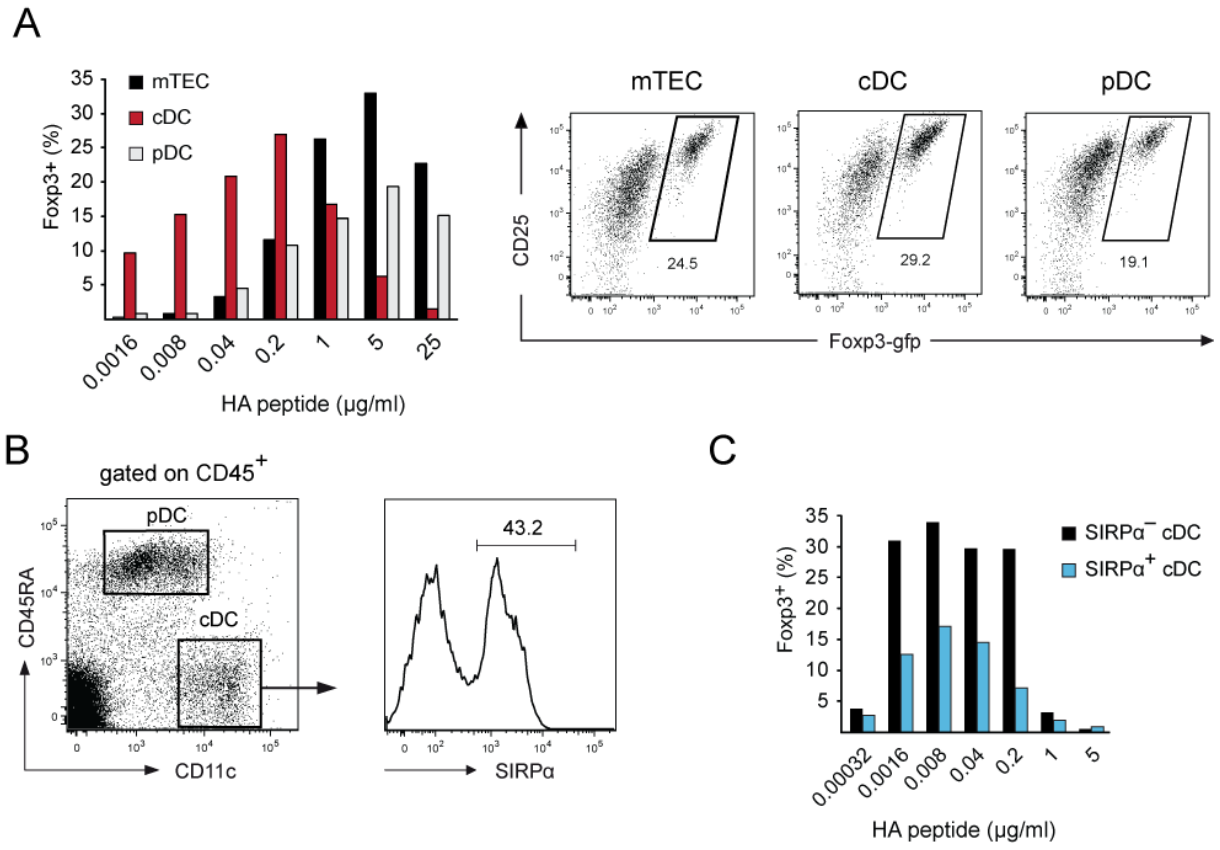

Figure 13 - Induction of T_{reg} cells by different thymic stromal APC *in vitro*. (A) *TCR-HA Rag2$^{o/o}$* CD4 SP thymocytes were co-cultured with mTEC, thymic cDC and thymic pDC in the presence of increasing amounts of HA peptide. After five days T cells were analyzed for CD25 and Foxp3-gfp expression. The bar diagram shows the percentage of Foxp3$^+$ cells recovered. One representative plot for mTEC, cDC and pDC induced T_{reg} differentiation at their respective optimal antigen dose is shown. (B) Separation of thymic DC into pDC, Sirpα$^+$ cDC, and Sirpα$^-$ cDC subpopulations. The histogram shows a Sirpα$^+$ staining of gated CD11c^{high} thymic dendritic cells (C) Sirpα$^+$ and Sirpα$^-$ cDC were co-cultured with TCR-HA$^+$ CD4 SP cells in the presence of increasing amounts of HA peptide. The diagram shows the percentage of Foxp3$^+$ cells recovered after 5 days.

To address the ability of different thymic stromal APC to induce $T_{reg,}$ TCR-HA$^+$ CD4 SP cells were co-cultured with mTEC, CD11c^{high}CD45RA$^-$ conventional DC (cDC) and CD11c^{int}CD45RA$^+$ plasmacytoid DC (pDC) in the presence of titrated amounts of HA peptide. Remarkbly, this revealed that both mTEC and thymic DC populations supported the differentiation of T_{reg} cells from CD4 SP *in vitro,* though with markedly different dose response curves (Figure 13 A).

Thus, T_{reg} differentiation induced by mTEC operated most efficiently at peptide concentrations that exceeded the respective optimum of both subsets of cDC by about two orders of magnitude. The optimal peptide concentration for pDC-mediated T_{reg} induction was in between that of cDC and mTEC. Conventional thymic DC have been further subdivided into two populations, based upon expression of SIRPα. Sirpα$^-$ and Sirpα$^+$ cDC have been shown to arise from different precursors, with the former arising from intrathymic progenitors and the latter considered to be a migratory population of DC arising extrathymically (170). Since these populations were recently suggested to differ in their potency to induce T_{reg} we co-cultured CD4 SP cells with sorted subpopulations of cDC (Figure 13 B) and titrated amounts of HA peptide. Both populations readily induced T_{reg} differentiation with Sirpα$^-$ cDC consistently showing a higher potency in doing so. Dose optima were similar for both cDC populations (Figure 13 C).

5.7. Differential predisposition of immature and mature CD4 SP thymocytes for T_{reg} differentiation *in vitro*

In another series of *in vitro* experiments, we confirmed and extended our *in vivo* findings concerning the inverse correlation of T cell maturation and the propensity to

undergo T_{reg} differentiation. The relative and absolute yield of $Foxp3^+CD25^+$ cells with mTEC or cDC at their respective peptide optimum was consistently higher with immature $CD69^+$ CD4 SP responders as compared to mature $CD69^-$ cells (Figure 14 A). Along these lines, conversion of peripheral $CD4^+$ responders from *TCR-HA Rag2$^{o/o}$ Foxp3-gfp* mice was barely detectable.

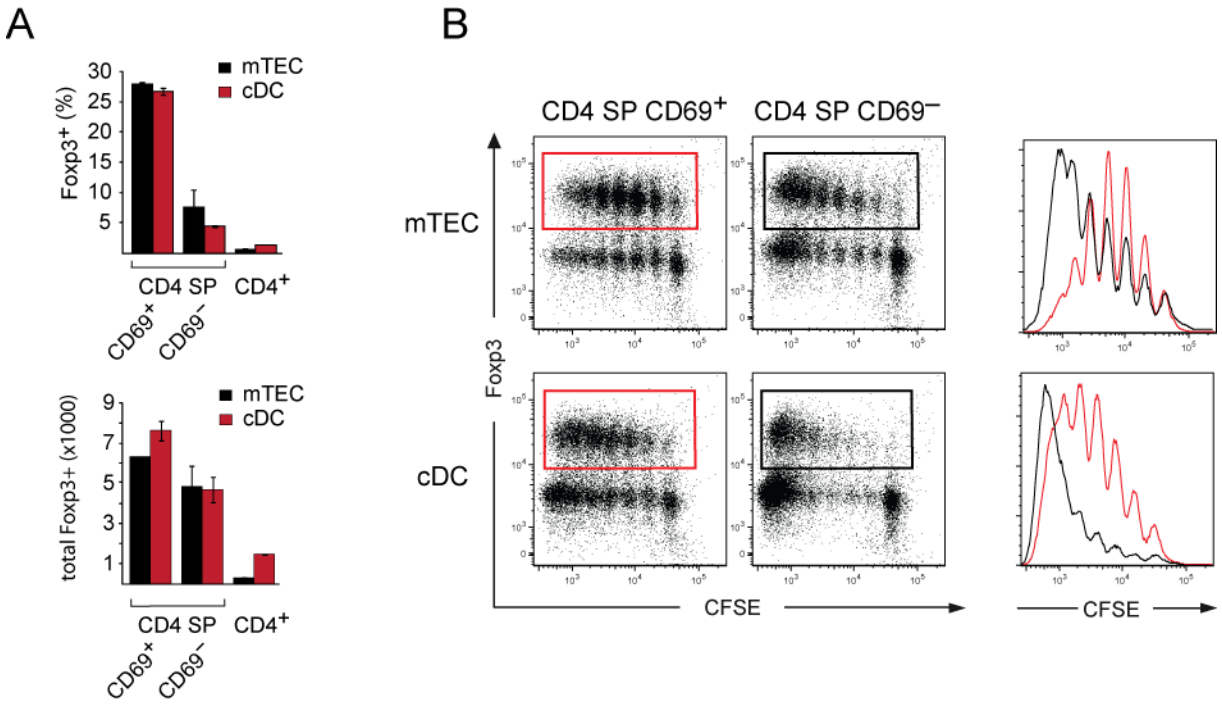

Figure 14 - T cell maturation impinges on the efficiency of T_{reg} induction *in vitro*. (A) The indicated T cell subpopulations were co-cultured with mTEC or cDC at their respective optimal peptide concentrations. The diagrams show the percentage (upper) or total number (lower) of $Foxp3^+$ cells recovered after 5 days. (B) Inverse correlation of T_{reg} induction efficiency and proliferation. CFSE labeled mature and immature CD4 SP cells from *TCR-HA Rag2$^{o/o}$ mice* were co-cultured with mTEC or cDC at their respective optimal peptide concentration and analyzed for Foxp3 expression and CFSE dilution after 5 days.

Whereas the predisposition for T_{reg} differentiation decreased with maturation of CD4 SP cells, the propensity to proliferate upon antigenic stimulation exhibited an inverse behavior (Figure 14 B). Thus, when CFSE-labeled $CD69^+$ or $CD69^-$ CD4 SP responder cells from *TCR-HA Rag2$^{o/o}$* mice were co-cultured with either mTEC or

cDC at the respective optimal peptide concentration, the progeny of mature cells went through a considerably higher number of cell cycles. This was seen with both types of APCs and irrespective of whether the T cells had acquired a $Foxp3^+$ phenotype or not (Figure 14 B).

Together, these findings not only confirmed the T cell intrinsic developmental control of T_{reg} development in an *in vitro* system of minimized complexity, but also revealed a surprising degree of redundancy among thymic stromal APCs in their principle capacity to support T_{reg} differentiation, given that APC-specific optimal doses of agonist ligand are provided.

5.8. T_{reg} differentiation of TCR transgenic and polyclonal thymocytes in an APC-free system

The largely redundant capacity of thymic APCs to orchestrate T_{reg} development may indicate that all of these cells similarly provide known and unknown critical co-stimulatory ligands or factors. Alternatively, T_{reg} differentiation of thymocytes may proceed with minimal requirements beyond a matching TCR stimulus and cytokine signaling. We therefore assessed T_{reg} differentiation in an APC-free system in which only signal 1 in the form of plate-bound anti-CD3 together with exogenous IL-2 would be present. Of several conditions tested (data not shown), anti-CD3 coated at 10µg/ml was found to induce the differentiation of significant numbers of $CD25^+Foxp3^+$ cells within *TCR-HA Rag2$^{o/o}$ Foxp3-gfp* thymocytes (Figure 15 A). Importantly, this APC-free system again recapitulated the gradual loss of competence for T_{reg} differentiation with progressive maturation within the CD4 lineage (Figure 14 A). Supporting the general relevance of our observations beyond

TCR transgenic systems, we observed an analogous behavior for polyclonal CD4 T cells of various developmental stages (Figure 15 B).

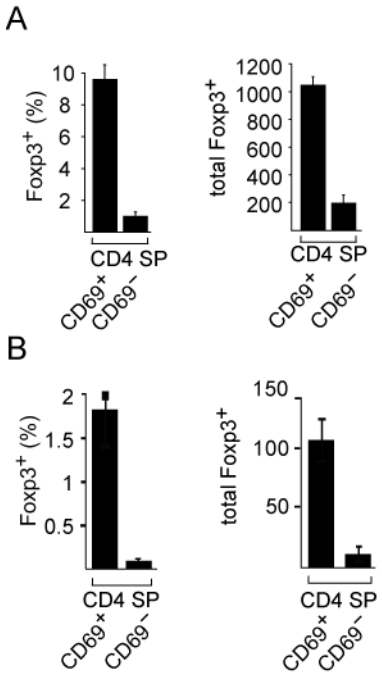

Figure 15 - T_{reg} induction in an APC-free system. (A) Immature and mature CD4 SP T cells from *TCR-HA rag$^{o/o}$ Foxp3-gfp* mice were sorted and cultured in the presence of plate-bound anti-CD3. Cells were analyzed for CD25 and Foxp3-gfp expression after 3 days. The percentage (left) and absolute numbers (right) of Foxp3$^+$ cells is depicted. (B) Immature (CD69$^+$CD62L$^-$) and mature (CD69$^-$CD62L$^+$) polyclonal CD25$^-$Foxp3$^-$ CD4 SP cells were sorted from *Foxp3-gfp* mice and cultured and analyzed as in A.

5.9. T_{reg} conversion of CD4 SP thymocytes requires TGFβ *in vitro*

Recent studies have cast doubt onto the commonly held view that the generation of "natural" T_{reg} in the thymus and the induction of "adaptive" T_{reg} from peripheral CD4$^+$ T cells follow fundamentally different rules regarding the requirement for TGFβ-signaling (137). To examine the contribution of TGFβ-signaling in our *in vitro* assays, we made use of both a monoclonal TGFβ-neutralizing antibody and the pharmacological inhibitor SB431542, which selectively inhibits activin-like kinase receptors. We first established the optimal conditions of TGFβ-inhibition that efficiently abrogated T_{reg} generation using splenic APC and saturating doses of exogenous TGFβ-1 (Figure 16 A and B). Using this mode of TGFβ-inhibition, we found a strong reduction of both mTEC- and thymic cDC-mediated T_{reg} conversion

(Figures 16 C and 16 D). Of note, the diminution of T_{reg} conversion, although profound, was not complete but rather shifted the dose response curve towards lower antigen doses, in particular with mTEC.

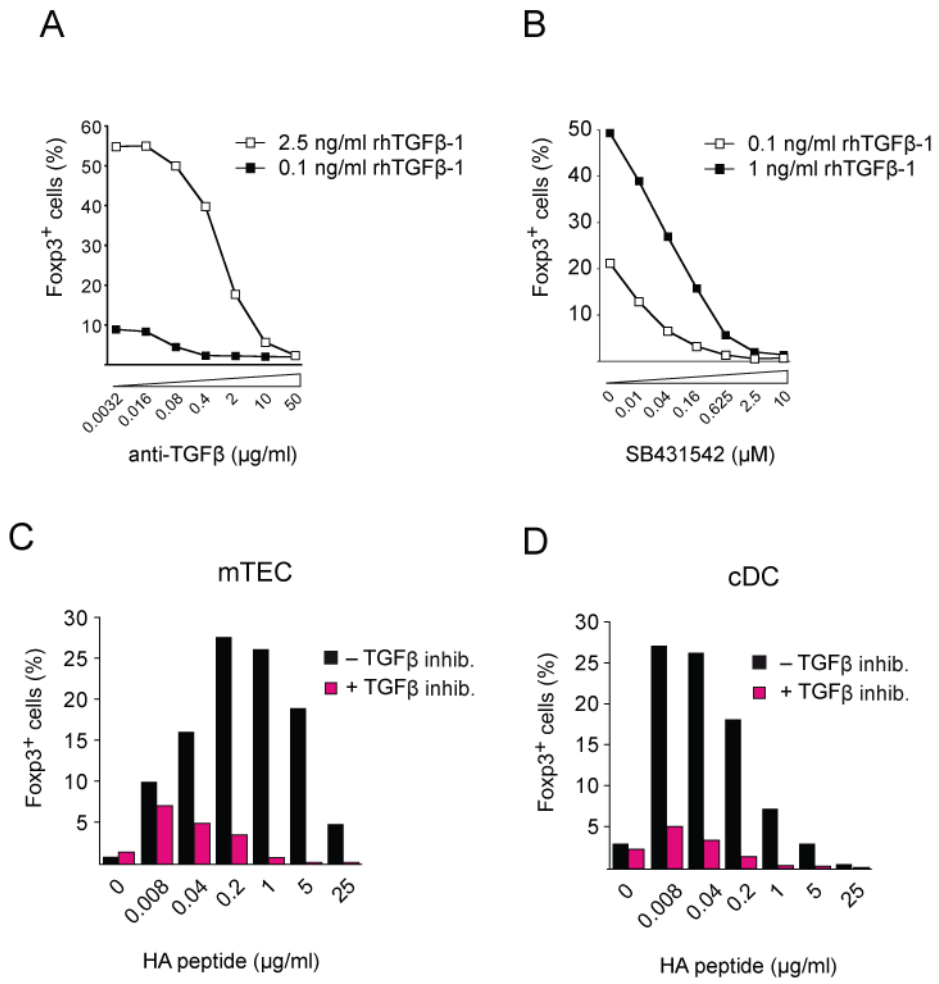

Figure 16 - Inhibition of T_{reg} induction by TGFβ-neutralizing antibody or the pharmacological inhibitor SB431542. (A) Naïve TCR-HA T cells were co-cultured for five days with splenic APC and 5µg/ml HA-peptide in the presence of 0.1 or 2.5 ng/ml TGFβ-1 and increasing concentrations of a TGFβ-neutralizing antibody. (B) Naïve TCR-HA T cells were co-cultured with splenic APC and 5µg/ml HA-peptide in the presence of 0.1 or 1 ng/ml rhTGFβ-1 and increasing concentrations of SB431542. The percentage of $Foxp3^+$ cells versus the concentration of either TGFβ-neutralizing antibody (A) or SB431542 (B) is shown. Data are representative of two independent experiments.(C, D) Effect of TGFβ-inhibition on T_{reg} differentiation *in vitro*. TCR-HA^+ CD4 SP cells were co-cultured with WT mTEC or WT

thymic cDC in the presence of the indicated concentrations of HA peptide with or without inhibition of TGFβ-signaling (TGFβ-neutralizing mAb plus the inhibitor SB431542 at concentrations of 10µg/ml and 3µM, respectively). T cells were analyzed for Foxp3-gfp and CD25 expression after five days. The percentage of Foxp3-gfp^{+} cells induced by mTEC (C) or thymic cDC (D) in the presence or absence of TGFβ-inhibition is shown. Data are representative of at least three independent experiments.

6. Discussion

The two-step model of T_{reg} development subdivides T_{reg} development into a first "instructive" phase requiring TCR engagement and a second "maturation/consolidation" phase relying solely on cytokine signals (165). The heterogeneity and developmental potential of TCR-HA$^+$ CD4 SP cells in the TCR-HA × AIRE-HA thymus was strikingly reminiscent of pre-Foxp3 stages of polyclonal T_{reg} development. Thus, a fraction of TCR-HA$^+$CD25$^-$GITR$^+$Foxp3$^-$ and TCR-HA$^+$CD25$^+$GITR$^+$Foxp3$^-$ thymocytes differentiated into TCR-HA$^+$CD25$^+$ GITR$^+$Foxp3$^+$ cells when transferred into a WT thymus, i.e. in the absence of further encounter of cognate antigen, emphasizing that this TCR transgenic model faithfully recapitulates intrathymic "natural" T_{reg} development.

Consistent with a confinement of Foxp3 function to late stage T_{reg} differentiation (120-122), we found that the characteristic hypomethylated state of the TSDR within the Foxp3 locus is established only rather late during intrathymic T_{reg} development. Expression of Foxp3 mRNA preceded loss of methyl marks, indicating that these epigenetic modifications at the Foxp3 locus are the consequence rather than the cause of commitment to the T_{reg} lineage. Pre-Foxp3 stages of steady state T_{reg} differentiation were characterized by low but significant levels of cell-cycling. Similarly, essentially all Foxp3$^+$ cells emerging upon i.t. injection of CD4 SP cells had gone through a low number of cell cycles. By contrast, excessive cycling appeared incompatible with T_{reg} differentiation *in vivo*. This scenario bears some resemblance to the inverse correlation of cell division and rate of T_{reg} conversion of peripheral CD4$^+$ T cells by suboptimal antigen doses (171) and to the role of cell-cycling during early thymocyte development, where at least one cell-cycle is thought to be

necessary for the DN-DP transition (172). It remains to be established whether changes in the methylation status of the TSDR are brought about by active demethylation or result from a lack of *de novo* methylation during cell division.

It is controversial whether the presumed instructive TCR stimulus that initiates T_{reg} differentiation needs to be delivered at a distinct stage of thymocyte development, e.g. the $CD4^+CD8^+$ DP or the CD4 SP stage (87, 146, 155-158). Here, using the intrathymic transfer of truly "antigen-inexperienced" CD4 SP cells we found that T_{reg} differentiation can efficiently be initiated at the CD4 SP stage. The nature and timing of phenotypic changes that accompanied T_{reg} differentiation ensuing from i.t. injection of TCR-HA^+ CD4 SP cells correlated well with the heterogeneity and developmental potential of CD4 SP cells present in the steady-state TCR-HA × AIRE-HA thymus. Thus, intrathymic T_{reg} induction can occur in a manner that is fully dissociated from positive selection and CD4/CD8 lineage commitment.

Thymectomy of 3-day-old mice leads to severe autoimmunity which has been attributed to a lack of T_{reg} cells (reviewed in (111)). The appearance of $Foxp3^+$ cells during ontogeny is delayed as compared to "mainstream" T cells. It was suggested that the neonatal paucity of T_{reg} is caused by the transient absence of an as yet undefined medullary niche facilitating T_{reg} induction (87). The kinetics of T_{reg} induction from CD4 SP cells reported here offer an alternative explanation. Thus, acquisition of the $Foxp3^+$ mature T_{reg} phenotype occurs only about three days after the instructive TCR signal. This indicates that even if all requirements for T_{reg} induction were met once the ontogenetically earliest wave of CD4 SP cells enters the medulla during the perinatal phase, emergence of "mature" T_{reg} cells would not be expected before another three days.

Initiation of T_{reg} differentiation at the CD4 SP stage implies a critical role of medullary APC, i.e. mTEC and/or DC. We found that T_{reg} induction in the adoptive transfer system was independent of presentation of the cognate antigen by thymic DC, consistent with our earlier observations in the steady state (155). Moreover *ex vivo* isolated mTEC supported the differentiation of T_{reg} from naïve CD4 SP cells *in vitro*. However, the comparison of thymic cDC, pDC and mTEC in the *in vitro* system revealed that antigen presentation by either cell type is compatible with T_{reg} induction, provided that the "optimal" antigen dose is available. Intriguingly, although we found earlier that mTEC and thymic DC deliver a similarly strong "signal one" at a given concentration of exogenously provided peptide as determined with a co-stimulation independent T hybridoma (155), the optimal concentration of antigen for T_{reg} induction *in vitro* was about two orders of magnitude higher for mTEC as compared to cDC. This discrepancy indicates that TCR-pMHC avidity is not the sole determinant of T_{reg} induction, and implies that a combination of quantitative and qualitative aspects of antigen recognition orchestrates T_{reg} differentiation. Irrespective of the latter considerations, our findings offer an explanation for why neither lack of MHCII on hematopoietic cells nor defective development of mTEC were reported to promote obvious perturbations in the polyclonal T_{reg} pool (20, 156), and may reconcile apparently conflicting results concerning the involvement of particular thymic stromal cell types in T_{reg} development (155, 163). We suggest that the similarly large thymic T_{reg} population in the absence of antigen presentation on either mTEC or thymic DC (20, 147, 155, 156) is a reflection of homeostatic mechanisms acting downstream of *bona fide* differentiation processes, rather than an indication of true redundancy at the level of TCR specificities. With the help of large scale sequencing and fixed TCRβ chains, this may be a testable hypothesis (119, 173).

Is the emergence of rapidly cycling $CD25^+$ cells that do not upregulate Foxp3 an artifact of intrathymic injection of CD4 SP cells? Studying polyclonal thymocytes, Lio et al. found that less than half of the CD4 SP $CD25^+Foxp3^-$ thymocytes differentiate into $CD25^+Foxp3^+$ cells upon injection into a WT thymus (165). While the presence of heterogeneous specificities among polyclonal $CD25^+Foxp3^-$ cells might explain this observation, we prefer an alternative interpretation. Thus, even when we i.t. injected a monoclonal cohort of T cells that was purified to phenotypic homogeneity (e.g. CD4 SP $CD69^+CD24^{hi}$) into an environment that was principally permissive for T_{reg} induction, the progeny of these cells segregated into $CD25^+Foxp3^-$ and $CD25^+Foxp3^+$ cells. We propose that these rapidly cycling $CD25^+Foxp3^-$ cells are the equivalent of a large fraction of the polyclonal $CD25^+Foxp3^-$ thymocytes. Indeed, polyclonal $CD25^+Foxp3^-$ cells also contain a fraction of cycling cells (our unpublished observation). A subset of these cells may engage in abortive proliferation, as evidenced by the increased frequency of Annexin-V^+ cells. However, it is also tempting to speculate that at least some of these $CD25^+Foxp3^-$ cells produce cytokines, most likely IL-2, to facilitate terminal differentiation of T_{reg} cells in the thymus and/or are direct precursors of peripheral $CD25^{lo}Foxp3^-$ cells, which have been implicated in the production of IL-2 necessary for T_{reg} homeostasis (174).

T_{reg} are commonly subdivided into so called natural T_{reg}, which arise during intrathymic selection, and adaptive T_{reg}, which are products of peripheral conversion (175). This distinction implies fundamentally different principles underlying the emergence of either class of T_{reg}. For example, TGFβ was commonly believed to be dispensable for intrathymic generation of a diverse T_{reg} repertoire (176), but essential for the conversion of mature $CD4^+$ T cells (124, 177, 178). However, this concept has recently been challenged and TGFβ may indeed serve a more universal role in the

induction of both subgroups of T_{reg} (137). The results reported here cast further doubt onto a formal distinction between natural and adaptive T_{reg} with respect to mechanistic aspects of their differentiation. Of note, our data do not exclude that there is a low avidity window in which T_{reg} deviation of thymic and peripheral cells might occur in a TGFβ independent manner (179).

The question remains: What discriminates whether a developing CD4 T cell is subject to clonal deletion rather than deviation into the T_{reg} lineage? There is growing evidence that the thymic medulla represents a mosaic of stromal niches in which self-antigens, in particular so called “peripheral tissue antigens”, may not be homogeneously available (68, 180). As a consequence, serial contacts with thymic stromal cells may be required until an autoreactive CD4 SP thymocyte engages in a tolerogenic interaction with an APC presenting the respective self-antigen at appropriate levels. If and when during the four to five days residence time of CD4 SP cells in the medulla such a rendezvous occurs would be subject to stochastic fluctuations. It has been described more than a decade ago that during progressive maturation at the CD4 SP stage, thymocytes undergo a quite dramatic switch from “tolerance susceptibility” to “tolerance resistance”, whereby tolerance was used synonymously with clonal deletion at the time (104). How T_{reg} induction fits into this scenario remains to be established. For example, it remains unclear at which developmental stage (pre- or post-positive selection) the principle capacity to branch off into the T_{reg} lineage is acquired. Nonetheless, our data show that once established, this capacity persists well into the CD4 SP stage, yet gradually decreases during progressive maturation. Ultimately, “aged” peripheral CD4 T cells displayed a dramatically reduced propensity to differentiate into T_{reg} when exposed to conditions that efficiently convert CD4 SP thymocytes. We propose that thymocytes

that escape negative selection, e.g. due to limited access to self-antigen presented by rare stromal APC, immediately after losing the susceptibility to be deleted enter a phase of exquisite “susceptibility” to being deviated into the T_{reg} lineage. In other words, TCR triggering with the exact same avidity and in the exact same context may be integrated in different ways by CD4 T cells at particular developmental stages. We therefore argue that decision making by individual autoreactive thymocytes cannot sufficiently be explained solely on the basis of an avidity model, not even when limited cytokine resources are incorporated (164). Instead, we propose that any model of T_{reg} development also needs to consider the somewhat underexplored aspect of developmental progression within the CD4 SP compartment and an ensuing sliding scale of responsiveness (see Figure 17).

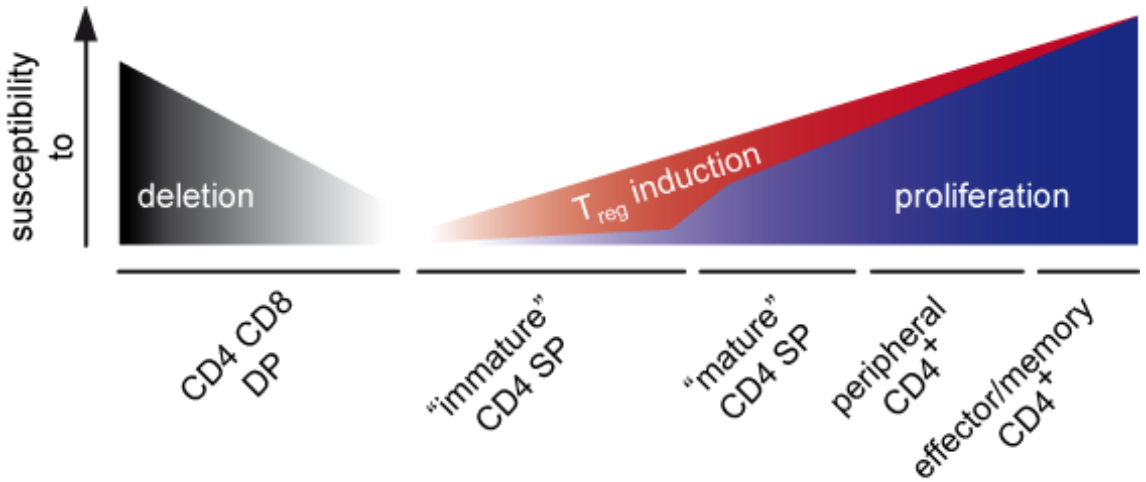

Figure 17 – Integration of TCR-triggering at distinct T cell developmental stages. Strong TCR-triggering results in fundamentally different outcomes in T cells of different maturity. While DP cells are efficiently deleted, maturation coincides with a reduction of susceptibility to undergo apoptosis that is virtually lost or has to be induced by different mechanisms in mature CD4 SP and peripheral cells. The loss of susceptibility to undergo apoptosis is followed by the gradual acquisition of strong

proliferative potential. In between these phases of preferential induction of apoptosis and the induction of proliferation cells pass through a stage of exquisite receptivity to T_{reg} induction. Most likely there are overlaps between phases of recessive or dominant tolerance induction with deletion and T_{reg} induction happening in parallel in populations of thymocytes as defined above. The lack of proliferative potential might favor T_{reg} induction as opposed to proliferation. The molecular basis for this difference is currently unknown, however.

7. Material and Methods

7.1. Animals

Mice were bred in the animal facility of the Research Institute of Molecular Pathology or in the animal facility of the Institute for Immunology of the LMU Munich in individually ventilated cages. TCR-HA, AIRE-HA, AIRE-HCO and DO11.10 mice have been described (155, 181, 182). Foxp3^{gfp} knock-in mice were kindly provided by Dr. Alexander Rudensky (88). Mice were backcrossed to BALB/c for at least five generations. All animal studies were approved by local authorities.

7.2. Antibodies and flow cytometry

Biotin-conjugated monoclonal antibodies (mAbs) to CD8 (53-6.7), CD24 (30-F1), GITR (DTA-1), Qa-2 (1-1-2), CD44 (IM7), CD62L (MEL-14), CD45RA (14.8), and CD69 (H1.2F3), PE-conjugated streptavidin, Annexin-V, and mAbs to Ki67 (B56), Qa-2 (1-1-2), PD-1 (J43), and GITR (DTA-1), CyChrome-conjugated mAb to CD8 (53-6.7), APC-conjugated CD45.1 (A20), and CD172a/Sirp α (P84), APC-Cy7 conjugated mAb to CD4 (GK1.5), and PE-Cy7 conjugated Streptavidin and mAb to CD25 (PC61) were obtained from Becton Dickinson. The mAbs to TCR-HA (6.5) and DO11.10 (KJ26) were purified and conjugated to Alexa647, PE, or biotin in our labaratory. Anti–mouse TGF-ß1/2 (1D11.16.8) was purified in our lab. Surface staining was done according to standard procedures at a density of 1 × 10^6 to 4 × 10^6 cells per 50µl, and volumes were scaled up accordingly. Ki67 and Annexin-V stainings were done according to the manufacturer's instructions. A FACSCanto

(Becton Dickinson) with FACSDiva software (Beckton Dickinson) was used for data acquisition, data analysis was done using FlowJo software (Tree Star Inc.).

7.3. Genotyping

For genotyping, mouse tail pieces were digested in 50 µl of tail buffer for 6 hours at 55°C, followed by Proteinase K inactivation at 95°C for 5 minutes. The following solutions/conditions were used for the tail digest:

Gitocher buffer (10x)

670mM Tris pH 8.8

166mM ammonium sulfate

65mM MgCl2

0.1% Gelatin

Digestion reaction

3mm mouse tail

3µl Proteinase K (10mg/ml stock)

2.5µl Triton (10% Stock)

5µl Gitocher Buffer (10x)

0.5µl β-Mercapto-ethanol

39µl H_2O

1 µl of the 50µl-digestion reaction mixture was used as a template for the genotyping-PCR reaction. The following buffers/solutions and conditions were used for the genotyping-PCR reactions:

PCR Buffer (5x)

250mM KCl

50mM Tris pH 8.3

43% Glycerol

7.5mM MgCl2

2mM Cresol Red

PCR reaction

1µl template

250nM primers (final)

200µM dNTP (final)

PCR Buffer (1x final)

Taq Polymerase

Ad 30µl with H_2O

PCR protocol

Temperature (°C)	duration (s)	cycles
94	**180**	
94	45	2
60	45	
72	60	
94	45	2
58	45	
72	60	
94	45	2
56	45	
72	60	
94	45	30
54	45	
72	60	
72	**300**	

Primers used for genotyping

	sequence		**amplicon**
fwd	ACAAGGTGGCAGTAACAGGA	**TCR-HA**	~800bp
rev	ACAGTCAGTCTGGTTCCTGA		
fwd	GCAACATGTTATCCAGTAGCCGGT	**RAG2**	~600bp WT
rev	TTGGGAGGACACTCACTTGCCAGT		~1000bp Rag2 ko
int	GTATGCAGCCGCCGCATTGCATCA		
fwd	CAGGAGGGATCCAGTGCCAGC	**DO11.10**	~290bp
rev	TGGCTCTACAGTGAGTTTGGT		
fwd	ACAGCCACTCCTGTCTTTGC	**AIRE-HA**	~1300bp
rev	CTCCGTCAGCCATAGCAAATTTCT		
fwd	ACAGCCACTCCTGTCTTTGC	**AIRE-HCO**	~1200bp
rev	GAATTGTTCGCATGGTAGCC		
fwd	AGACAGACCAGAGGTGTAGT	**Foxp3^{gfp}**	~200bp WT
rev	TCCTGGGGATGGGCCAAGGGCCAAGG		~800bp Foxp^{gfp}
fwd	AGACAGACCAGAGGTGTAGT	**Foxp3^{gfp}**	~200bp WT
rev	TCCTGGGGATGGGCCAAGGGCCAAGG		~800bp Foxp^{gfp}

7.4. Preparation of thymic stroma

Thymi from 3-week-old mice were cut into small pieces and were digested at 37°C in IMDM containing 0.2 mg/ml of collagenase (Roche), 0.2 mg/ml of dispase I (Roche), 2% (vol/vol) FCS, 25 mM HEPES, pH 7.2, and 25 µg/ml of DNase I. Cells were re-suspended every 5 min. After 25 min fresh medium was added and cells were digested for another 15 min, followed by the addition of EDTA (5mM final concentration) and incubation for 5 min. Cells were then washed and re-suspended in Percoll (ρ, 1.115; GE Healthcare). A discontinuous gradient was then generated by the addition of a layer of Percoll (ρ, 1.055) followed by a layer of IMDM on top of this cell suspension. Gradients were spun for 30 min at 1,350g at 4°C. To avoid disturbance of the gradient before and after centrifugation, the acceleration of the centrifuge was reduced and the brakes were switched off. After centrifugation, low-density cells were collected from the upper interface, washed and stained for cell sorting. Stromal cells were sorted according to CD45, Ly51, EpCAM, CD11c, CD45RA and Sirpα expression (mTEC = $CD45^{-}Ly51^{-}EpCAM^{+}$; pDC = $CD45^{+}CD11c^{int}CD45RA^{+}$; $Sirp\alpha^{+}$ cDC = $CD45^{+}CD11c^{high}CD172a^{+}$; $Sirp\alpha^{-}$ cDC = $CD45^{+}CD11c^{high}CD172a^{-}$). The following buffers/solutions were used for the preparation of thymic stromal cells:

Percoll gradient solutions

100ml high densitiy percoll (ρ = 1.115g/ml):

90ml Percoll (GE Healthcare, ρ = 1.134g/ml)

10ml 10x PBS

25mM HEPES pH 7.2 (final)

low densitiy percoll (ρ = 1.055g/ml):

20ml Percoll (GE Healthcare, ρ = 1.134g/ml)

26ml 1x PBS

25mM HEPES pH 7.2 (final)

optional:

low densitiy percoll (ρ = 1.045g/ml):

20ml Percoll (GE Healthcare, ρ = 1.134g/ml)

31.1ml 1x PBS

25mM HEPES pH 7.2 (final)

7.5. Purification of CD4 SP and CD4$^+$ peripheral cells

CD4 SP cells from TCR-HA *Rag2$^{o/o}$Foxp3^{gfp}* mice or subpopulations of TCR-HA × AIRE-HA Foxp3^{gfp} mice were purified by staining of thymocyte cell suspensions with biotinylated- anti-CD8 antibody, followed incubation of cells with streptavidin Microbeads (Miltenyi Biotech) and depletion of CD8$^+$ cells using LS-MACS columns (Miltenyi Biotech). 20µl of MACS Microbeads plus 180µl of MACS Buffer were routinely used for 10^8 cells. The CD8$^-$ fraction was stained for the indicated surface markers and cells were sorted using a FACSAria cell sorter (Beckon Dickinson).

Naïve peripheral cells were obtained by staining of a pooled suspension of Spleen and lymph node (mesenteric, axillary, brachial, inginual, popliteal and cervical) cells with biotinylated anti-CD4 antibody, followed by incubation of cells with streptavidin Microbeads and enrichment of CD4$^+$ cells using LS-MACS columns. The CD4$^+$

fraction was stained for the indicated surface markers and cells were sorted using a FACSAria cell sorter.

7.6. Intrathymic transfer

The indicated number of CD4 SP or peripheral cells was purified from donor mice (CD45.1) as described above, washed two times in PBS, re-suspended in PBS and intrathymically tranferred into one thymic lobe of congenic WT (CD45.2) or AIRE-HA (CD45.2) recipient mice using a Hamilton syringe. The cells were transferred in a volume of 3µl.At various time points after transfer mice were sacrificed, thymi were depleted of $CD8^+$ cells as described above, stained for surface markers and analyzed by flow cytometry using a FACS Canto II (Becton Dickinson).

7.7. *In vitro* differentiation assay

For *in vitro* differentiation assays thymic stromal APC and T cells of various developmental stages were purified as described above. Sorted thymocytes or peripheral T cells (5×10^4) from TCR-HA *Rag2*$^{o/o}$*Foxp3*^{gfp} mice were co-cultured with sorted mTEC, thymic cDC (SIRPα$^{+/-}$) or thymic pDC (1×10^4) of AIRE-HA or WT mice in the presence of the indicated amount of $HA^{107-119}$ peptide (SVSSFERFEIFPK, recognized by the TCR-HA in the context of I-E^d) and 100 U/ml recombinant IL-2 (Preprotech). TGFβ-signaling was inbibited by the addition of a TGFβ neutralizing antibody (1D11.16.8) and the activin receptor-like kinase inhibitor SB431542 (Sigma-Aldrich) at a final concentration of 10µg/ml and 3µM, respectively. Analysis was carried out after three or five days.

The following media was used for the differentiation assays:

7.8. Assessment of the methylation status of the TSDR

To assess the methylation status of the TSDR within the first intron of the Foxp3 gene the indicated populations of thymocytes of TCR-HA or TCR-HA × AIRE-HA were purified by FACS-sorting. Subsequently, genomic DNA was purified using the NucleoSpin Tissue XS Kit (Macherey-Nagel) according to the manufacturer's instructions. DNA methylation analysis was performed by bisulfite sequencing as described previously (126).

Media for primary T cell culture

IMDM (Gibco)

10% FCS

0.292mg/ml L-Glutamin

100U/ml Streptomycin

100U/ml Penicilin

50µM β-Mercaptoethanol

7.9. Bone marrow chimaeras

6 week old [BALB/c × BL/6]$_{F1}$ (H-2$^{b/d}$) AIRE-HA mice were lethally irradiated (2 × 450 rad split dose) and were reconstituted with 5 × 10^6 T cell depleted C57BL/6 or BALB/c bone marrow cells by injection into the lateral tail vein immediately after the second irradiation. Intrathymic transfer was carried out 6 weeks after bone marrow reconstitution as described above. After five days injected thymi were analyzed both for chimaerims, by digesting a piece of the thymus and subsequent staining for I-E^d

or I-A^b expression on thymic DC, and for the phenotype of donor derived cells as described above. The chimaerism was routinely greater than 97%.

7.10. RNA isolation and Real time PCR

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche) and transcribed into cDNA using the Superscript II Kit (Invitrogen) according to the manufacturer's instructions. Quantification of Foxp3 mRNA levels was carried out using a MyiQ cycler (Biorad) and the iQ SYBR Green Supermix (Biorad). Expression levels were normalized to the expression levels of thymic Treg and calculated using the formula $x = 2^{-\Delta\Delta Ct}$, where Ct represents the threshold cycle of a Foxp3 and ΔCt represents the difference between Ct values of Foxp3 and the housekeeping gene β-actin. ΔΔCt is the difference between the ΔCt values of the examined subpopulations and the ΔCt value of thymic Treg. Intron-spanning primers for Foxp3 (fwd: 5'-GGCCCTTCTCCAGGACAGA -3', rev: 5'- GCTGATCATGGCTGGGTTGT -3') and β-actin (fwd: 5'-GCCTTCCTTCTTGGGTAT -3', rev: 5'- GGCATAGAGGTCTTTACGG -3') were tested by recording a melting curve and by agarose gelelektrophoresis.

Program used for Real Time PCR

Temperature (°C)	duration (s)	cycles
94	**300**	
94	45	40
56	45	
72	60	
72	60	final elongation

Melting curves were recorded between 55 and 95°C (+0.5°C every 30s).

7.11. Statistical analysis

Statistical significance was assessed by the two-tailed Student`s t-test.

7.12. Additional Buffers

10 x PBS *(10l)*

800g NaCl,

20 g KCl

115g Na2HPO$_4$ · 2H$_2$O

20g KH2PO4, pH adjusted to 7.2 - 7.4

FACS Buffer

1 x PBS, 2% FCS, 1 mM EDTA

MACS Buffer

1 x PBS 0.5% BSA, 2mM EDTA (sterile filtered with 0.45µm filters)

8. References

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