Development of regulatory T cells in the human thymus

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ACADEMIC DISSERTATION

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To Viljami and Pietari
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ABSTRACT

The role of the immune system is to protect an organism against pathogens while maintaining tolerance against self. T cells are an essential component of the immune system and they develop in the thymus. The AIRE (autoimmune regulator) gene product plays an important role in T cell development, as it promotes expression of peripheral tissue antigens in the thymus. Developing T cells, thymocytes, which recognize self-antigens with high affinity are deleted. However, this deletion process is not perfect and not all autoreactive T cells are destroyed. When the distinction between self and non-self fails, tolerance breaks and the immune system attacks the host’s own tissues. This results in autoimmunity.

Regulatory T cells contribute to the maintenance of self-tolerance. They can actively suppress the function of autoreactive cells. Several populations of cells with regulatory properties have been described, but the best characterized population is the natural regulatory T cells (Treg cells), which develop in the thymus and express the transcription factor FOXP3. The thymic development of Treg cells in humans is the subject of this thesis.

Thymocytes at different developmental stages were analyzed using flow cytometry. The CD4^+CD8^- double-negative (DN) thymocytes are the earliest T cell precursors in the T cell lineage. My results show that the Treg cell marker FOXP3 is up-regulated already in a subset of these DN thymocytes. FOXP3^+ cells were also found among the more mature CD4^+CD8^- double-positive (DP) cells and among the CD4^- and CD8^- single-positive (SP) thymocytes. The different developmental stages of the FOXP3^+ thymocytes were isolated and their gene expression examined by quantitative PCR. T cell receptor (TCR) repertoire analysis was used to compare these different thymocyte populations. My data show that in humans commitment to the Treg cell lineage is an early event and suggest that the development of Treg cells follows a linear developmental pathway, FOXP3^+ DN precursors evolving through the DP stage to become mature CD4^+ Treg cells.

Most T cells have only one kind of TCR on their cell surface, but a small fraction of cells expresses two different TCRs. My results show that the expression of two different TCRs is enriched among Treg cells. Furthermore, both receptors were capable of transmitting signals when bound by a ligand. By extrapolating flow cytometric data, it was estimated that the majority of peripheral blood Treg cells are indeed dual-specific. The high frequency of dual-specific cells among human Treg cells suggests that dual-specificity has a role in directing these cells to the Treg cell lineage.

It is known that both genetic predisposition and environmental factors influence the development of autoimmunity. It is also known that the dysfunction or absence of Treg cells leads to the development of autoimmune manifestations. APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) is a rare monogenic autoimmune disease, caused by mutations in the AIRE gene. In the absence of AIRE gene product, deletion of self-specific T cells is presumably disturbed and autoreactive T cells escape to the periphery. I examined whether Treg cells are also affected in APECED.

I found that the frequency of FOXP3^+ Treg cells and the level of FOXP3 expression were significantly lower in APECED patients than in controls. Additionally, when studied in cell cultures, the suppressive capacity of the patients’
Treg cells was impaired. Additionally, repertoire analysis showed that the TCR repertoire of Treg cells was altered. These results suggest that AIRE contributes to the development of Treg cells in humans and the selection of Treg cells is impaired in APECED patients.

In conclusion, my thesis elucidates the developmental pathway of Treg cells in humans. The differentiation of Tregs begins early during thymic development and both the cells’ dual-specificity and AIRE probably affect the final commitment of Treg cells.

Autoreaktivisia soluja saattaa kuitenkin päästää elimistoön, jolloin ne voivat aiheuttaa autoimmuneuitautajeja. T-solujen toimintaa ommel elmiöstöä vastaan voivat estää säätelijä- eli regulatoriset T-solut (Treg-solut). Tämä vääjöskirjatutkimus selvittää Treg-solujen kehitystä ihmisessä.


Väitöskirjatutkimukseni tulokset antavat uutta tietoa Treg-solujen kehityksestä ihmisen kateenkorvassa. Treg-solujen erilaistuminen alkaa hyvin varhaisessa vaiheessa, ja AIRE-geeni sekä kahden eri T-solureseptorin pintaekspressiio vaikuttavat niiden kehitykseen.
ABBREVIATIONS

AIRE    autoimmune regulator
APC    antigen presenting cell
APECED   Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal
dystrophy
CDR    complementarity determining region
cTEC   cortical thymic epithelial cell
CTLA-4    cytotoxic T lymphocyte antigen 4
DC    dendritic cell
DN    double-negative CD4\(^{-}\)CD8\(^{-}\) thymocyte
DP    double-positive CD4\(^{+}\)CD8\(^{+}\) thymocyte
eTAC    extrathymic Aire-expressing cells
ETP    early thymic progenitor
FACS   fluorescence-activated cell sorting
FOXP3   forkhead box P3
\(\gamma_c\)    common \(\gamma\) chain
GFP    green fluorescent protein
GITR   glucocorticoid-induced tumor necrosis factor receptor
HLA    human leukocyte antigen
HSC   hematopoietic stem cells
IDO    indoleamine 2,3-dioxygenase
IFN-\(\alpha\)    interferon alpha
IFN-\(\gamma\)    interferon gamma
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<tr>
<td>IPEX</td>
<td>Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked</td>
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<td>ISP</td>
<td>immature single-positive</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
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<td>LAG3</td>
<td>lymphocyte activation gene 3</td>
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<td>LT-α</td>
<td>lymphotoxin alpha</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cells</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PTA</td>
<td>peripheral tissue-antigens</td>
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<tr>
<td>pTα</td>
<td>pre T cell receptor α chain</td>
</tr>
<tr>
<td>RAG 1&amp;2</td>
<td>recombination activating genes 1&amp;2</td>
</tr>
<tr>
<td>SP</td>
<td>single positive CD4⁺ or CD8⁺ thymocyte</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>thymic epithelial cell</td>
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<td>Term</td>
<td>Description</td>
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<tr>
<td>Th1</td>
<td>T helper 1 cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2 cell</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 cell</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<td>TREC</td>
<td>T cell receptor excision circle</td>
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<tr>
<td>Treg cell</td>
<td>regulatory T cell</td>
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<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
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<tr>
<td>TSP</td>
<td>thymus seeding progenitor</td>
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ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by Roman numerals.


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REVIEW OF THE LITERATURE

1 T CELL SUBSETS

T lymphocytes or T cells are a central component of cell-mediated immunity. The T cells compose the adaptive immune system in conjunction with the B cells. The T cells recognize their specific antigens through a T cell receptor (TCR). The majority of the T cells express an accessory molecule on their cell surface, either CD4 or CD8. The T cells are divided into two main subsets according to the expression of these molecules: The CD4+ T cells recognize antigens presented by antigen presenting cells (APC) expressing major histocompatibility complex (MHC) class II molecules, while the CD8+ T cells recognize antigens in MHC class I molecules (Andersen et al. 2006). These molecules are discussed in depth later.

1.1 CD8+ T cells

Upon activation, the CD8+ T cells differentiate into CD8+ cytotoxic T cells, which kill their target cells by inducing apoptosis. The CD8+ T cells are particularly important in immunologic defense against viral infections, and also against other intracellular pathogens. Further, they recognize dysfunctional or malignant host cells. The elimination of infected or dysfunctional cells without damaging the healthy tissue requires that the cytotoxic mechanisms are targeted accurately. The CD8+ T cells can be activated directly by dendritic cells (DC). Usually, however, additional assistance is required. It is provided by the CD4+ T cells, which recognize related antigens on the surface of the same APC (Janeway et al. 2008).

The CD8+ T cells utilize two different mechanisms in mediating the apoptosis, both require direct cell-to-cell contact. Once the cytotoxic T lymphocyte has recognized its antigen on the surface of the target cell, it releases cytotoxic granules into the intercellular space. The cytotoxic granules contain cytotoxins: perforin, granzymes, and in humans, granulysin. Perforin acts in the delivery of the contents of the granules through the target cell membrane, and thereupon the granzyme molecules released from the granules to the cytosol trigger the apoptosis by activating the caspase pathway. Granulysin has antimicrobial effects and can induce apoptosis.

Another mechanism by which the CD8+ T cells can induce apoptosis is Fas-mediated killing. The CD8+ T cells have a Fas ligand (CD95L or CD178) on their cell surface. When it binds to the Fas molecule (CD95) on the target cell, apoptosis is induced (Andersen et al. 2006, Harty et al. 2000). The third mechanism by which the cytotoxic T cells are known to act is through cytokines, for example interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and lymphotoxin-α (LT-α). The IFN-γ enhances the expression of MHC class I molecules and other proteins involved in the peptide loading of the MHC class I molecules. Consequently, the presentation of endogenous peptides increases, as well as the chance that infected cells will be recognized. TNF-α and LT-α induce apoptosis through TNF receptor I (TNFR-I). They also act synergistically with the IFN-γ in activating macrophages. The IFN-γ also inhibits viral replication and viruses can thus be removed so that the infected cell survives (Harty et al. 2000).
1.2 CD4+ T cells

The majority of the CD4+ T lymphocytes function as helper T cells. The CD4+ T cell population also includes the subset of regulatory T cells (Treg cells) (Sakaguchi et al. 2008). The most important function of the activated CD4+ T cells is to activate and control the immune response and the cells taking part in it. The CD4+ T cells influence other immune cells, both with molecules on the cell surface and by secreting cytokines. For example, the interaction of the co-stimulatory molecule CD40L (CD154) on a CD4 T cell with CD40 on the APC activates the APC and induces diverse downstream effects depending on the target cell type (Jiang and Chess 2006).

After activation, the CD4+ T cell population can be separated to various subsets. The fate of the progeny of a naïve CD4+ T cell is largely regulated by signals provided by the local cytokine milieu. Each naïve CD4+ T cell has the potential to differentiate to any of the T helper subsets and the pathway to which they commit depends on the signals received during activation. Activation of the transcription factor of a certain subset confirms the differentiation to that lineage. Activated effector T cells provide feedback and reinforce the priming of naïve T cells. Cytokines produced by a given T cell subset function as inducers of that subset and inhibit the differentiation of other subsets. The variety of cytokines affects substantially the incipient immune response as a whole (Janeway et al. 2008, Weaver et al. 2006).

1.2.1 Th1 Cells

The differentiation to T helper 1 (Th1) cells is favored when macrophages, DCs and NK cells secrete IFN-γ and IL-12. These cytokines induce expression of the transcription factor T-bet (Szabo et al. 2000), which is an essential regulator of Th1 commitment. Activation of T-bet in turn triggers IFN-γ production and expression of IL-12 receptor β2 subunit, both further directing the cell to the Th1 lineage. Th1 cells participate in defence against intracellular pathogens, particularly viruses. They activate especially macrophages, NK cells and CD8+ T cells. In addition, Th1 cytokines help to activate B cells and, in humans, transmit signals for immunoglobulin class switching to IgG1 and IgG3, which opsonize pathogens (Zhu and Paul 2008, Kondo and Martin 2001).

1.2.2 Th2 cells

The Th2 cells contribute to humoral immunity by activating B lymphocytes. They take part in the extermination of parasites and extracellular bacteria. The Th2 cells develop when the predominant cytokine is IL-4. The initial source of IL-4 is still unclear, but once the effector Th2 cells are generated, they promote the differentiation to the Th2 lineage by secreting IL-4 themselves. Consequent signaling leads to the activation of the transcription factor GATA-3 (Ouyang et al. 2000) in the T cell and production of cytokines IL-4, IL-5, IL-10 and IL-13. The Th2 cells induce immunoglobulin class switching, particularly to IgG2 and IgG4, and in addition conversion to IgE and IgA. Production of eosinophils and mast cells is also increased (Zhu and Paul 2008, Kondo and Martin 2001).
1.2.3 Th17 cells
The most recently discovered Th cell subset is the Th17 cells. Cytokines inducing the differentiation to Th17 cells are IL-6 and TGF-β in mice (Stockinger et al. 2007), and in humans the crucial cytokines seem to be IL-6 and IL-1β (Acosta-Rodriguez et al. 2007). Also IL-23 appears to be important for Th17 cell development and expansion, but it is not required for the Th17 commitment. The key regulator of Th17 development is RORγt (Ivanov et al. 2006) and a characteristic of these cells is the abundant production of cytokines belonging to the IL-17 family, hence the name Th17. The Th17 cells act against extracellular pathogens and they appear to boost the acute inflammatory response of innate immunity. They induce epithelial surfaces or fibroblasts, epithelial cells and keratinocytes to secrete molecules to recruit neutrophils and augment the production of neutrophils and macrophages from the bone marrow (Korn et al. 2009).

1.2.4 Natural Treg cells
There are also several subsets of T cells with suppressive capacity. These are called regulatory T cells (Roncarolo and Levings 2000). The best-known population is the CD4+CD25+ natural regulatory T cells (Sakaguchi et al. 1995a, Baecher-Allan et al. 2001, Stephens et al. 2001), which express the transcription factor forkhead box P3 (FOXP3) (Fontenot and Rudensky 2005, Ziegler 2006). These cells commit to the regulatory phenotype during their development in the thymus. They are actively involved in maintaining immune tolerance (Sakaguchi et al. 2008). These cells are the focus of this thesis and are discussed in depth later. It has been reported that there are also thymus-derived CD8+CD25+FOXP3+ cells (Cosmi et al. 2003), but their significance in the periphery is still unknown.

1.3 Other regulatory T cells
In contrast to the natural FOXP3 expressing regulatory T cells, adaptive regulatory T cells develop from naïve CD4+ T cells in the periphery. Th3 cells are probably activated in the mucosa and they seem to control or suppress responses on the mucosal surfaces. Like Th2 cells, they produce IL-4 and IL-10, but they also secrete TGF-β (Weiner 2001, Inobe et al. 1998). Tr1 cells are induced in vitro when stimulated with high concentrations of IL-10. They produce IL-10 and TGF-β, but to make a distinction with Th3, they do not secrete IL-4 (Groux et al. 1997). The activity of these cells is antigen-specific (Roncarolo and Levings 2000). Further, a population of induced regulatory T cells expressing FOXP3 has been described. FOXP3 is induced when naïve CD4+ T cell are under influence of TGF-β instead of IFN-γ, IL-12 or IL-4 (Chen et al. 2003, Kretschmer et al. 2005). Retinoic acid enhances the TGF-β mediated induction of FOXP3 (Coombes et al. 2007, Sun et al. 2007). In mice these cells produce TGF-β and have other suppressive mechanisms. Additionally antigen presentation under suboptimal conditions induces FOXP3+ suppressor cells (Kretschmer et al. 2005, Apostolou and von Boehmer 2004). The features of the regulatory populations may overlap and the distinction between different CD4+ Treg subsets is sometimes difficult (Shevach 2006).

In addition there are some other T cell subpopulations which have been shown to have regulatory properties. NKT cells express both TCR and NK cell receptor. Their TCR repertoire diversity is limited, a part of them expressing the invariant
Vα24Jα18/Vβ11 TCR. They recognize lipids and glycolipids presented by CD1d molecules. Upon activation, they produce large quantities of cytokines (IL-4, INF-γ, TNF) and they also exhibit cytolytic activity (FasL, perforin). Deficiency or dysfunction of NKT cells is reported to cause autoimmune manifestations. Additionally they seem to have a role in tumor surveillance and infectious diseases (Roncarolo and Levings 2000, Bendelac et al. 2007).

γδ T cells comprise less than 5% of peripheral blood T cells, but they are enriched in epithelia. As γδ T cells do not require MHC molecules for antigen recognition, they do not usually express CD4 or CD8 co-receptors either. The antigens they recognize are poorly known, but they include pathogen-derived antigens and self-antigens up-regulated on stressed cells. γδ T cells have many effector functions and they are suggested to stand at the border between innate and adaptive immunity. They are thought to have an activating role and capability to elicit a rapid response in the beginning of immune reaction, especially in the vicinity of epithelia (gut, skin). On the other hand a regulatory role in modulating the immune responses has also been suggested for γδ T cells (Carding and Egan 2002).

Some peripheral CD8+ and CD4+CD8− double-negative (DN) cells have also been reported to have regulatory properties (Roncarolo and Levings 2000, Shevach 2006, Strober et al. 1996).

2 ANTIGEN RECOGNITION AND T CELL ACTIVATION

2.1 T cell receptor complex

The antigen recognition of the T lymphocytes occurs through the T cell receptor (TCR). TCR is a heterodimeric surface protein consisting of α and β chains. This is the case in 95% of T cells. The residual 5% bear alternative polypeptide chains, designated γ and δ. Each glycoprotein chain of TCR is composed of variable (V) and constant (C) domains. The outer V domains of α and β chains form the antigen binding sites, the complementarity determining regions (CDR). CDR 1 and 2 bind the MHC molecule and CDR 3 is the antigen-binding site. In both chains there is also a transmembrane domain spanning the lipid bilayer, and a short cytoplasmic tail (Moss et al. 1992).

The cytoplasmic tail of the receptor is too short to transmit signals inside the cell. Hence TCR is always expressed on the T cell membrane with the CD3 molecule. CD3 is a signal transduction complex composed of various chains and it enables T cell activation after its TCR has encountered an antigen. The cell-surface receptor complex is also associated with a homodimer of intracytoplasmic ζ-chains, which signal to the interior of the cell upon antigen binding. The intracytoplasmic parts of the CD3 complex and ζ-chains contain the immunoreceptor tyrosine-based activation motifs (ITAMs), and the phosphorylation of the tyrosines in the ITAMs serves as the first intracellular signal indicating that the lymphocyte has detected its specific antigen. The phosphorylated ITAMs engage ZAP70, the activation of which starts a pathway that eventually conducts signals from the cell membrane to the nucleus (Smith-Garvin et al. 2009).

The receptor structure of the γδ TCR is similar to the αβ receptor and it is also associated with CD3. In spite of the structural similarities of the αβ and the γδ TCRs,
antigen recognition between these receptors differs remarkably (Carding and Egan 2002, Moss et al. 1992).

2.2 Antigen recognition by T cells

In most cases, the αβ TCR recognizes antigens only when peptides are bound to MHC molecules. In humans, the MHC molecules are also called human leukocyte antigens (HLA). The MHC molecules are on the cell surface of the APCs, therefore cell to cell contact is required when αβ T cells recognize antigens. During antigen recognition, the T cell receptor makes contact with both the MHC molecule and the antigen peptide and therefore the T cell responses are called MHC restricted (Moss et al. 1992).

There are two classes of MHC molecules, MHC class I and MHC class II. The MHC class I molecule is a heterodimer. Nearly all nucleated cells express MHC class I molecules. It consists of a membrane spanning α chain, which is composed of three domains (α1-α3) and which is non-covalently associated with β2-microglobulin. The peptide-binding cleft is formed on the surface of the molecule by α1 and α2 domains.

The MHC class II molecule consists of two transmembrane proteins, the α and β chains and the peptide-binding cleft is thus formed by two different chains. It is expressed only on the cell surface of specialized APCs i.e. dendritic cells, macrophages, B lymphocytes and thymic epithelial cells (Rudolph et al. 2006).

The three-dimensional structure of both the MHC classes is very similar. The major difference is that the peptide-binding cleft of the MHC class II molecule is more open. Hence, the ends of a peptide in the MHC class I are substantially embedded within the molecule, whereas in the MHC class II they are not. Consequently, the MHC class II molecules can bind longer peptides. The MHC molecules are very unstable when peptides are not bound. Peptide is bound as an integral part of the MHC molecule’s structure and the binding is solid. The MHC molecules are exceedingly polymorphic, with major differences in the peptide-binding cleft. Each molecule can bind stably many different peptides. Genetic variation influences the repertoire of the antigens presented to T cells (Rudolph et al. 2006).

Antigens presented in the MHC class I molecules are typically synthesized inside the presenting cell itself. They are derived from intracellular proteins, which are degraded in the cytosol by proteasomes and transported through the transporter associated with antigen processing (TAP1&2) in the endoplasmic reticulum. Thereafter the antigen peptide binds the MHC molecule and the complex is transported to the cell surface. Class I molecules can signal to T cells that there is an intracellular infection, and recognition of foreign antigens associated to the MHC class I molecules leads to the killing of the presenting cell (Andersen et al. 2006, Germain and Margulies 1993). Additionally, DCs can process exogenous antigens into MHC class I molecules and present them to the cytotoxic CD8+ lymphocytes. This is referred to as cross-presentation. This is an important mechanism for generating immunity against viruses, for example in viral infections of the epithelial cells. Cross-presentation is also associated with tolerance induction (Bevan 1975, Bevan 2006, Heath and Carbone 2001).

The antigens presented in the MHC class II molecules originate from outside the cell. Endosomes containing extracellular antigens fuse with the vesicles containing the MHC class II molecules. The MHC class II associated invariant chain directs the newly synthesized MHC class II molecules to these acidified intracellular
vesicles. Additionally the invariant chain prevents the MHC class II molecules from binding prematurely to the cell’s own peptides, or the peptides transported into the endoplasmic reticulum, and thus allows the MHC class II molecules to bind only peptides degraded in endosomes. The resulting peptide-MHC class II complex proceeds to the cell surface (Germain and Margulies 1993).

2.3 Co-receptors
In order to obtain an effective response during antigen recognition, accessory molecules CD4 or CD8 are associated on the T cell surface with the T cell receptor and bind nonpolymorphic sites on the MHC molecules. The intracellular parts of co-receptors strengthen the signalling of the TCR-CD3 complex, thus the sensitivity of a T cell to the antigen presented is markedly increased.

CD4 is a monomeric transmembrane protein composed of four immunoglobulin-like domains (D1-D4). It binds the MHC class II molecules during antigen recognition and this occurs mainly through a region located on the outermost first domain, D1.

CD8 molecules combine with the MHC class I molecules. The CD8 is a dimer consisting usually of a pair of α and β chains, both members of the immunoglobulin superfamily. The CD8α chains can also form homodimers when the CD8β is not present. These homodimers may have a function in recognizing the nonclassical MHC class I molecules. The CD8α domain binds weakly to the α3 portion of the Class I MHC molecule (Janeway et al. 2008).

Associated with the cytoplasmic domain of the CD4 and CD8 is the Lck tyrosine kinase which helps to activate the T cell, ZAP70 being one of the prime targets. The CD4 molecules bind significantly more Lck than the CD8 molecule, producing stronger signals. Ultimately, transcription factors, for example the nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of the activated B cells (NFκB) family members, are activated leading to initiation of the genetic program for T cell activation (Smith-Garvin et al. 2009).
2.4 T cell activation

2.4.1 First signal
Activation of a resting T cell during the immune response requires contact between the T cell and APC presenting foreign peptide/MHC complex with sufficient affinity. The activation occurs in lymph nodes or in other organized lymphoid tissues (Tseng and Dustin 2002).

2.4.2 Second signal
When a naïve T cell recognizes its specific ligand on APC, a conformational change occurs on cell-adhesion molecules and causes cells to bind with higher affinity to each other. To trigger an effective response to the antigen presented, the resting T cells need also other co-stimulatory signals besides the CD4/CD8 engagement with the TCR-MHC complex. To ensure that activation occurs only in appropriate situations, only professional APCs can give these additional signals. The most important co-stimulatory receptor in T lymphocytes is CD28. Its ligands on the APC surface are B7-1 (CD80) and B7-2 (CD86). The B7 molecules are found only on the surface of cells that can stimulate the T cell proliferation. Co-stimulation must be delivered by the same APC on which the T cell recognizes its antigen. The CD8+ T cells seem to require stronger co-stimulatory signals than the CD4+ T cells (Andersen et al. 2006, Janeway et al. 2008).

2.4.3 Third signal
The additional signals that are required during activation are transmitted by cytokines, which strengthen and guide the response. The nature of the pathogen contributes to the outcome of what kinds of cytokines are secreted by the APCs. For example, the IL-12, IL-18 and IFN-α are secreted by the DCs and macrophages in response to viral infections and direct the T cells towards cell-mediated immunity, whereas IL-4 guides towards humoral immunity. After induction, the T cells themselves start to secrete cytokines as well, thus further reinforcing the immune response.

2.4.4 Consequences of activation
Upon activation, the T cell proliferates and differentiates into an effector T cell. The effector T cells are capable of synthesizing all the molecules required for exerting their specialized functions. Once activated, the effector T cells do not require co-stimulation to act (Janeway et al. 2008).
Antigen recognition by T lymphocytes occurs through the TCR, which on the cell surface is always expressed with the CD3 molecule. The αβ T cells recognize antigens bound to the MHC molecules. The accessory molecule CD4 associates with the TCR-CD3 complex and binds to a MHC class II molecule while the CD8 binds to a MHC I molecule. The co-receptors CD4 and CD8 strengthen the signaling of the TCR-CD3 complex. Effective activation of a naïve T cell requires also a second signal, which must be delivered by the same APC. The most important co-stimulatory molecule on T cells is CD28. Its ligands on the APC are CD80 (B7-1) and CD86 (B7-2). Effective activation happens only if the APC presents both the specific antigen and a B7 molecule. Activation of the T cell is also controlled by cytokines secreted by the APC.

3 T CELL DEVELOPMENT AND CENTRAL TOLERANCE

3.1 Thymus structure
The development of T cells occurs in the thymus via a series of intermediate stages. The thymic stroma develops early during the embryonic development from the endodermal layer. The contribution of the ectodermal epithelium to the development of thymus is ambiguous (Rodewald 2008). The thymus consists of numerous lobules, each of which comprises a medulla and a cortex. Thymic epithelial cells (TECs) can be characterized by their expression of keratin. The TECs of the cortex and the medulla are functionally and phenotypically different, supporting different stages of T cell maturation. Most of the T cell development occurs in the cortex, thus the bulk of the immature thymocytes are within the cortex and thymocytes enter the medulla only after they are single-positive. The network of medullary TECs (mTECs) is compact, with the mature thymocytes interspersed within it (Chidgey et al.). The thymic medulla of humans also contains enigmatic structures called Hassall’s corpuscles, characterized by keratinized epithelial cells (Lobach et al. 1985). In addition to the maturing T cells or thymocytes and a network of epithelial cells, the thymus consists of intrathymic DCs, macrophages and B cells. Interactions between the thymic stroma and differentiating thymocytes are fundamental to the development of T cells. Moreover, lymphostromal interactions are required to maintain the functional potential of the stromal compartment (Janeway et al. 2008, Chidgey et al., Klaus 2001).
Figure 2. Thymic Structure

A. The thymus consists of several lobules separated by connective tissue septae. Each lobule consists of a cortex and a medulla. The cortex is rich in immature thymocytes (blue) and scattered cortical thymic epithelial cells (TECs; dark blue). Additionally, macrophages (yellow) are interspersed throughout the cortex. The macrophages eliminate apoptotic thymocytes. The medulla comprises numerous medullary TECs (purple) and mature thymocytes (blue). The macrophages and the thymic DCs (green) are also distributed throughout the medulla. Hassall’s corpuscles (pink), formed from keratinized epithelial cells, are also found in the medullary region.

Hematopoietic stem cells enter the thymus from the bloodstream at the cortico-medullary junction. First, T cell precursors migrate to the subcapsular region of the thymic cortex. As the thymocytes mature, they migrate through the cortex towards the medulla and the most mature single-positive cells are found in the medulla. Positive selection of thymocytes occurs during the DP stage at the cortex and negative selection can occur at any
time after the TCR is expressed on the cell surface, either in the cortex or in the medulla. The mature T cells leave the thymus and enter the bloodstream.

B. Hematoxylin-eosin staining of thymic tissue. Connective tissue septae separate the different lobules. The cortical areas are darkly stained and the medulla is light. The large, pink structures in the medullary areas are Hassall’s corpuscles.

3.2 Entry into thymus and early stages of T cell development

T lymphocytes develop from hematopoietic stem cells (HSC) originated from the bone marrow. The HSCs differentiate to common lymphoid precursor cells (CLP) having the CD34+CD38− (CD45RA+) phenotype. The thymus seeding progenitor (TSP) enters the thymus and evolves into an early thymic progenitor (ETP). These CD34+CD38low cells form the most immature population in the thymus. It is known that at least part of the seeding precursor cells are still multipotential, being able to differentiate also to B cells, NK cells and DCs. This indicates that the T cell commitment takes place within the thymus (Blom and Spits 2006). The ETPs are CD1a−, and as the development proceeds, differentiating cells become CD34+CD1a+. CD1a is a useful marker for identification of immature thymocytes in humans and up-regulation of CD1a marks T cell lineage commitment. The CD1a+ cells begin to undergo rearrangements in the TCR β, γ, and δ loci. At this stage, maturing thymocytes do not express either CD4 or CD8 and are called double-negative thymocytes (DN). Consecutive stages in the development are marked by changes in cell-surface molecules (Chidgey et al., Plum et al. 2008, Spits 2002).

The transcription factor Notch is essential for the T cell lineage decision and development. IL-7 is also indispensable for T cell development. It is important for proliferation and survival of the early T cell precursors, as already ETPs express IL-7Ra (Blom and Spits 2006).

3.3 β-rearrangements

Rearrangements occur during early stages of T cell development in the order δ>γ>β>α, but almost simultaneously in the β, γ and δ loci. Here I concentrate on the α and β gene arrangements. As the most immature T cells are double-negative, the next stage consists of CD4 immature single positive (ISP) cells, which start to express also first the CD8α chain and thereafter the CD8β chain, developing to double-positive (DP) CD4+CD8+ T cells. The rearrangement of the TCR β-chain locus begins during these stages (Janeway et al. 2008, Spits 2002).

The function of recombination activating genes (RAG1&2) is fundamental to the gene rearrangements (Sadofsky 2004). The first gene segments to be rearranged are DJβ with Jβ, and this is succeeded by Vβ rearranging with D-Jβ. Diversity of the junctional regions is increased by adding P- and N-nucleotides. The junctional regions of the α and β chains are most variable. They contribute to CDR3s, which form the antigen binding site. If the thymocyte fails to make a successful β-recombination, the cell dies, unless the γ and δ rearrangements take place and rescue the cell to continue development on the γδ T cell lineage (Chidgey et al., Spits 2002, Schatz et al. 1992). After productive, in-frame TCRβ rearrangements, the TCRβ protein pairs with a pre T cell receptor α chain (pTα) and is expressed with the CD3 molecule on the cell surface. The PreTα protein belongs to the immunoglobulin superfamily. Its
expression is developmentally regulated and restricted to lymphoid cells. Onset of the PreTα expression precedes β chain rearrangements, and the transcription is highest in the CD4 ISP and CD4⁺CD8⁺ DP thymocytes (Ramiro et al. 1996, von Boehmer and Fehling 1997, Carrasco et al. 2002).

Cells expressing the preTCR complex are selected for further differentiation. This β-selection occurs in variable stages of the early T cell development where expression of the CD4 and CD8 differ. First cells express β-chain even before the CD4 expression, and some cells start β rearrangements only when both the CD4 and CD8α are already expressed. The pre-T cell receptor signals to the cell to abort further β chain rearrangements (Spits 2002, von Boehmer et al. 1998).

Before starting the rearrangements in α locus, the DP thymocytes proliferate intensely. The DP cells constitute the biggest thymocyte subset. DP thymocytes may also have an influence on early thymocyte precursors, thus contributing to the thymic crosstalk (Janeway et al. 2008, Spits 2002, Takahama 2006).

Figure 3. Developmental stages of αβ T cells in the human thymus

Hematopoietic stem cells originate from the bone marrow and when they enter the thymus they are still multipotent. The next stage after the early thymic precursor cells (ETP) is already committed to the T cell lineage. After commitment to the T cell lineage, the double-negative (DN) cells begin to rearrange their TCR β, γ and δ loci. If the successful TCR γ and δ rearrangements occur prior to β-selection, the cell will likely develop into a γδ T cell.

The β-selection and expression of the co-receptors, first CD4 and then CD8, begin at the same time. Recombination of the α chain occurs during the double-positive (DP) stage. Positive and negative selection may occur when the thymocyte expresses a functional αβ TCR on the cell surface. The CD4/CD8 lineage choice is made during the positive selection.

3.4 α-rearrangements

The TCRα locus contains only V and J gene segments. The gene segments encoding the δ chain are entirely within the TCRα locus, hence any rearrangement in the α locus causes the deletion of the δ locus (Krangel et al. 1998). After the proliferative phase, the RAG-genes are again up-regulated in the DP thymocytes and the RAG proteins join Vα to Jα. Nucleotides are inserted in the V-J junctions of the α chains as well. Because of the numerous Vα and Jα segments, multiple sequential
rearrangements can occur until the productive rearrangement takes place. Thus a developing T cell that already has a functional β-chain can be rescued from cell death even when early α-rearrangements fail. The α-rearrangements continue until the self-peptide:self-MHC complex induces the TCR transmitted signals through the αβ TCR, or the cell dies (Starr et al. 2003).

Figure 4. TCR rearrangements

Sequential gene rearrangements in the TCR α and β loci. The first gene segments to be arranged are Dβ with Jβ, followed by the joining of Vβ to DJβ. The joining of the V, D and J segments involves the looping-out and deletion of the intervening DNA between the genes to
be joined. Recombinations are carried out by the recombination activating gene (RAG) 1 and 2 proteins. The genes are expressed in developing lymphocytes only when the TCRs are assembled. The rearrangements start in the DN stage (upper part of the figure). When successful rearrangements occur, the β-chain is expressed on the cell surface with preTα (not shown). This preTCR complex signals to stop further β-chain rearrangements. The DP thymocytes proliferate before starting rearrangements in the α locus. The TCR α locus contains only V and J segments (the lower part of the figure). Additionally, the δ locus is entirely within the TCR α locus, and any rearrangements in the α locus delete the δ locus (not shown). Because of the large number of Vo and Ja segments, rearrangements in the α locus can proceed for several cycles. After a productive α rearrangement, the α and the β chain pair and they are expressed on the cell surface as αβ TCR. Nucleotides are inserted by the deoxynucleotidyl transferase (TdT) in the Dβ–Jβ, Vβ–DJβ and Va–Ja junctions to increase the diversity of the TCR.

3.5 Allelic exclusion
Allelic exclusion is a process where protein from only one allele is expressed and the other allele is silenced (Malissen et al. 1992). In other words, when a functional β chain is rearranged, it hinders further β-chain gene recombination in the other locus. The α locus, instead, lacks allelic exclusion. Both alleles of the α chain gene simultaneously rearrange until the α chain pairs with the β chain and receptor engagement to self-peptide:self-MHC complex occurs (Borgulya et al. 1992). Positive selection switches off the RAG expression. That is, T cells can have many rearranged α chains from both chromosomes which are tested for self-peptide:self-MHC recognition. This, in turn, enables existence of T cells which express two different TCRs with the same β chains and different α chains (Casanova et al. 1991).

3.6 Dual-specific T cells
Lack of allelic exclusion in the α locus challenged the old view of one cell bearing only one receptor specificity. Although the frequency of two in-frame α-rearrangements is reported to be quite high, there are mechanisms limiting the frequency of two different α chains expressed on the cell surface. One factor limiting the surface expression is the preferential association of some α chains with the β chain, others being unable to pair at all (Malissen et al. 1992, Kuida et al. 1991, Corthay et al. 2001). The post-translational mechanisms affecting the surface expression are called phenotypic allelic exclusion. Cells expressing two α chains are much more frequent in the developing TCRlow thymocytes, and it has been shown that the phenotypic allelic exclusion occurs quite simultaneously with positive selection. As dual-TCR cells are more prevalent among the resting population, the phenotypic allelic exclusion may be regulated by TCR signaling (Boyd et al. 1998, Gascoigne and Alam 1999, Niederberger et al. 2003).

The mechanisms inhibiting the expression of two different TCRs on a cell surface are incomplete, and there is still a noticeable population of cells in the periphery that are dual-specific. It has been reported that up to 30% of human or mouse peripheral T cells may express two TCR α chains (Casanova et al. 1991, Padovan et al. 1993, Heath et al. 1995). It was originally thought that the second receptor would not function, as it has not been positively selected and therefore it would not recognize MHC molecules (Malissen et al. 1992, Corthay et al. 2001, Boyd...
et al. 1998, Niederberger et al. 2003, Heath et al. 1995, Gascoigne and Alam 1999, Lacorazza and Nikolich-Zugich 2004). However, the TCR chains are inherently biased towards MHC recognition (Zerrahn et al. 1997), so that even before the selection a large proportion of the TCRs may interact with the MHC and hence be functional. If the dual-specific T cell passes positive selection through one receptor, the other, unselected, receptor could potentially be autoreactive. The escape from negative selection may also be facilitated if the second receptor is expressed at low density (Zal et al. 1996). In the periphery, the dual-specific T cell may be activated through either of the receptors. In addition, mouse models have shown that the second (self-specific) receptor can function, even when the cell has been activated initially through the other receptor (Zal et al. 1996).

Several studies have indeed addressed the role of dual-specific T cells in autoimmunity (Elliott and Altmann 1995, Sarukhan et al. 1998). In a TCR transgenic system the expression of a second TCR could save autoreactive T cells from deletion (Zal et al. 1996). Other studies, however, have demonstrated that the dual-specific cells do not increase susceptibility to autoimmunity and may even have a protective role (Corthay et al. 2001, Van de Keere and Tonegawa 1998, Olivares-Villagomez et al. 1998, Itoh et al. 1999, Kawahata et al. 2002, Hori et al. 2002). The studies of dual-specific cells are based mainly on transgenic models, and the effects of dual-specificity in normal immune system, if any, are unknown.

3.7 Positive selection
In order to mature as CD4 or CD8 single positive (SP) T lymphocytes, DP thymocytes have to receive adequate signals through their TCR. Several elegant experiments using mouse models have elucidated the selection process. As the antigen recognition of T cells is MHC restricted, the immature T cells must recognize self-MHC. Positive selection occurs in the cortex where the cortical TECs (cTECs) introduce self-peptide:self-MHC complexes to maturing thymocytes. Thus the MHC molecules expressed in the environment where T cells develop determine which MHC molecules the mature T cells recognize as self (Starr et al. 2003). The TCR Vα chain possibly has a more important role in the TCR-MHC interaction (Gascoigne and Alam 1999).

The co-receptor expressed on a single-positive thymocyte is also defined in the course of or after positive selection. The decision of phenotype depends on which MHC molecule the TCR recognizes, MHC class I recognition leading to CD8 expression and MHC class II to CD4 expression. How the TCR specificity dictates the CD4/CD8 choice has been difficult to resolve and is still under debate. It is unclear whether the decision is stochastic, or dependent on the strength or duration of TCR signaling. These propositions are however at least in part contradictory (Singer et al. 2008).

At the moment the CD4/CD8 lineage choice is best explained by the kinetic signaling model, proposing that the TCR-signal duration, in concert with the cytokines of the common γ-chain receptor family, such as IL-7, determine the co-receptor. According to this model, both the CD4 and CD8 committed cells down-regulate the CD8 after positive selection. Should the positively selecting signals be MHC class II restricted, the TCR signaling persists in the CD4⁺CD8⁻ cells. This in turn impairs the IL-7 signaling and induces the differentiation into a mature CD4⁺ T cell. However, if the positively selecting TCR signaling is MHC class I restricted, it is disrupted in the CD4⁺CD8⁻ cells and the continuous IL-7 signaling enables co-
receptor reversal and differentiation into the CD8+ cells. The lineage decision must induce functional programming, so that different gene expression is induced for cytotoxic CD8+ cells and for cytokine producing CD4+ cells (Takahama 2006, Singer et al. 2008). CD69, a molecule associated with the TCR-mediated signaling, is up-regulated after positive selection (Hare et al. 1999).

3.8 Negative selection
After being positively selected, the thymocytes migrate towards the thymic medulla. The TCR repertoire of the developing T cells has to be extensive. Recognition of vast amounts of foreign antigens is required, while recognition of self should be avoided. Thus the T cells with TCR specific to self-antigens must be destroyed. Developing cells are introduced to the MHC molecules complexed with self-peptides. Antigens are presented by DCs, macrophages and the specialized stromal cells known as mTECs. Because the DCs and the macrophages are able to activate mature T cells effectively in the periphery, they are presumably the most efficient mediators of negative selection also in the thymus.

It is still uncertain what precisely mediates the balance between survival and cell death. There are currently two predominant hypotheses. The first model states that the selection is determined by the affinity of the TCR to peptide/MHC complex, providing different signal strengths intracellularly. This means that the binding of self-peptide:self-MHC with low affinity signals survival for thymocytes, while strong binding leads to apoptosis. The other model suggests that intracellular signals, generated during the binding of TCR to self-peptide-MHC complex, differ qualitatively during positive and negative selection. Negative selection may occur at any stage of thymic differentiation beginning at the double-positive stage where expression of the αβ TCR originates and extending to nearly mature SP αβ thymocytes. The elimination of potentially self-reactive T cells during T cell development is referred to as central tolerance (Klaus 2001, Takahama 2006, Palmer 2003)

3.9 AIRE and central tolerance
It has recently become clear that in order to facilitate negative selection, tissue-specific antigens are actively transcribed in the thymus (Derbinski et al. 2001, Linsk et al. 1989) and presented by mTECs and DCs. This ectopic transcription is not fully understood, but one factor promoting it is a gene called AIRE (AutoImmune REgulator). The AIRE gene has the ability to induce the expression of an extensive selection of peripheral tissue-antigens (PTA), and in that way to contribute to negative selection of autoreactive thymocytes (Anderson et al. 2002, Liston et al. 2003). It has been estimated that the AIRE may influence the expression of several hundred, or more likely several thousand genes (Derbinski et al. 2005). However, it should be noted that, firstly, there is also AIRE-independent antigen presentation (Kuroda et al. 2005, Niki et al. 2006) and consequently other genes or mechanisms also promote expression of PTAs and, secondly, AIRE has also other functions besides ectopic transcription (Mathis and Benoist 2009).

Proper thymic microenvironment is required for AIRE expression. It has been reported that AIRE expression is decreased under conditions in which normal thymic architecture is disturbed (Zuklys et al. 2000). Additionally, AIRE itself seems to have
a role in the differentiation of mTECs, and PTAs are expressed only in terminally differentiated mTECs (Hamazaki et al. 2007, Yano et al. 2008, Gray et al. 2007).

Figure 5. Selection of developing thymocytes

Maturing thymocytes have to receive adequate signals through their TCR to survive. If the TCR of the thymocyte does not recognize the self-MHC/self-peptide complex on the surface of a thymic antigen-presenting cell (APC), the thymocyte will die. This is referred to as death by neglect.

If the self-MHC/self-peptide complex is recognized with a low affinity, the thymocyte survives and the cell is positively selected. A high-affinity interaction with the self-MHC/self-peptide complex leads to negative selection of the developing thymocyte. It has also been suggested that the recognition of self antigen with intermediate to high affinity may promote generation of FOXP3+ Treg cells.

In order to facilitate negative selection, peripheral tissue antigens are presented to the developing thymocytes in the thymic medulla by mTECs and dendritic cells. The transcription of these peripheral antigens is partly under the control of the transcription factor AIRE.

4 DEVELOPMENT OF NATURAL REGULATORY T CELLS

4.1 Identification of natural regulatory T cells

Regulatory T cells (Treg cells) represent ~5-10 % of CD4+ cells in the periphery. The Treg cells actively maintain tolerance and immunologic homeostasis (Sakaguchi 2004). Depletion of the Treg cells leads to severe immunological disturbances both in humans and mice (Sakaguchi 2004, Wraith et al. 2004). The existence and importance of these cells were questioned for a long time, but at present the significance of this population is undisputed.

In mice, the Treg cell development is delayed compared to non-regulatory T cells during ontogeny and the Treg cells start to emerge only after birth. It was at first discovered that in mice, thymectomy on day three after birth led to severe
autoimmune manifestations, which could be prevented by transfer of total lymphocytes or CD4⁺ lymphocytes (Nishizuka and Sakakura 1969, Sakaguchi et al. 1982, Penhale et al. 1973, Penhale et al. 1975, Sakaguchi 2004). These observations demonstrated that autoreactive T cells exist in normal mice, and that there are cells capable of suppressing these pathogenic cells. After the discovery of CD25 as a specifying marker for these regulatory cells (Sakaguchi et al. 1995), more specific studies have confirmed this suppressive effect. The transfer of CD4⁺ T cells devoid of CD25⁺ cells produces autoimmune disease in athymic, T cell deficient nude mouse, while the co-transfer of CD25⁺CD4⁺ cells inhibits autoimmunity (Asano et al. 1996). In vitro assays have also demonstrated that the CD4⁺CD25⁺ cells co-cultured with CD4⁺CD25⁻ are able to suppress the proliferation of the CD25⁻ T cells (Thornton and Shevach 1998).

In contrast, the human Treg cells are detected in the thymus already on week 13 of gestation, simultaneously with conventional T cell subsets and a few weeks later also in the periphery (Darrasse-Jeze et al. 2005). The difference between mice and humans is probably explained by the substantially longer gestation of humans. Additionally, functional adaptive Treg cells can be induced already in the fetal periphery (Mold et al. 2008).

4.2 FOXP3 gene

4.2.1 Significance of FOXP3 to Treg cells

The key marker for natural Treg cell population is the transcription factor forkhead box P3 (FOXP3) (Khattri et al. 2003, Fontenot et al. 2003) This gene is considered to be involved in the development of natural Treg cells, and is seen as essential for the function of these thymus-derived natural regulatory T cells. Continuous expression of the gene is required to maintain the differentiated phenotype (Williams and Rudensky 2007, Lopes et al. 2007). Retroviral gene transfer of FoxP3 reprograms murine T cells to become Treg cells (Hori et al. 2003, Fontenot et al. 2003). Mutation of FOXP3 gene leads to a severe autoimmune syndrome, IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked) in humans (Bennett et al. 2001, Wildin et al. 2001, Chatila et al. 2000) and analogous lymphoproliferative disease in Scurfy mice (Brunkow et al. 2001). Autoimmune manifestations include, among others, massive lymphoproliferation, diabetes, eczema and severe diarrhea (Torgerson and Ochs 2007). In addition, experiments in mice have revealed that the mutation of FoxP3 leads to a massive proliferation of T cells specific to both self and non-self, and a concomitant activation and expansion of myeloid cells, including DCs and granulocytes (Ziegler 2006). Moreover, the level of FOXP3 expression is important. Reduced FoxP3 expression leads to an autoimmune disease in mice (Wan and Flavell 2007). Similarly in humans, the FOXP3 level correlates with suppressive function (Allan et al. 2008, Wang et al. 2007).

4.2.2 Control of FOXP3 expression

TCR mediated signals are considered to be central in the FOXP3 up-regulation during development (Fontenot et al. 2005). The TCR stimulation has been observed to affect directly the FOXP3 promoter (Mantel et al. 2006). Also several epigenetic factors are important in controlling the FOXP3 expression.
CpG motifs in the FOXP3 promoter are nearly totally demethylated in the Treg cells. Additionally, there is a stronger association of the FOXP3 promoter with acetylated histones in Treg cells than in conventional T cells, suggesting that the FOXP3 promoter is more accessible in the Treg cells. A TGF-β sensitive element, containing binding sites for transcription factors, has also been identified in the FOXP3 locus. In addition, there is another highly conserved region in the FOXP3 locus which has also been observed to be completely demethylated in the Treg cells and methylated in the conventional T cells. Demethylation of this area is assumed to affect the stability of FOXP3 expression (Huehn et al. 2009).

In addition to epigenetic modifications, FOXP3 has been reported to have interactions with chromatin remodeling enzymes, histone acetyltransferases and histone deacetylases, which result in the modification of the FOXP3 binding sites, facilitating FOXP3 function. Acetylation of the forkhead domain of FOXP3 is also associated with optimal Treg function. Small non-coding RNAs, microRNAs (miRNAs) involved in the regulation of gene expression have recently turned out to have a role also in the Treg cell biology. The miRNAs, especially miR155, are involved in the Treg cell development in the thymus and seem also to be essential for homeostasis and the function of Treg cells (Sakaguchi et al. 2008, Huehn et al. 2009).

### 4.2.3 Functions of transcription factor FOXP3

FOXP3 is a member of the forkhead/winged-helix family of transcriptional regulators. There are 11 exons in the FOXP3 gene. It acts both as a transcriptional activator and repressor with numerous target genes. It likely regulates gene expression both directly and indirectly. FOXP3 affects other transcription factors, for example a direct interaction and inhibition of NFAT is observed. FOXP3 actively regulates the expression of many characteristic surface markers of Treg cells. It also down-regulates production of IL-2, IL-4 and IFN-γ, and ablation of FoxP3 restores the ability to produce IL-2. FOXP3 regulates IL-2 only after TCR stimulation, whereas surface molecules CD25 and CTLA-4 are constantly up-regulated by FOXP3 (Zheng et al. 2007). FOXP3 seems to affect also TCR signaling. The early signaling events are reduced, including phosphorylation of CD3ζ and subsequent recruitment of ZAP70 (Tsang et al. 2006). In addition actin reorganization has also been reported to be affected (Tsang et al. 2006). Actin reorganization is required for sustained TCR signaling (Miletic et al. 2003) and thus FOXP3 action may lead to diminished TCR signaling in Treg cells. In vitro, Treg cells proliferate poorly (Baecher-Allan et al. 2001, Itoh et al. 1999, Li et al. 2005).

In humans FOXP3 is expressed as three isoforms, the full-length version, an isoform lacking the exon 2 (FOXP3Δ2) (Allan et al. 2005) and a third splice variant that lacks both exon 2 and exon 7 (FOXP3Δ2Δ7) (Smith et al. 2006). It was recently reported that the full-length form of FoxP3 inhibits RORα (Du et al. 2008) and RORγt (Ichiyama et al. 2008) mediated transcriptional activation. RORα and RORγt expression up-regulates some Th17 cell related genes. The FOXP3Δ2 isoform is unable to interact with RORα and RORγt, because the binding site is located in exon 2. Thus only the full-length form of FOXP3 may suppress the induction of Th17 differentiation.

FOXP3Δ2Δ7 fails to induce Treg cell associated surface markers CD25 and CTLA-4 and it does not confer suppressive capacity to transduced CD4+CD25+ cells.
FOXP3Δ2Δ7 may have a role in regulating the function of the other FOXP3 isoforms and it may be involved in cancer pathogenesis (Mailer et al. 2009).

4.3 Surface markers of Treg cells

Albeit FOXP3 is at the moment the most specific marker for Treg cells, as an intracellular transcription factor its usage in Treg cell identification is restricted. Many cell surface markers have been identified to recognize Treg cells among effector T cells. As many markers are expressed equally by activated effector cells and Treg cells, the differentiation of these two populations is often difficult. Although the markers are used for the characterization of this cell population, they are not only markers, but have also have functional significance.

Most commonly used cell surface marker is CD25, which is richly expressed on Tregs (Sakaguchi et al. 1995). A complicating factor is that effector T cells also up-regulate CD25 upon activation, in spite of that Tregs are generally identified as CD4\(^+\)CD25\(^{hi\text{gh}}\) cells. As CD25 is the \(\alpha\) chain of IL-2R, its expression is pertinent for regulatory T cells since IL-2 is crucial for the maintenance and function of regulatory T cells (Furtado et al. 2002, Malek and Bayer 2004). Cytotoxic T Lymphocyte Antigen 4 (CTLA-4, CD152) is constitutively expressed both intra-cellularly and on the cell surface of Treg cells. CTLA-4 is involved in regulating immune tolerance (Takahashi et al. 2000, Read et al. 2000). In humans, another characteristic for Treg cells is the low expression of IL7-R\(\alpha\) or CD127 (Liu et al. 2006, Seddiki et al. 2007, Hartigan-O’Connor et al. 2007), although usability of this feature has also been disputed, since CD127 is down-regulated early during T cell activation. Glucocorticoid-induced tumor necrosis factor receptor (GITR) has also been reported to be expressed by the majority of Treg cells, but it is also expressed by effector T cells. It is a co-stimulatory molecule, which activates effector T cells and modulates Treg cells. It has been reported that GITR engagement can inhibit Treg cells or render effector T cells more resistant to Treg-mediated suppression (Shimizu et al. 2002, Nocentini and Riccardi 2005). Several other molecules have also been identified on the cell surface of Treg cells, e.g. L-selectin (CD62L), CD45RO, CD69, LAG-3, neuropilin-1, CD103 and OX-40 (Qu and Zhao 2007). However the selectivity of these markers is arguable, especially in humans.

FOXP3 regulates the transcription of many Treg-specific markers. For example CD25, CTLA4 and GITR are induced by FOXP3 (Chen et al. 2006) and CD127 is respectively negatively regulated (Liu et al. 2006).

4.4 Commitment to Treg cell lineage

The developmental pathway through which Treg cells arise is still ambiguous. The precise phase of development when regulatory T cells diverge from other thymocytes is unclear. The original view has been that TCR/MHC interactions are essential for the up-regulation of FOXP3 and Treg cells diverge from the CD4-lineage rather late during thymic development (Fontenot et al. 2005, Liston and Rudensky 2007).

4.4.1 FoxP3\(^+\) thymocyte subsets

Findings in mice have shown that the majority of FoxP3\(^+\) cells are found at the CD4\(^-\)CD8\(^-\) SP stage. Additionally, a small population of CD4\(^-\)CD8\(^-\) DP thymocytes expressing FoxP3 has been described in several publications (Fontenot et al. 2005,
Liston et al. 2008, Cabarrocas et al. 2006, Wan and Flavell 2005). Nevertheless, FoxP3 expression is clearly detected also in some DN and CD8+ SP thymocytes (Cosmi et al. 2003, Fontenot et al. 2005). Most mature CD4+CD25+FoxP3+ thymocytes already share phenotypic and functional characteristics of their peripheral counterparts (Liotta et al. 2005). Expression of other characteristic Treg markers, such as GITR and intracellular CTLA4, are also detected from DP CD25+ cells (Cosmi et al. 2003, Annunziato et al. 2002). The relationship of these DP cells to the more mature Treg cells however remains unclear, some stating a developmental relationship between DP and SP Treg cells while others contest it. In addition, human CD8+CD25+ thymocytes expressing FOXP3 have been reported to share many features with the CD4+CD25+ regulatory thymocytes. Similarly they could suppress the proliferation of both CD4+CD25+ and CD8+CD25+ T cells (Cosmi et al. 2003). In other studies the expression level of CD25 on CD8+FOXP3+ was lower than that of CD4+ thymocytes (Cupedo et al. 2005). The developmental pathway and relationship of CD8+CD25+ regulatory T cells with other FOXP3+ subsets is unknown.

4.4.2 Evidence for commitment in SP stage
The majority of the FoxP3+ thymocytes have been shown to localize in the medulla and the expression of FoxP3 is largely restricted to the CD4+ SP cells. Hence the major events of Treg cell development are thought to take place in the thymic medulla. Specifially, it has been argued that there is no preceding FoxP3+ DP population in mice, but FoxP3+ DP cells emerge in parallel with FoxP3+ SP cells, thus no progression from the FoxP3+ DP to FoxP3+ SP population takes place (Fontenot et al. 2005). It is thought that the high affinity interactions with mTECs and thymic DC direct T cells to Treg lineage (Bensinger et al. 2001, Aschenbrenner et al. 2007). It has also been suggested that in addition to TCR mediated signals cytokines are needed to induce the up-regulation of FOXP3 and complete the commitment to the Treg cell lineage in the thymic medulla (Burchill et al. 2008, Lio and Hsieh 2008).

4.4.3 Evidence for commitment in DP stage
An increasing number of studies argues that also cortical epithelial cells are sufficient for supporting Treg cell differentiation. CCR7-/- mice, which have impaired trafficking of developing thymocytes from cortex to medulla, export mature T cells to the periphery directly from the cortex (Kurobe et al. 2006). Thus the thymic cortex appears to be capable of supporting the development of mature thymocytes. It is proposed that the cortex is at least capable of inducing the development of Treg cells, since FoxP3 is up-regulated already at the double-positive cell stage. However the final commitment may occur in the medulla (Bensinger et al. 2001, Pacholczyk et al. 2002).

Alternatively, it has been recently published that a considerable proportion of FoxP3+ SP cells acquired FoxP3 expression as cortical DP cells (Liston et al. 2008, Ribot et al. 2007). Then cortical DP Foxp3+ cells migrate rapidly to the medulla (Liston et al. 2008). Another study also showed a clear increase of Treg cell precursors already at DP cell stage. Moreover developing thymocytes in these double-transgenic mice, which express a neo-self-Ag for hemagglutinin(HA) in thymic stroma and transgenic TCR specific for HA in T cells, could acquire Treg markers CD25 and FoxP3 before negative selection, implying divergence to Treg lineage before the entrance to the medulla (Cabarrocas et al. 2006, Ribot et al. 2007). A recent
study showed that in reaggregate cultures Treg precursors were competent to express FoxP3 immediately after β rearrangements (Relland et al. 2009).

4.4.4 Evidence for commitment in DN stage
There are also data suggesting that the commitment to Treg lineage may occur already before FoxP3 is up-regulated. Recent reports in mice demonstrated that when FoxP3 expression was disrupted by insertion of GFP (green fluorescent protein) into the FoxP3 locus, the GFP+ cells shared many features with their FoxP3+ counterparts in the absence of FoxP3 expression. The precursors of FOXP3+ cells had their Treg characteristics at an early stage and up-regulation of FOXP3 stabilized the phenotype, thus the Treg cell lineage commitment seem to occur already before up-regulation of FOXP3 (Gavin et al. 2007, Lin et al. 2007). Another recent report proposed that DN precursors, which have not yet rearranged TCR genes, are affected by DP cells, some precursors becoming more susceptible to differentiate to Treg cells after additional signals (Pennington et al. 2006).

4.5 Selection and TCR specificities of Treg cells
The TCR repertoire of Treg cells seems to be polyclonal and more diverse than the repertoire of other CD4+ T cells (Pacholczyk et al. 2006). Current understanding has strongly favored the idea that Treg cells bear self-reactive TCRs. The main theory is that the commitment to Treg cell-lineage is defined by an increased affinity of the TCR (Kawahata et al. 2002, Apostolou et al. 2002, Walker et al. 2003, D’Cruz and Klein 2005). Thus thymocytes recognizing self-peptide:self-MHC complexes with intermediate to high affinity or affinity nearly at the level that would normally induce negative selection/deletion would direct cells to Treg-lineage. The model is based on observations of numerous mouse models in which the numbers of Treg cells increase when TCR transgenic thymocytes encounter their transgene-encoded ligand in thymic stroma (Jordan et al. 2001, Hsieh et al. 2004, Romagnoli et al. 2002).

Supporting data for the hypothesis that TCRs used by Treg cells are self-reactive were acquired from experiments where TCRs of activated CD4+CD25+ T cells from FoxP3-deficient TCRβ-transgenic mice were compared with TCRs of regulatory or non-regulatory T cells from FoxP3+ TCRβ-transgenic mice. There was an obvious over-representation of TCRs usually found in Treg cells in the repertoire of activated CD4+CD25+ FoxP3-deficient population. Thus conventional T cells share autoreactive TCR-specificities with Treg cells, but FoxP3+ Treg cells normally keep these self-reactive T cells in check (Hsieh et al. 2006). Again, to study the specificity of TCR expressed by Treg cells, transgenic mice expressing Treg-TCR were generated. Over 90% of CD4+ T cells were deleted indicating that the TCR was specific to some naturally expressed self-peptide in the thymus. The deletion occurred rather late during the development suggesting that the ligand for TCR existed and the selection took place in the medulla (DiPaola and Shevach 2009). However, the expression of Treg-TCR did not increase the development of FoxP3+ CD4 T+ cells.

Since the thymic medulla is reported as the most important site for Treg cell differentiation and Treg cells are profiled as self-specific, mTECs promiscuously expressing peripheral tissue antigens doubtless play an important role. In addition DCs can present antigens captured from mTECs. Recent study showed evidence that PTAs, expressed under control of AIRE, affect to the TCR repertoire of Treg cells (Aschenbrenner et al. 2007).
Despite the several published observations stating that Treg cells have TCRs specific for self-peptide:self-MHC complexes, contradictory results have been presented. Instead of enhanced formation of Treg cells, there are challenging reports suggesting selective survival for Treg cells when encountering their cognate ligand. It has been reported that increase of regulatory T cells, while interacting with variable amounts of their cognate antigen, does not occur or is minimal. Instead CD4¬CD25+ cells seem to be more resistant to deletion and this way appear to be enriched in the presence of their ligand (van Santen et al. 2004). Another recent report demonstrated that the Treg TCR repertoire is shaped by affinity-based selections, similar to that of conventional T cells, and FoxP3 induction is an independent process. Both FoxP3+ and FoxP3- cells were negatively selected when confronting high affinity ligands, the extent of deletion being significantly lower among the FoxP3+ thymocytes, which again suggests that developing Treg precursors are more resistant to negative selection (Relland et al. 2009). The ligands, that mediate negative selection and FOXP3 induction of Treg cells may be different as well as the APCs that mediate each process.

There are several reports arguing that Treg cells also recognize foreign antigens. A recent study discovered that autoreactive TCRs specific for abundant self-MHC-peptide complexes were rare on all CD4+ T cells, yet more common among Treg cells than conventional T cells (Pacholczyk et al. 2007). In other studies where the TCR repertoires of FoxP3+ regulatory T cells were compared with other CD4+ T cells, the repertoires proved to be more or less overlapping, with similarities ranging from 10% to 42% (Pacholczyk et al. 2006, Hsieh et al. 2006, Wong et al. 2007, Fazilleau et al. 2007). The widely diverse repertoire helps to explain how Treg cells, in addition to self antigens, recognize foreign antigens as well.

4.6 Cytokines and additional signals

Albeit TCR signaling is considered to be essential, development of Treg cells is influenced by cytokines. IL-2, though not produced by Treg cells themselves, is indispensable for their development and peripheral maintenance. Numerous experiments performed using mouse models where some component of IL-2 signaling, IL-2, CD25, IL-2Rβ, or STAT5 is disrupted have shown that IL-2 is specifically required for the development and maintenance of Treg cells (Ziegler 2006). Recent studies have proposed that signaling through IL-2 receptor would elicit STAT5, which in turn is capable of inducing FOXP3 (Yao et al. 2007, Burchill et al. 2007). However, results showing that IL-2 is not crucial for FOXP3 expression and the establishment of Treg cells during thymic development, have also been reported (D’Cruz and Klein 2005, Fontenot et al. 2005). Instead, other common γ chain (γc) dependent cytokines can compensate for the absence of IL-2. Common γc-deficient mice have complete deficiency of FoxP3+ Treg cells (Fontenot et al. 2005). For example IL-15 is capable of inducing FoxP3 expression, but its potency is much weaker than IL-2 (Imamichi et al. 2008).

IL-7 promotes proliferation and survival of all thymocytes as well as mature peripheral T cells. Additionally, thymic stromal lymphopoietin (TSLP) can also signal through IL-7 receptor (IL-7R). IL-7R (CD127) is richly expressed on thymocytes and naïve T cells generally. However, Treg cells seem to develop and function normally in IL-7-deficient mice and Treg cells express low levels of IL-7R (Bayer et al. 2008). It has also been speculated that IL-7R signaling affects thymic T cell progenitors early during differentiation, at a stage distinct from which IL-2 functions
(Mazzucchelli et al. 2008). In total, analyses of mice indicate that neither IL-7 nor IL-15 are required when IL-2R signaling is possible (Bayer et al. 2008).

The co-stimulatory CD28 molecule has shown to be essential for thymic Treg development (Sansom and Walker 2006). The role of TGF-β in the thymic development of Treg cells is still under debate, but TGF-β and CD28 seem to have importance in the maintenance and homeostasis of natural Treg cells in the periphery (Tang et al. 2003, Marie et al. 2006, Marie et al. 2005, Li et al. 2006). Also the CD40/CD40L interaction is reported to contribute to homeostasis of Tregs in the periphery (Guiducci et al. 2005).

Recently a two-step model of Treg cell differentiation has been generated. According to this model, signaling through TCR and co-receptor CD28 is initially required, but additional signaling through cytokines completes the differentiation that triggers the expression of FOXP3 (Burchill et al. 2008). In accordance with this model, recent data suggest that cells among CD4⁺ SP thymocytes, which are recently activated through TCR, become CD25high. These cells also express GITR and CD69 and are immediate precursors of Treg cells. In addition, the TCR repertoire of Treg cells was more similar to CD25high GITRhigh cells than CD25low GITRint proposing that CD25high cells were already selected by TCRs characteristic for Tregs (Lio and Hsieh 2008).

Probably several cell types can influence Treg cell development in the thymus, since cTECs, mTECs and DCs all have exhibited possible effects on Treg cell development. Hassall’s corpuscles have been shown to support the development of Treg cells. TSLP is expressed by Hassall’s corpuscles and activates CD11c-positive immature DCs to up-regulate MHC class II molecules and B7 co-stimulatory molecules. These primed DCs were thereafter capable of inducing proliferation and differentiation of CD4⁺CD8⁺CD25⁺ thymic T cells into CD4⁺CD25⁺FOXP3⁺ Treg cells in humans (Watanabe et al. 2005). In mice, TSLP also supports Treg cell development, but unlike in humans, the cytokine seems to act directly on FoxP3⁺ CD4⁺ SP thymocytes (Jiang et al. 2006).

5 PERIPHERAL TOLERANCE

In spite of the negative selection during the T cell development some autoreactive cells always escape to the periphery. There are several mechanisms to keep these cells in check, the Treg cells probably being among the essential mediators of peripheral tolerance.

5.1 Natural Treg cell function

It has been well established that Treg cells are able to suppress the proliferation of other T cells. Fundamental to the Treg cell function is that the suppression is TCR-dependent, that is, the CD4⁺CD25⁺ Treg cells must be stimulated via TCR to exert suppression. Additionally, the Treg cells are highly sensitive to antigenic stimulation, and much lower antigen quantities are sufficient for activation when compared to conventional T cells. However, once the Treg cells are stimulated by a specific antigen, they can suppress both the CD4⁺ and CD8⁺ T cells, and the suppression they mediate is antigen-nonspecific (Sakaguchi 2004).
The hallmark for Treg cell function has been the dependence of cell-to-cell contacts. Nonetheless, it has been questioned whether actual contact is required or whether close proximity of the cells is sufficient. Natural Treg cells are shown to suppress naïve and effector T cells, as well as affect the function of NK cells, NK-T cells, B cells, DCs and macrophages. They have various mechanisms of suppression. It has also been suggested that there are subpopulations of the FoxP3$^+$ Treg cells with different functional properties (Sakaguchi et al. 2008, Vignali et al. 2008, Gupta et al. 2008).

5.1.1 Cellular contacts
Several studies clearly indicate that the regulation of effector T cells occurs in part through the regulation of APCs. Because APCs are required for the activation of effector T cells, these cells are at a fundamental point of control. It has been suggested that Treg cells affect both the maturation and function of DCs. It was recently published that, in mice, CTLA-4 on the cell surface of Treg cells mediates down-regulation of B7 molecules on APCs, thus limiting the potency of APCs to activate T cells (Wing et al. 2008). Treg cells can also induce up-regulation of the IDO enzyme (indoleamine 2,3-dioxygenase) in DCs. IDO catalyzes breaking down of tryptophan and deprivation of tryptophan inhibits the entry of the cell to cell cycle. Additionally, tryptophan metabolites, kynurenines, are toxic and induce apoptosis. Consequently proliferation of T cells is inhibited. Also up-regulation of IDO appears to be CTLA-4 dependent. LAG3 (lymphocyte activation gene 3), which is up-regulated upon Treg activation, is suggested to suppress the maturation of DCs and it has also been proposed to engage and inhibit effector T cells. Direct suppression of effector T cells through CTLA-4 may also happen (Vignali et al. 2008, Gupta et al. 2008, Tang and Bluestone 2008, Maggi et al. 2005).

5.1.2 Cytokines
Suppression of effector T cells through inhibitory cytokines is one of the well established means. IL-10 and TGF-β are thought to function as suppressor molecules of Treg cells. However, contribution of these cytokines on the function of thymus-derived Treg cells is unclear, since these cytokines are used by peripherally induced Treg cells as well. Treg cells also modulate cytokine production by DCs. TGF-β may function also in a cell contact-dependent manner, since Treg cells may have it bound on their cell surface as well (Vignali et al. 2008, Tang and Bluestone 2008). In mice, a third important cytokine mediating suppression is the recently described cytokine IL-35, which has been stated to be required for maximal suppression (Collison et al. 2007). However, human Treg cells were not found to express detectable amounts of IL-35 (Bardel et al. 2008).

Another way for regulating effector T cells is thought to occur through changes in the metabolism of effector cells. It has been suggested that inhibition of the production of cytokines, especially IL-2, by Tregs results in apoptosis of effector cells. In addition, pericellular adenosine or intracellular cAMP introduced to the effector cells is described to inhibit their function. Treg cells are able to suppress target cells also by killing through cytolysis. Human Treg cells have been noted to secrete granzyme A and perforin, in mice granzyme B has been detected (Vignali et al. 2008).
5.1.3 Suppressive role of Treg cells in immune responses
Regulatory T cells are reported to have diverse effects on immune responses and are important to the maintenance of tolerance to many tissue-specific antigens. Mutations in FOXP3 gene cause the complete absence of Treg cells, leading to multiorgan inflammation and destruction or IPEX. This disease is described in more detail in 4.2. Several more common autoimmune conditions have also been linked to defects in Treg cells. These include colitis, inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis. Treg cells can also play a role in modulating immune responses against microbes. They limit inflammation and prevent the development of excessive immune responses. On the other hand, they may promote persistence of the pathogen and thereby allow a chronic infection. They may also dampen antitumor immunity and prevent the eradication of malignant cells (Piccirillo 2008, Sakaguchi et al. 2008, Vignali et al. 2008). Recently it was also reported that the number of Treg cells is reduced in abdominal fat of obese mice and Treg cells influence the inflammatory state of adipose tissue and insulin resistance (Feurer et al. 2009, Winer et al. 2009).

5.1.4 Activating role of Treg cells in immune responses
In contrast to their suppressive role, it was lately published that instead of suppression Treg cells contributed to early immune responses against local virus infection. It was shown that the ablation of Treg cells impaired the migration of antiviral effector cells to the infection site. In addition in the absence of Tregs there was an increased pathogen replication and altered chemokine milieu in secondary lymphoid organs (Lund et al. 2008). Thus in some situations Treg cells seem also able to promote defence against pathogens.

5.2 Peripheral conversion of Treg cells
Natural Treg cells develop in thymus, yet cells with regulatory properties are also converted from conventional T cells in the periphery. Peripheral development of Treg cells likely generates also cells specific to foreign antigens. However, there is some disagreement whether activation-induced Treg cells can act as suppressors and are functionally stable.

5.2.1 Peripheral induction of FOXP3+ Treg cells
The cytokine milieu in which T cells are activated plays an important role in determining the phenotype of induced suppressor or effector cells. TGF-β is reported to participate in the peripheral generation of Treg cells. Retinoic acid, produced by DCs, enhances the differentiation to FoxP3+ Tregs as retinoic acid inhibits IL-6 driven programming to Th17 cells. Especially CD103+ DCs in the mesenteric lymph nodes and the small intestine lamina propria promote induction of FoxP3+ Treg cells through TGF-β and retinoic acid-dependent mechanism. These induced Treg cells are thought to contribute to mucosal tolerance and homeostasis (Benson et al. 2007, Coombes et al. 2007, Sun et al. 2007, Mucida et al. 2007). Also IL-2 is reported to be important for peripheral Treg generation, since it inhibits induction towards Th17 cells (Laurence et al. 2007). The relationship of peripheral induction between Th17 cells and Treg cells seems to be intimate. Additionally it has been shown in both mice
and humans that IL-17 producing cells can develop from FoxP3+ Treg cells (Voo et al. 2009, Xu et al. 2007). Further, cells co-expressing both FoxP3 and RORyt have been reported (Zhou et al. 2008).

Recent studies analyzing TCR repertoires have shown a clear overlap between the TCR repertoires of thymic and peripheral FoxP3+ cells, or respectively between thymic and peripheral conventional TCR repertoires. This indicates that a considerable proportion of peripheral Treg cells in mice are of thymic origin and are not recruited from conventional T cells in the periphery upon contact with self-antigens (Hsieh et al. 2006). Another report also stated that peripheral conversion had no prominent contribution to the normal Treg cell population, with the conversion being, however greater in a lymphopenic environment (Relland et al. 2009). Several TCRs from converted Treg cells in lymphopenic hosts could also be found in the normal thymic Treg cell population. These data suggest that peripheral conversion is not an automatic consequence of T cell activation, but it is the TCR specificity that determines the capability of the cell to undergo conversion (Lathrop et al. 2008). The extent of the peripheral conversion thus remains controversial, being probably more common in humans.

In humans it has been reported that a fraction of the regulatory population is generated from rapidly dividing, highly differentiated memory CD4+ T cells. A comparison of the TCR repertoires of the regulatory CD4+CD25high and memory CD4+CD25- T cells suggested that these populations are derived from the same clonal precursors (Vukmanovic-Stejic et al. 2006).

5.2.2 Nonregulatory induction of FOXP3

Activation induced up-regulation of FOXP3 is not necessarily associated with suppression and is widely seen in humans. However, the level of FOXP3 is then transient and substantially lower when compared to natural Treg cells (Allan et al. 2008, Walker et al. 2005, Yagi et al. 2004, Walker et al. 2003, Allan et al. 2007). Surface molecules expressed by these activated FOXP3+ cells are similar to natural Treg cells. The production of cytokines, for example IL-2 and IFN-γ, by recently activated FOXP3+ cells continues and it is reported to diminish over time. It is suggested that a prolonged expression of FOXP3 is required for suppression of cytokine secretion and, at least in humans, regulatory properties also require prolonged expression of FOXP3 (Allan et al. 2007, Gavin et al. 2006, Roncador et al. 2005). However, in vitro studies of human T cells showed that stimulation of CD4+CD25+ cells with IL-15 or IL-2 and without TCR stimulation, led to sustained high levels of FOXP3 and CD25, but not to suppressive capacity (Imamichi et al. 2008).

5.3 Other mechanisms of peripheral tolerance

In addition to active suppression, there are also other mechanisms to maintain tolerance. Activation of T cells is restricted to secondary lymphoid tissues, mainly to lymph nodes, where antigens are presented, typically by DCs. In addition DCs have to be activated by a pathogen, since immature DCs are not very effective in stimulating naïve T cells (Mellman and Steinman 2001). Naïve T cells circulate predominantly between blood and lymph nodes and no entry to tissues occur without activation. Thus naïve T cells do not recognize their antigens if antigens are not properly
presented with costimulatory molecules. Physical boundaries, for example the blood-brain barrier, are another mechanism to avoid T cell activation (Simpson 2006).

In addition to the negative selection during thymic development, T cells are deleted also in the periphery. Sometimes during the antigen presentation by APCs, mainly dendritic cells, T cells are directed to die through programmed cell death instead of activation. At first, a naïve T cell however gets activated and also proliferates, but during the clonal expansion it is directed to apoptosis. The mechanisms that lead DCs to give different signals are unclear. DCs can take up and process antigens originating from nearby tissues and present them in MHC class I molecules. This is referred to as cross-presentation and it is thought that cross-presentation supports signals leading to deletion. Particularly deletion of CD8+ T cells is considered as an important tolerance mechanism (Heath and Carbone 2001).

Yet another way to prevent unsuitable immune responses is anergy. Anergic T cells are unresponsive to their specific antigen. It is a consequence of unsuccessful activation. Anergy arises when a T cell does not receive appropriate co-stimulation during antigen recognition or the antigen recognized triggers inadequate signaling through TCR. Anergic T cells are incapable of producing IL-2 (Mellman and Steinman 2001, Schwartz 2003, Zouali 2007, Van Parijs and Abbas 1998).

6 FAILURE OF TOLERANCE: APECED AS A MODEL

Autoimmunity ensues, when tolerance to self breaks. Autoimmune diseases affect 3-5% of the population. These diseases are divided into systemic and organ-specific or localized autoimmune disorders. Some are caused by autoantibodies, for example myasthenia gravis, and some by T cells, for example type 1 diabetes, although also antibody-mediated pathways cause tissue injury in diabetes (Greeley 2002). The background of autoimmunity is complicated, both genetic and environmental factors have role in the disease’s induction (Marrack et al. 2001, Santamaria 2001).

Some rare monogenic diseases have been recognized. Since APECED (Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy) results from a mutation of a single gene, it is an interesting and valuable disease model (Perheentupa 2006), especially since AIRE (AutoImmune Regulator) is linked to thymic development, and natural Treg cell development occurs in the thymus.

6.1 Human disease
APECED or Autoimmune Polyendocrine Syndrome type I (APS-1) is a recessively heritable monogenic autoimmune disease, caused by mutation in the AIRE gene (Finnish-German APECED Consortium 1997, Nagamine et al. 1997). It belongs to the Finnish disease heritage. This rare disease is enriched also in Iranian Jews and Sardinians. Approximately 90% of the Finnish patients carry the Finnmajor mutation, R257X (Perheentupa 2006), which is also the most common mutation worldwide.

The clinical picture varies, but the most common manifestations, the classic triad, are mucocutaneous candidiasis, hypoparathyroidism and adrenocortical failure (Addison’s disease). Candidiasis occurs at least occasionally in all patients and it is often the first symptom of APECED. Other components of the disease are e.g. gonadal failure, type 1 diabetes, pernicious anemia, malabsorption, enamel hypoplasia, alopecia, nail dystrophy and keratoconjunctivitis. The onset of the disease
occurs usually in childhood and new components may evolve throughout life. Adult patients have on average five disease components, but some individuals suffer from up to ten components (Perheentupa 2006, Ahonen et al. 1990, Perheentupa 2002). Clear correlations between the type of mutation and the phenotype have not been shown. The manifestations vary substantially, as much between siblings than between other patients, hence one can assume that there are other genetic and environmental factors besides the AIRE mutation that affect the phenotype (Ahonen et al. 1990). Yet, it has been noted that patients lacking the Finnmajor mutation have a decreased prevalence of chronic mucocutaneous candidiasis (Halonen et al. 2002).

In the absence of a functional AIRE gene product, autoreactive T cells may escape to the periphery because of the disturbed negative selection, due to the lack of expression of PTAs in thymus (Anderson et al. 2002, Liston et al. 2003). Various autoantibodies against components of the target tissues are also present. The autoimmune tissue destruction appears similar to that observed in the same tissues in non-APECED autoimmunity including tissue infiltrations of lymphocytes in the affected organs. The appearance of specific autoantibodies often precedes the clinical disease of the target tissue. Antibodies specific for type 1 interferons are prevalent and since they are specific to APECED patients, they can be used as diagnostic markers for the disease (Meager et al. 2006, Meloni et al. 2008).

6.2 Aire knock-out mouse and ectopic transcription
Several mouse models of APECED have been generated. The suggestion that the transcription factor Aire, at least in part, induces ectopic transcription of PTAs in mTECs, has originated from studies in Aire−/− mice (Anderson et al. 2002, Liston et al. 2003). The absence of AIRE leads to lack of broad repertoire of PTAs, and thus the negative selection of thymocytes in the thymic medulla is impaired. In consequence autoreactive T cells escape to the periphery and autoimmune manifestations ensue.

However, the Aire−/− mouse does not mimic APECED too successfully. This suggests that the failure of negative selection is not enough to explain the disease manifestations seen in APECED. Different kinds of mutations have been targeted and different mouse backgrounds have been used. The background of mice has been shown to have influence on the phenotype, with the autoimmune-prone nonobese diabetic (NOD) mouse getting the most severe autoimmune characteristics (Niki et al. 2006, Jiang et al. 2005). In most backgrounds the phenotype of the mice is mild and they are clinically fairly healthy.

There are, nonetheless, signs of immunological dysregulation. T cells are hyperreactive and there are disturbances of the TCR repertoire. In addition, tissue infiltrates and autoantibodies have been detected (Anderson et al. 2002, Ramsey et al. 2002, Kuroda et al. 2005). However, the symptoms and target tissues differ from the manifestations of APECED, with the exception of perturbations of fertility. Additionally, Aire-deficient mice do not seem to be susceptible to candidiasis (Hubert et al. 2009).

Treg cells are reported to develop and function normally in Aire−/− mice (Liston et al. 2003, Kuroda et al. 2005, Anderson et al. 2005). Mice lacking both Aire and Foxp3 show pronounced autoimmune manifestations compared to mice with only one mutation (Chen et al. 2005). These results suggest that in mice functional Treg cells may curtail potentially pathological immune reactions caused by Aire-deficiency and keep the phenotype of Aire−/− mice mild.
6.3 Other functions of AIRE

AIRE is localized mainly in the cell nucleus (Björses et al. 1999, Heino et al. 1999, Rinderle et al. 1999). The AIRE protein contains several functional domains, including a DNA binding domain (Kumar et al. 2001, Purohit et al. 2005), and its structure suggests a role as a transcriptional regulator. E3 ubiquitin ligase activity has been suggested, but these findings have been challenged (Uchida et al. 2004, Bottomley et al. 2005). Since major patient mutations occur in the plant-homeodomain (PHD) zinc fingers, these regions are probably important for AIRE (Björses et al. 2000).

Several observations suggest that AIRE has other functions in addition to ectopic transcription. Aire-deficient mice have shown autoimmunity against antigens, which are retained in the thymus in the absence of Aire (Kuroda et al. 2005, Niki et al. 2006). It has been shown that Aire-deficient mTECs present antigens less efficiently. This suggests that Aire contributes also to the expression of some genes related to antigen processing and presentation (Anderson et al. 2005, Johnnidis et al. 2005). Additionally, rapid apoptosis of Aire-mTECs is detected. This may be a mechanism to promote the cross-presentation of the peripheral-tissue antigens they produce (Gray et al. 2007).

In addition mTECs, it has been reported that AIRE is expressed at low levels on B cells and some thymocytes, mainly DP cells (Suzuki et al. 2008). In one report Aire-deficiency is reported to cause developmental block in the late stage differentiation of CD4 SP cells in mice, although the mechanism remained undetermined (Li et al. 2007). However, later studies have shown that Aire affects thymic organization and maturation of mTECs (Yano et al. 2008), and thus the altered thymic microenvironment in Aire-deficiency may affect the maturation of thymocytes.

Aire is detected also to a lesser extent in DCs (Anderson et al. 2002, Anderson et al. 2005, Hubert et al. 2008). Aire is described to affect the differentiation and function of DCs. Aire-/- DCs induced stronger stimulation of T cells, which was in part influenced by the increased expression of VCAM-1 on APCs (Ramsey et al. 2006). In APECED patients, DCs are shown to be functionally impaired, responding poorly to microbial stimuli (Pontynen et al. 2008).

It was recently published that Aire is also expressed in secondary lymphoid tissues of mice, in cells referred to as extrathymic Aire-expressing cells (eTACs). These cells were capable of interaction with naïve T cells, followed by deletion of self-specific T cells. They had characteristics of professional APCs, but were non-DC and resembled in part mTECs. eTACs were MHC class II+, but lacked the expression of B7 co-stimulatory molecules. AIRE-regulated expression of PTAs was detected, but the set of antigens differed from the antigens presented in the thymus, suggesting a supplementary role in maintaining immune tolerance (Gardner et al. 2008). The effects of AIRE thus seem to extend also beyond the thymus.
AIMS OF THE STUDY

Regulatory T cells are important in the maintenance of tolerance. Disturbances in the development or function of the Treg cells are linked to autoimmune manifestations of varying severity. Aims of this study were:

I To characterize the earliest precursors expressing FOXP3 in the human thymus

II To compare and define the developmental relationship of different thymocyte populations expressing FOXP3 in the human thymus

III To examine regulatory T cells with APECED as a model autoimmune disease
SUMMARY OF MATERIALS AND METHODS

The materials and methods of this study are described in more detail in the original publications I-IV.

1 Samples

1.1 Healthy donors (I-IV)
Blood samples were collected from healthy adult volunteers and APECED patients. Additionally, when large amounts of peripheral blood mononuclear cells (PBMCs) were needed, buffy coats obtained from blood donors were used (Finnish Red Cross Blood Service, Helsinki, Finland). PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). If not used immediately, PBMCs were cryopreserved in 10% DMSO and 90% FCS. Thymic tissue was obtained from children undergoing cardiac surgery. Thymocytes were released from the tissue samples by mechanical homogenization and used fresh.

1.2 APECED patients (IV)
We studied 26 APECED patients (12 males, 14 females). The mean age of the patients was 39.8 years (range 26–60). The AIRE mutation was verified by sequencing, as described (Halonen et al. 2002). Twenty-one of the patients carried the homozygote Finn-major mutation (R257X) in the AIRE gene. The HLA typing of the patients has been previously published (Halonen et al. 2002b) and the HLA-matched control was identified among donors studied in Mustonen et al. 1996.

Table I The most common disease components in the patients studied

<table>
<thead>
<tr>
<th>Disease Component</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mucocutaneous candidiasis</td>
<td>26/26</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>22/26</td>
</tr>
<tr>
<td>hypoparathyroidism</td>
<td>19/26</td>
</tr>
<tr>
<td>hypogonadism</td>
<td>13/26</td>
</tr>
<tr>
<td>hypothyroidism</td>
<td>9/26</td>
</tr>
<tr>
<td>diabetes</td>
<td>6/26</td>
</tr>
</tbody>
</table>

1.3 Mice (IV)
Aire deficient C57BL/6 mice were constructed using a targeted disruption of the murine Aire gene, as described in Ramsey et al. 2002. The controls were parental, syngeneic wild-type (WT) mice. The mice were kept at specific pathogen free conditions at the National Public Health Institute, Helsinki, Finland. They were sacrificed at the age of 5-6 months. Perfused organs were frozen in liquid nitrogen and homogenized in a Tripure reagent (Roche, Basel, Switzerland) using an Ultra-Thurrax apparatus (Janke & Kunkel, Staufen, Germany).
2 Cell separation and flow cytometry (I-IV)

2.1 Immunomagnetic sorting
Selected subsets were isolated using monoclonal antibodies (mAbs) and magnetic beads (Dynal, Oslo, Norway), as instructed by the manufacturer. The bound cells were directly lysed. Alternatively, when necessary, the cells were detached either by overnight incubation in +37°C or by using DETACHaBEADS (Dynal). The efficiency of the cell isolations was confirmed by flow cytometry.

2.2 Flow cytometry
Anti-CD3, CD4, CD5, CD8, CD25, CD45RA, CD45RO, CD127, CTLA-4, EpCAM, GITR, HLA-DR, αβ TCR and γδ TCR mAbs were direct conjugates and purchased from Becton Dickinson (San Jose, CA). FITC- or PE-labeled anti-mouse Ig second step reagents and isotype-matched control mAbs were also from Becton Dickinson. mAbs against human TCR Vα2 and Vα12 were from Pierce (Rockford, IL), and Vα24 from Beckman Coulter (Fullerton, CA). The anti-preTα mAb was a gift from Dr. M. Toribio (Universidad Autonoma de Madrid, Madrid, Spain). Anti-AIRE mAb was from P. Peterson (University of Tartu, Tartu, Estonia). Anti-human FOXP3 mAb conjugated to PE (clone 236A/E7) was purchased from eBioScience (San Diego, CA) and anti-FOXP3 clone 150D/E4 (unlabeled) a gift from Dr. A. Banham (University of Oxford, Oxford, UK). A mix containing anti-human CD14, CD19, and CD56 mAb was purchased from Dynal.

Cell permeabilization for intracellular protein detection was carried out by using a Fix&Perm kit (Caltag, Burlingame, CA) or by using a FOXP3 Permeabilization and Fixation kit from eBioscience, both according to the manufacturers’ instructions.

The flow cytometric analysis was done with a FACScan or a FACSaria (Becton Dickinson). For cell sorting, a FACSaria or a FACStar (Becton Dickinson) was used.

2.3 Control staining
Some of the subsets studied were present at extremely low numbers. Thus several control stainings were carried out to exclude significant levels of nonspecific staining. The binding of the secondary reagents in the absence of primary mAb was tested. Fluorescently labeled nonspecific mouse antibodies were also tested.

3 Cell culture (III, IV)
The cells were cultured in an RPMI medium (Life Technologies, Paisley, UK), supplemented with 10 % fetal calf serum (FCS) or 10 % heat-inactivated human AB serum (Finnish Red Cross Blood Service), 20 μM HEPES, 2 μM L-glutamine, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin. For stimulation, platebound anti-CD3 mAb, heat-killed Candida albicans at 2.5 μg/ml, phytohemagglutinin (PHA) (Sigma Aldrich) at 5 μg/ml or human insulin (Orion Pharma,
Espoo, Finland) at 10, 2, or 0.4 μg/ml was used. The cells were cultured in duplicate in 96-well plates for 5 days at +37°C in 5% CO₂. For the last 6h the cells were incubated with 1 μCi of ³H-thymidine/well. The cells were then harvested with a Skatron harvester (Newington, New Hampshire), and thymidine incorporation was analyzed with a liquid scintillation counter (Wallac, Turku, Finland).

IFN-γ levels were determined using an ELISA with anti-human IFN-γ mAb (Endogen, Woburn, MA), as described (Skarsvik et al. 2005).

4 PCR

4.1 RNA/DNA isolation and cDNA synthesis (I-IV)
Total RNA was isolated from the cells lysed with a Tripure reagent by using an RNeasy Mini or Midi Kit (Qiagen, Crawley, UK). First-strand cDNA was synthesized by using an oligo-dT-primer (Sigma, St. Louis, MO) and an AMV-reverse transcriptase (Finnzymes, Espoo, Finland). DNA was isolated using a QIAamp Blood kit (Qiagen).

4.2 Quantitative PCR (I-IV)
Quantitative PCR was carried out using primer-probe-assays and a TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA), the reactions were analyzed using an iCycler-IQ instrument (Biorad, Hercules, CA) or an ABI 7900HT (Applied Biosystems). With human samples, commercially available intron-spanning primer-probe assays (Applied Biosystems) were used. The TCR Cα and RAG-2 assays were assay-by-design products. The sequences are found in the original articles. The assay for the murine TCR Cα was not designed to span an intron and therefore the mouse RNA was treated with DNase I (Sigma). The absence of genomic DNA was verified by real-time PCR.

Reactions were run in duplicate and results were normalized against β-actin, or GAPDH when indicated. A relative quantitation was done by comparing the results to a standard dilution curve.

The amount of TCR excision circles (TRECs) was estimated by a quantitative PCR-ELISA assay as described (Al-Harthi et al. 2000).

4.3 TCR repertoire analysis (II- IV)
The TCR repertoire analysis was performed as described (Pannetier et al. 1995). A set of Vα or Vβ-specific primers was used with a Cα or Cβ-specific primer to amplify the total Vα or Vβ repertoire, followed by a run-off reaction with an internal FAM-labeled Cα or Cβ primer. All primers were synthesized by Sigma and Amplitaq Gold enzyme was used for the PCR (Applied Biosystems). The fluorescent amplification products were separated and detected with an ABI377 or ABI3730 sequencer (Applied Biosystems). For quantitation of similarity, comparative methods described in original articles II and IV were used.
5 Immunohistochemistry (IV)

Thymic tissue was fixed in formalin or frozen in liquid nitrogen. For immunohistochemistry, 5 μm sections of the formalin-fixed tissues were dewaxed, heated in a microwave oven for 10 min for antigen retrieval, and stained with the monoclonal anti-AIRE antibodies and with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase. For immunofluorescence, acetone-fixed, 5 μm cryostat sections were stained with the AIRE mAb, followed by FITC-labelled second-step reagents.

The densities of the AIRE⁺ cells in the thymic medulla at or in the near vicinity of Hassall’s corpuscles and at more distant medullary areas were estimated in sections stained by the indirect immunohistochemistry or immunofluorescence techniques. For analysis, an Olympus BX50 microscope equipped with a CCD-camera (Hamamatsu Photonics, Hamamatsu, Japan) was used.

6 Statistical analyses

Results are shown as mean ± SD. P-values were calculated with Student’s two-tailed t-test, correlations with Pearson’s correlation coefficient, with a limit of P < 0.05 for significance. Mann-Whitney U test was used for P-values of AIRE⁺ cell densities in the different areas of the thymus. Confidence intervals were calculated at the 95 % level.

7 Ethical considerations

The study has been evaluated and approved by the ethical committee of the Helsinki University Hospital. All samples were collected in accordance with the Helsinki Declaration. Informed consent was obtained from the patients, adult volunteers and the parents of the children.

The study using Aire knock-out mice has been approved by the ethical committee of animal use at the National Public Health Institute and the University of Helsinki as well as the State Provincial Offices of Southern Finland.
RESULTS

1 Up-regulation of FOXP3 in thymus (I)

1.1 FOXP3 expression in DN thymocytes

FOXP3 is currently considered the best candidate for determination of CD4$^+$ CD25$^+$ Treg cells (Fontenot and Rudensky 2005, Ziegler 2006). The present opinion on Treg cells of thymic origin is that upregulation of FoxP3 requires TCR-mediated signals (Fontenot et al. 2005, Hsieh et al. 2004, Picca et al. 2006). In mice, FoxP3 is currently thought to be up-regulated at the CD4$^+$CD8$^+$ DP stage, following the surface expression of αβ TCR (Cabarrocas et al. 2006, Wan and Flavell 2005). The flow cytometric analysis showed that in the human thymus 8.6 ± 2.5 % (range 4.6 - 13.2 %) of the CD4$^+$ SP population and 0.4 ± 0.2 % (range 0.1 - 0.8 %) of the DP population expressed FOXP3. Of all FOXP3$^+$ cells, 69.9 ± 12.8 % belonged to the CD4$^+$CD8$^+$ SP subset, and 61.2 % of these cells expressed CD25 as well. According to earlier studies, these cells are functionally mature and indistinguishable from circulating CD4$^+$CD25$^{high}$ Treg cells (Maggi et al. 2005). Of the rest FOXP3$^+$ thymocytes, 17.9 ± 7.7 % belonged to the DP and 11.0 ± 7.2 % to the CD8$^+$CD4$^+$ SP populations. However, a small subset of FOXP3$^+$ cells was also found in the CD4$^+$CD8$^+$ DN subset in every thymus studied, accounting for 1.4 ± 1.1 % of all DN cells. The intensity of FOXP3 in the DN cells was similar to the FOXP3$^+$ CD4$^+$ SP cells. However, only 22.3 % of the FOXP3$^+$ DN cells were CD25$^+$. Any nonspecific staining was excluded.

To ensure that the DN thymocyte population expressed FOXP3, the amount of FOXP3 mRNA in the isolated DN thymocytes was measured using quantitative PCR. Conformant with the FACS data, there was no significant difference between thymocytes in general and the DN cells. As a comparison, the level of FOXP3 mRNA in the DN thymocytes was 30 times higher than in γδ T cells of peripheral blood, which are not reported to express FOXP3. Taken together, these data attest that a small population of DN thymocytes express both FOXP3 mRNA and protein.

1.2 TCR expression in FOXP3$^+$ thymocytes

The DN thymocytes represent the earliest precursors of T cells, since they have not yet finished TCR gene rearrangements and therefore cannot express αβ TCR on their cell surface (Blom and Spits 2006). We analyzed TCR expression in the FOXP3$^+$ thymocyte subsets using FACS. The majority of all FOXP3$^+$ thymocytes expressed αβ TCR. In the FOXP3$^+$ CD4$^+$ SP population, almost all of the cells were TCR$^{high}$, whereas in the FOXP3$^+$ DN population less than one third of the cells expressed αβ TCR, and the TCR expression level was significantly lower than in the CD4$^+$ SP cells (mean fluorescence intensity (MFI) of TCR staining 129 ± 36 vs. 256 ± 48; P < 0.01). Intracellular staining showed that lack of TCR on the surface of the FOXP3$^+$ DN cells was not an artifact caused by TCR internalization. Expression of pTα was not observed. These results demonstrate that a subset of FOXP3$^+$ DN cells lacking TCR
expression exists in the normal human thymus. Immunofluorescence staining showed that the TCR FOXP3+ cells were localized in cortical areas of the thymus (Fig 6D).

Most of the FOXP3+ DN thymocytes expressed CD2, an early T/NK lineage marker (Tangye et al. 2000), supporting the fact that the FOXP3+ DN thymocytes belonged already to the T cell lineage. Further analysis showed that the FOXP3+ DN thymocytes were not $\gamma\delta$ T cells, B cells, NK cells or monocytes, excluding the possibility of ectopic FOXP3 expression in some other subset belonging to the DN thymocytes. Neither did the epithelial cells express any FOXP3.

Figure 6. DN thymocytes express FOXP3 in the absence of TCR

A, Flow cytometric analysis shows that the majority of FOXP3+ DN thymocytes do not yet express TCR on their cell surface. B, Immunohistochemical double-staining with FOXP3 and CD3 showed that the FOXP3+ thymocytes are still CD3 negative. Double-stained thymus section with red (FOXP3) and green (CD3) filter on. C, The same thymus section only with the green filter on, showing lack of CD3 expression in a FOXP3+ cell. D, HE-staining showed that FOXP3+ CD3- thymocytes are localized to the cortical areas of the thymus.
1.3 FOXP3+ DN thymocytes have characteristics of Treg cells

The FOXP3+ DN cells were CD127low, which is a feature of human peripheral blood Treg cells (Liu et al. 2006, Hartigan-O’Connor et al. 2007, Seddiki et al. 2006). Another characteristic marker of Treg cells, the intracellular expression of CTLA-4 (Sakaguchi 2004) could be detected in most FOXP3+ thymocytes, as well as in DN FOXP3+ cells. There were no significant differences in the expression of either CD127 or CTLA-4 when comparing the FOXP3+ DN and FOXP3+ CD4+ SP cells. However, the expression pattern was clearly different between the FOXP3+ CD4+ SP thymocytes and DN thymocytes. The Treg cell-associated molecules GITR and CD39 were expressed by a subset of FOXP3+ CD4+ SP cells, but no expression was found in the FOXP3+ DN thymocytes.

Of relevance was the finding that the expression of CD69, a marker associated with TCR-mediated signaling and positive selection in developing thymocytes (Hare et al. 1999, Hare et al. 2002), differed in the two FOXP3+ subsets. Of the FOXP3+ CD4+ SP cells 80.8% ± 4.7% were CD69+, and of FOXP3+ DN cells only 13.7% ± 6.3%, indicating that the FOXP3+ DN cells have not received TCR-mediated signals. Similarly, the expression of CD5 was analyzed to study the maturational stage of DN cells. Expression of CD5 has been reported to correlate with the strength of TCR-mediated signals in thymocytes (Azzam et al. 1998). We found differing expression levels of CD5, MFI of CD5 was 32 ± 20 in the FOXP3+ CD4+ SP cells, and only 3 ± 1 in the FOXP3+ DN cells.

We tried to isolate FOXP3+ DN thymocytes indirectly by depleting other cells in the absence of suitable surface markers. After depletion the average frequency of FOXP3+ DN cells was 38.6% (range 10.3% - 80.2%). However, when the sorted cells were co-cultured with autologous CD4+ SP thymocytes or PBMC, no suppressive effect or IL-10 production was observed. The incapability to sort out viable FOXP3+ cells also prevented cell cultures and differentiation assays, and it was thus not possible to track the developmental role of the FOXP3+ DN thymocytes.

2 FoxP3 expression in DP cells (II)

2.1 CD25+ DP thymocytes express as much FOXP3 as CD25+ CD4+ SP thymocytes

During the thymic T cell development the DN thymocytes mature to DP cells. Similarly to the DN thymocytes, the immature DP population, which accounts for 70-90% of all thymocytes (Spits 2002), already has a noticeable expression of FOXP3. The level of FOXP3 mRNA in DP cells mimics the FOXP3 level of thymocytes in general. Yet, a small population, 0.7 ± 0.6% (0.1-1.7%), of the DP thymocytes expressing also CD25 had a high expression of FOXP3 mRNA. The level of FOXP3 mRNA in the CD25+ DP thymocytes was virtually equivalent to the isolated CD4+CD25+ SP thymocytes, which are considered functionally mature Treg cells (Stephens et al. 2001, Liotta et al. 2005, Annunziato et al. 2002, Cupedo et al. 2005, Wing et al. 2005). By comparison, FOXP3 mRNA was expressed at a low level in the CD4+CD25+ SP thymocyte population.

Flow cytometric analysis established that CD25 expression was strongly associated with FOXP3 in both the DP and CD4+ SP thymocytes, arguing for the use of CD25 as a substitute for the isolation of FOXP3+ cells. Nonetheless, a clear subset of CD25+FOXP3+ cells could also be detected in both the CD4+ SP and DP populations. That the FOXP3 protein levels, analyzed regardless of CD25 expression,
were similar in DP and CD4\(^+\) SP cells (MFI 57 ± 16 and 62 ± 18, respectively, difference not statistically significant) is noteworthy.

![Figure 7. FOXP3 expression in thymocytes of different developmental stages.](image)

Four-color flow cytometric analysis of surface CD25 and intracellular FOXP3 protein. The frequency of FOXP3\(^+\) thymocytes in indicated gates is shown. Note that FOXP3\(^+\) DN cells are mainly CD25\(^-\) and that in CD8\(^+\)FOXP3\(^+\) cells the CD25 expression is low or absent.

2.2 Markers of positive selection on FOXP3\(^+\) DP thymocytes

To test whether the FOXP3\(^+\) DP thymocytes have already been positively selected, we determined their TCR expression level. As shown in the original article I, high levels of αβ TCR were expressed by the majority of CD4\(^+\) SP thymocytes, whereas the DP thymocytes contained large populations of TCR\(^-\) and TCR\(^{low}\) cells as well. However, the FOXP3\(^+\) fraction of DP thymocytes already expressed high TCR levels,
comparable to that of the FOXP3⁺ CD4⁺ SP thymocytes. Similarly to the FOXP3⁺ DN thymocytes, both FOXP3⁺ DP and FOXP3⁺ CD4⁺ SP cells expressed CD127 at clearly lower levels than the FOXP3⁻ thymocytes. Lastly, the FOXP3⁺ DP thymocytes expressed CD69, while the majority of FOXP3⁻ DP thymocytes were still CD69⁻. The majority of the CD4⁺ SP thymocytes, both FOXP3⁻ and FOXP3⁺, was CD69⁺. Thus the FOXP3⁺ DP subset clearly differed from the majority of the DP thymocytes having a phenotype of already positively selected cells.

2.3 FOXP3⁺ DP thymocytes have features of immature cells
Similar FoxP3⁺ thymocyte subsets have been described in mice, but the developmental relationship of these thymocyte populations remains uncertain (Fontenot et al. 2005, Wan and Flavell 2005, Fontenot et al. 2005, Pacholczyk et al. 2006). Notably, it has been argued that the FOXP3⁺ CD25⁺ DP thymocytes are not precursors of the more mature FOXP3⁺ CD4⁺CD25⁺ SP Treg cells (Fontenot et al. 2005).

To further study the developmental stage of the FOXP3⁺ DP thymocytes the CD5 expression was reviewed. CD4⁺ SP thymocytes, both FOXP3⁺ and FOXP3⁻, had a high expression of CD5. The FOXP3⁺ DP cells had higher expression of CD5 than the other DP thymocytes, but the level was significantly lower in comparison with the CD4⁺ SP subsets.

Subsequently, we quantified the expression of RAG-2 mRNA in the thymic subsets. As an indicator of ongoing recombination activity in the α loci (Spits 2002), the RAG-2 mRNA levels were high in the DP cells. The CD25⁺ DP cells also expressed high levels of RAG-2. No significant difference was detected between them and other DP thymocytes. Some RAG-2 was expressed by both CD4⁺CD25⁺ and CD4⁺CD25⁺ SP thymocytes, but the levels were significantly lower than in the DP populations.

2.4 TCR repertoires of CD25⁺ DP and CD4⁺CD25⁺ SP cells
We determined the TCR β chain repertoire of the CD25⁺DP, CD4⁺CD25⁺ SP and CD4⁺CD25⁺ SP populations for comparison of the thymocyte subsets expressing FOXP3. (For a detailed description of the method, see original article II.) Most Vβ genes displayed a polyclonal pattern in all three thymocyte subsets, as previously reported (Kasow et al. 2004). Numerous similarities were discovered between the CD25⁺DP and CD4⁺CD25⁺ SP thymocytes, but there were even more differences. In many instances, the repertoire of the CD25⁺DP cells was actually closer to that of the CD4⁺CD25⁺ SP thymocytes. An additional quantitative comparison of the CD25⁺DP cells with CD4⁺CD25⁺ SP cells was made, but no evident congruity was observed. (Detailed description in the original article II.)

In addition, we studied whether the frequency of the FOXP3⁺ DP thymocytes predicted the frequency of FOXP3⁻ cells in the CD4⁺ SP subset, as might be expected if the FOXP3⁺ DP cells are on the brink of maturing into SP cells. No correlation was observed. To summarize, these data imply that the FOXP3⁺ DP thymocytes have been positively selected but are still immature, in that they still express molecules associated with TCR rearrangements.
2.5 FOXP3 expression in CD8+ SP thymocytes
Also the CD8+CD25+ SP thymocytes have been reported to express FOXP3 mRNA and to have suppressive capabilities (Cosmi et al. 2003). Contrary to earlier data, the FOXP3 mRNA level in the CD8+CD25+ SP subset was significantly lower than in the CD25+DP or CD4+CD25+ SP thymocytes (P < 0.05 in both cases), but still higher than in CD8+CD25+ thymocytes. Intracellular detection of FOXP3 protein showed that 3.3 ± 1.4 % of the CD8+ SP thymocytes expressed FOXP3. Flow cytometry confirmed that the CD8+ SP thymocytes expressed significantly less FOXP3 than either the DP or CD4+ SP thymocytes (MFI 47 ± 10, P < 0.005 when compared with either of the two other subsets). Also the level of CD25 was clearly lower in the FOXP3+CD8+ SP thymocytes than either in the DP or CD4+ SP thymocytes, corresponding to a previous report (Cosmi et al. 2003).

3 Dual-Specificity of Treg cells (III)

3.1 Increased TCR Cα mRNA levels in CD4+CD25+ SP thymocytes
When normalizing the PCR data to the TCR Cα gene, we noted that the CD4+CD25+ SP thymocytes consistently expressed more Cα mRNA than the CD4+CD25- SP subset. The immature DP cells also expressed higher levels of TCR Cα mRNA when compared to the CD4+CD25+ cells. To double-check the results, we reanalyzed the samples using GAPDH for normalization in substitute for β-actin, which was used initially. The results remained unchanged. By flow cytometry, we measured if the higher Cα mRNA levels reflected an increased expression of the αβ TCR proteins on the surface of individual cells. In contrast to the quantitative PCR results, the CD4+CD25+ subset had the highest intensity of TCR, CD4+CD25+ cells expressing significantly less TCR proteins (MFI 71 ± 5 and 52 ± 6, P=0.0024), nonetheless more than the DP cells (24 ± 2).

Since the αβ mAb we used reacts only with the whole αβ TCR, we next analyzed the thymocyte subsets using a Vα12 mAb. We also permeabilized the cells in order to detect intracellular TCR α protein uncoupled to TCR β and CD3. The MFIs of CD4+CD25+ and CD4+CD25- cells were equivalent to the results obtained with the anti-αβTCR mAb. Thus, the differences of αβTCR expression levels did not explain our results of increased levels of TCRα mRNA in CD4+CD8+CD25+ thymocytes.

3.2 Expression of two different TCR Vα genes in CD4+CD25+ thymocytes
The above results, along with the fact that the TCR α locus lacks allelic exclusion, suggested that the CD4+CD25+ thymocytes might transcribe both TCR α loci simultaneously (Padovan et al. 1993). The CD4+CD25+ subset had a higher frequency of Vα12+ than the CD4+CD25- subset, as would be expected if the CD4+CD25+ subset included more cells expressing two TCRs. Similar results were obtained when using another Vα gene, Vα2. We then isolated CD4+CD8+ thymocytes and analyzed the frequency of CD4+CD25+ and CD4+CD25- cells expressing both Vα2 and Vα12 chains. Expression of two TCRs was significantly more common among the CD4+CD25+ cells. Similarly, isolation and subsequent analysis of Vα2+ thymocytes
showed that the frequency of cells simultaneously expressing Vα12 was again significantly higher in the CD4⁺CD25⁻ thymocytes.

3.3 Dual-specific cells express more FOXP3
Next we wanted to define whether the mature Treg cells maintained the expression of two TCRs. CD4⁺CD25⁻ and CD4⁺CD25⁺ cells isolated from blood had no significant difference in the TCR Cα mRNA levels. In the thymus, the CD25 intensity varied. However, in the periphery the T cells up-regulate CD25 also upon activation, and when studying PBLs, only CD4⁺ cells expressing high levels of CD25 are considered to be Treg cells (Baecher-Allan et al. 2001). In direct analysis of cells expressing simultaneously both Vα2 and Vα12, the dual-specific cells were more common in the CD4⁺CD25⁺ high subset than in the CD4⁺CD25⁻ subset. Because the Vα2⁺Vα12⁺ population is so small, we subsequently isolated the Vα2⁺ PBL population. The prevalence of Vα2⁺ cells also expressing Vα12 was on the average four times higher in the CD4⁺CD25⁺ high population compared to the CD4⁺CD25⁻ population. When measuring the frequency of the TCR Vα2⁺ and Vα12⁺ cells in the CD4⁺CD25⁺ high and CD4⁺CD25⁻ subsets, the frequency of the Vα⁺ cells was significantly higher in the CD4⁺CD25⁺ high subset, invariably.

To make certain that the dual-specific CD4⁺CD25⁺ high cells were Treg cells instead of activated T cells, we analyzed the FOXP3 expression in cells bearing one or two TCRs. Sorted cells expressing both Vα2 and Vα12 were compared with CD4⁺ cells or cells expressing either Vα2 or Vα12 alone. Significantly more FOXP3 mRNA was detected in cells expressing two surface TCRs than in the control populations. An intracellular staining with anti-FOXP3 mAb attested these results. FOXP3⁺ cells were significantly more common among cells with two TCRs than among cells with a single TCR or the whole CD4⁺ population. Similarly, purified Vα2⁺ cells, expressing also Vα12 were significantly more frequent in the CD4⁺FOXP3⁺ subset than in the CD4⁺FOXP3⁻ subset (1.5 ± 0.5 vs 0.7 ± 0.4, P=0.0149). These data affirm that the dual-specific cells are highly enriched in human CD4⁺CD25⁺ high FOXP3⁺ Treg cell population.
A, FOXP3$^+$ cells are significantly more common among dual-specific T cells than among cells with one TCR or the whole CD4$^+$ population. $*** = P < 0.001$. B, Isolated V$\alpha$2$^+$ cells expressing also a second V$\alpha$, V$\alpha$12, were substantially more common among the CD4$^+$FOXP3$^+$ cells (R2) than in the CD4$^+$FOXP3$^-$ subset (R1). The purity of the V$\alpha$2$^+$ population was 99.0%.

3.4 V$\alpha$2 and V$\alpha$12 are comparable with the whole repertoire
In our donors, V$\alpha$2 or V$\alpha$12 represented only 5-7% of the whole repertoire. Hence detailed analysis was required to ascertain that these V$\alpha$ chains are representative of the TCR $\alpha$ repertoire. Flow cytometric analysis showed that the V$\alpha$12$^+$ cells were more common among CD8$^+$ cells, while V$\alpha$2$^+$ were equally represented within the CD4$^+$ and CD8$^+$ subsets. The frequency and intensity of the CD25 expression as well as the CD45RO expression was similar in V$\alpha$12$^+$ and V$\alpha$2$^+$ cells to that of cells expressing other V$\alpha$ genes within the CD4$^+$ population. The amount of FOXP3 mRNA and protein was comparable in V$\alpha$2$^+$ cells and all T cells. V$\alpha$12$^+$ cells had a somewhat lower level of expression, probably due to the preference for CD8. Isolated V$\alpha$2$^+$ CD4$^+$CD25$^{\text{high}}$ and V$\alpha$12$^+$ CD4$^+$CD25$^{\text{high}}$ cells expressed as much FOXP3 as the CD4$^+$CD25$^{\text{high}}$ cells in general. In addition, isolated CD4$^+$CD25$^{\text{high}}$ cells expressing V$\alpha$2, V$\alpha$12, or both suppressed stimulated T cells as efficiently as the CD4$^+$CD25$^{\text{high}}$ cells generally. The TCR repertoire analysis of V$\alpha$2$^+$ and V$\alpha$12$^+$ cells showed similar polyclonal profiles as other V$\alpha$ chains. The TCR $\beta$ repertoire of
isolated V\(\alpha\)2\(^+\) or V\(\alpha\)12\(^+\) cells essentially resembled the repertoire profiles of other T cells as well. Except for the biased MHC class I recognition by the V\(\alpha\)12\(^+\) subset, V\(\alpha\)2 or V\(\alpha\)12 expressing cells do not differ from T cells expressing other V\(\alpha\) genes.

3.5 Most human Treg cells express two functional TCR \(\alpha\) chains

Because only a few V\(\alpha\)-specific antibodies are available and a small number of cells can be examined, the evaluation of cells expressing two TCRs is troublesome. However, the produced calculations of the frequency of dual-specific cells, made using three complementary methods, were in close agreement (see description in original article III). Approximately 20% of the CD4\(^+\)CD25\(^-\) population, comparable to T cells in general, was dual-specific. Frequency of cells with two TCRs in the CD4\(^+\)CD25\(^{high}\) subset ranged from 50% to 99%. These calculations firmly indicate that the majority of human CD4\(^+\)CD25\(^{high}\) Treg cells are dual-specific.

Finally we wanted to test whether both of the TCRs on the surface of the dual-specific Treg cells were functional. We studied the down-regulation of the surface TCR in V\(\alpha\)2\(^+\) single- and V\(\alpha\)2\(^+\)V\(\alpha\)12\(^+\) dual-specific cells when bound by a ligand, V\(\alpha\)2 mAb. The MFI of the TCR, in cells expressing only V\(\alpha\)2, was reduced to 64% of the original in CD4\(^+\) cells and to 65% in the CD4\(^+\)CD25\(^{high}\) cells, while the intensity of the non-engaged V\(\alpha\)12, used as a negative control, remained stable (94% in both cases). Dual-specific T cells gave similar results, and so did experiments carried out using V\(\alpha\)12 mAb as ligand. Dual-specific CD4\(^+\)CD25\(^{high}\) Treg cells seem to transmit TCR-mediated signals as competently as single-specific T cells.

4 Treg cells in APECED (IV)

4.1 Impaired function of Treg cells

AIRE is an important regulator of the thymic T cell development (Mathis and Benoist 2009), thus we wanted to study if the AIRE-mutation has an effect on the development of Treg cells in humans. No differences were detected in the frequency or intensity of CD4\(^+\)CD25\(^{high}\) cells when comparing APECED patients and controls, hence we studied whether the CD4\(^+\)CD25\(^{high}\) Treg cells of the patients were functioning properly. Polyclonal stimulation with anti-CD3 mAb or PHA resulted in equal proliferation of unfractionated cells in both groups. Co-culture of CD4\(^+\)CD25\(^{high}\) cells and polyclonally stimulated CD4\(^+\)CD25\(^-\) cells, (at ratios of 1:3 or 1:1) resulted in normal suppression of proliferation in controls, while the CD4\(^+\)CD25\(^{high}\) cells from patients failed to significantly suppress the response of CD4\(^+\)CD25\(^-\) cells. At the same time, an antigen-specific stimulation using *Candida albicans* evoked suppression by CD4\(^+\)CD25\(^{high}\) cells in both groups. This observation demonstrated that T cells from the APECED patients respond competently to suppression. This was also confirmed by an experiment where isolated CD4\(^+\)CD25\(^{high}\) Treg cells from healthy blood donors were cultured together with T cells from APECED patients or controls (at a ratio of 1:1). Treg cells were able to suppress anti-CD3-induced proliferation equally in patients and controls (a decrease of 79.5 ± 12.2% and 73.8 ± 23.2%, respectively).
4.2 Activation profile of Treg cells

When comparing the activation status of the CD3\(^+\) cells of the patients and controls, no significant differences were detected in the expression of HLA-DR or CD62L. When expression of CD45RO was checked in addition to the aforementioned markers, no major differences in the CD4\(^+\)CD25\(^{\text{high}}\) populations between the patients and controls were observed. As described above, the patients and controls had a similar response to *C. albicans*. Because the patients are suffering from chronic candidiasis, we contemplated that the CD4\(^+\)CD25\(^{\text{high}}\) population in the patients might be enriched by a significant fraction of activated effector T cells. In that case, a result of the depletion of the CD4\(^-\)CD25\(^{\text{high}}\) cells would be a decreased response to *Candida*. However, no decrease of the response was observed, nor did the depletion of the CD4\(^-\)CD25\(^{\text{high}}\) cells have any significant effect on polyclonal, anti-CD3-induced responses.

Lastly, we determined the TREC level of PBL from patients and controls. There was considerable variation in both groups, but no clear differences were observed in the TREC content between the patients and controls, suggesting a similar proliferation history in the periphery.

4.3 FOXP3 expression is diminished

When quantifying the FOXP3 mRNA from PBMC, the level was double in the controls compared with the patients. Next we sorted the CD4\(^+\)CD25\(^{\text{high}}\) cells and, as expected, the sorted cells expressed FOXP3 mRNA at a much higher level than the total PBMCs in both patients and controls. The CD4\(^+\)CD25\(^{\text{high}}\) cells from the patients, however, expressed approximately four times less FOXP3 than the CD4\(^+\)CD25\(^{\text{high}}\) cells from the controls. In conclusion, the expression of FOXP3 mRNA was congruently lower in patients, both in the CD4\(^-\)CD25\(^{\text{high}}\) subset and in the total T cell population.

Intracellular staining of FOXP3 protein showed that the frequency of FOXP3\(^+\) cells in the patients was significantly lower than in the controls, although the percentage varied and overlapped. Additionally, the MFI of FOXP3 was reduced in cells of the patients when compared with the controls.
Figure 9. Expression of FOXP3 in APECED patients and controls.

**A**, Flow cytometric analysis of surface CD25 and intracellular FOXP3 protein. Patients expressed FOXP3 with decreased intensity: MFI of patient 1, 12 and of patient 2, 16 when compared with the controls: MFI of control 1, 31 and of control 2, 38. The percentages indicate frequency of FOXP3+ in gated CD4+ cells.

**B**, Relative expression of FOXP3 mRNA in CD25$^{high}$ cells. The mRNA levels were determined by quantitative PCR and are shown on a relative scale as mean (range).
4.4 TCR repertoire of Treg cells

Next we performed a TCR β chain repertoire analysis of the CD4⁺CD25<sup>high</sup> cells. Since the HLA type can influence the TCR V gene usage, we looked for and found a patient-control pair matching each other at the HLA class II locus. We then sorted the same number of CD4⁺CD25<sup>high</sup> cells from the patient and the control. The samples were also adjusted by quantitative β-actin amplification. Although the sample size was just 50,000 sorted cells, the repertoire of the Treg cells was polyclonal and included most Vβ genes. However, patient–control comparison showed significant differences in the TCR diversity. The repertoire of the control showed clonal expansions in most Vβ genes. In the patient the repertoire was much less skewed and the average number of different CDR3 lengths within individual Vβ genes was significantly higher. Additionally, compared to the average repertoire derived from 11 cord blood samples, the repertoire of the APECED patient was significantly closer to that of the naïve repertoire than the repertoire of the control. Analysis of 4 HLA-non-matched patient-control pairs supported the results. These results indicate that, in APECED patients, there might be a failure in the selection of the TCR repertoire of the Treg cells.

4.5 AIRE localization in human thymus

As Hassall’s corpuscles are reported to take part in the positive selection of human Treg cells (Watanabe et al. 2005), we investigated the relationship of AIRE<sup>+</sup> cells and Hassall’s corpuscles in the human thymus. Immunohistochemical techniques demonstrated that the corpuscles were mostly AIRE<sup>-</sup>. The AIRE<sup>+</sup> cells were dispersed within the thymic medulla and more abundantly at the margins of Hassall’s corpuscles. (For detailed description see original article IV). Thus the PTAs controlled by AIRE at this location may contribute to the Treg cell selection, and the lack of AIRE function may affect the observed defect in the Treg cells of APECED patients respectively.

The role of Hassall’s corpuscles in T cell development remains puzzling. They are well developed in human thymus, but interestingly in mice these structures are poorly represented (Farr et al. 2002). We analyzed FoxP3 mRNA levels to define if there is a failure of the Treg cell development in Aire<sup>−/−</sup> mice. No significant differences in either the thymus or the spleen were observed. In accordance with previous reports (Liston et al. 2003, Kuroda et al. 2005, Anderson et al. 2005), our data demonstrate that in Aire<sup>−/−</sup> mice, there is no evident Treg cell defect analogous to APECED patients. Consequently, there may be some mechanistic difference in the development of Tregs between mice and men.
DISCUSSION

1 Treg cells and transcription factor FOXP3

1.1 FOXP3 as a marker for Treg cells
This study mostly used FOXP3 as the surrogate marker in identifying Treg cells. Indeed, when the transcription factor FOXP3 was originally discovered, it was believed to be an exclusive, Treg lineage-determining molecule. The absence of Treg cells, responsible for the characteristic phenotype of the Scurfy-mouse and IPEX-patients, was found to be due to a mutation in the transcription factor FOXP3 gene. Consequently it was thought that the expression of FOXP3 equals the Treg phenotype (Ziegler 2006, Khattri et al. 2003, Fontenot et al. 2003, Hori et al. 2003). This judgement has recently turned out to be inexact.

The peripheral increase in FOXP3 expression seems to be common phenomenon in humans and it may be constant or transient (Walker et al. 2005, Walker et al. 2003, Yagi et al. 2004). Of note is that FOXP3 expression does not in all circumstances mean gain of suppressive capacity (Wang et al. 2007, Allan et al. 2005, Gavin et al. 2006). Besides the up-regulation of FOXP3, the expression must be maintained in order to obtain the Treg characteristics. The amount of FOXP3 expression has been reported to vary, and only high and continuous expression confers with the regulatory phenotype (Allan et al. 2008). It has been proposed that transient, activation-induced FOXP3 expression might be a mechanism to attenuate the activation or dampen the response of these cells.

Although FOXP3 is recognized as a characteristic of CD4+ T lymphocytes, there are also studies suggesting that FOXP3 is expressed in epithelial cells, among others in the thymus, lungs and mammary glands (Chen et al. 2008). Two recent reports demonstrated that FOXP3 acts as a breast cancer suppressor gene, repressing the oncogenes ErbB2/HER-2 and SKP2 and suppressing tumor growth (Zuo et al. 2007b, Zuo et al. 2007a). A mutation of FoxP3 in the thymic stroma has also been demonstrated to lead to defective thymopoiesis (Chang et al. 2005). However, controversy, particularly concerning the expression of FOXP3 in thymic epithelium, remains (Liston et al. 2007).

At the moment the transcription factor FOXP3 is considered as a characteristic, but not a unique, marker of the Treg cells. Although its role in the development of natural Treg cells has become ambiguous, its impact on the functions of these cells is beyond dispute. The continuous expression of FOXP3 contributes to the phenotype seen in Treg cells, for example the expression of CD25 and CTLA-4 (Sakaguchi et al. 2008, Lopes et al. 2007). Our analyses of FOXP3 in the human thymus showed FOXP3 expression at different developmental stages of T cells. FOXP3 is especially suited for the identification of Treg precursors in the thymus.

1.2 Factors affecting the up-regulation of FOXP3
Knowledge of the factors or molecules triggering the up-regulation of FOXP3 during thymic development is incomplete, but the dominant view is that TCR signaling is essential (Fontenot et al. 2005), although supplementary signaling is also required (Burchill et al. 2008, Lio and Hsieh 2008). Our results, however, show that in the
human thymus there clearly is a population of immature DN thymocytes that already express FOXP3, yet mostly in the absence of TCR. Additionally, these cells share the expression of some characteristic Treg cell markers with their more mature thymic CD4⁺CD25⁺ counterparts. The FOXP3 expression level is also similar to the FOXP3⁺ CD4⁺ SP cells. These results dispute the necessity of TCR signaling in the induction of FOXP3.

Indeed, increasing evidence has given support to the possibility of pre-Treg cell existence prior to the thymic selections. It is also suggested that Treg cell development is initiated already at DN stage in mice. According to this theory, the DP thymocytes interact with DN cells before the recombination of the TCR genes. A subset of cells that are not exposed to this ‘trans-conditioning’ are more disposed to mature to Treg cells later on during development (Pennington et al. 2006). Furthermore, some recent studies have demonstrated that the induction of the genetic program of Treg cells may be an early event, preceding the FOXP3 up-regulation. The cells that were destined to develop into Treg cells but lacking a functional FOXP3 protein, acquired several Treg characteristics excluding regulatory properties (Lin et al. 2007, Gavin et al. 2006).

In a recent report it appeared that the expression of transgenic Treg-TCR did not enhance Treg development (DiPaolo and Shevach 2009). Although the lack of increased induction of Treg cells may be due to some factor in this transgenic setting, it may also suggest that Tregs are not, indeed, induced simply by the signals received through a self-reactive TCR, but instead have to be pre-committed to the lineage already earlier during the development in order to survive through the selection bearing such autoreactive receptors.

Thus it appears that, presumably, there are other factors affecting the commitment to Treg lineage already in the early stages of development independently of TCR. This view is supported by our results, as we see FOXP3 already in DN human thymocytes. The commitment to the Treg lineage and/or initial up-regulation of FOXP3 in the DN thymocytes may thus be induced by cytokines or signals derived from some thymic cell population if not all stochastic.

2 Thymic development of natural regulatory T cells

2.1 Relationships of FOXP3⁺ precursors

If FOXP3 is up-regulated already in the DN thymocytes, then one may suggest that the differentiation of Treg cells progresses through the DP stage to mature SP Treg cells. Conflicting views have however been presented. Although a few DP thymocytes already express FoxP3 in mice as well, these cells are not considered precursors of more mature FoxP3⁺ CD4SP cells. Instead the favored hypothesis is that lineage commitment to Treg cells occurs in SP thymocytes (Fontenot et al. 2005). However, recent studies, also in mice, have suggested that the Treg lineage commitment takes place already in the thymic cortex or during positive selection (Liston et al. 2008, Cabarrocas et al. 2006).

The first selection step after the TCR rearrangements is the positive selection, confirming recognition and restriction to self-MHC molecules. CD5 expression, correlating with the strength of TCR-signaling (Azzam et al. 1998) was scant on the FOXP3⁺ DN cells. Furthermore, the expression of CD69, a marker associated with TCR-mediated signaling and positive selection (Hare et al. 1999), was low on
FOXP3⁺ DN cells as expected if under one third of those cells expressed TCR. A fraction of the developmentally more mature DP thymocytes examined, contained FOXP3 at levels comparable to CD4⁺CD25⁺ thymocytes, and also expressed CD25. The association of CD25 expression with FOXP3 was pronounced in the DP and CD4⁺ SP thymocytes. CD25 was expressed by the majority, but not all FOXP3⁺ cells. There was also a clear subset of CD25⁻ FOXP3⁺ thymocytes. Of the FOXP3⁺ DN cells less than one-fourth expressed CD25. Thus, the CD25 expression in thymocytes seems to be associated with signal transduction through TCR. In fact, a two-step model of Treg development in mice also suggests that CD25 up-regulation is induced after the recognition of self–antigens (Lio and Hsieh 2008).

In contrast to the FOXP3⁺ DN cells, FOXP3⁺ DP cells were CD69⁺ and TCR<sup>high</sup> and most likely already positively selected. Consistent with our results it has been reported that in humans the CD25<sup>hi</sup> DP thymocytes detected were CD69⁺ suggesting that the commitment to the regulatory lineage occurs during positive selection (Darrasse-Jeze et al. 2005).

Nevertheless, our results showed that the CD25⁺ DP thymocytes were still immature. They expressed CD5 more than the other DP cells, but less than mature SP cells. Secondly the DP FOXP3⁺ thymocytes still expressed high levels of RAG-2, indicating immaturity and potential to improve their TCR (Spits 2002)

To define the developmental relationship between the CD25⁺ DP and CD4⁺CD25⁺ SP thymocytes, a comparison of the TCRβ repertoires of these populations was made. Regardless of the phenotypic similarities of FOXP3⁺ DP and mature FOXP3⁺CD4⁺CD25⁺ thymocytes, there was a clear disparity of repertoires between these two populations. There are several possibilities to explain these repertoire differences. If FOXP3⁺ DP and SP cells represent a continuous developmental lineage, possibly not all of the FOXP3⁺ DP cells mature into SP cells. Some of the FOXP3⁺ DP cells may still be deleted during selection. Up-regulation of FOXP3 in itself does not appear to provide protection from deletion (Hsieh et al. 2006), although there are also conflicting results. Additionally, part of the DP cells may still undergo rearrangements of the TCRα chains, thus altering the repertoire. FOXP3⁺ DP cells indeed still express RAG-2, enabling modifications of α chains. On the other hand, some thymocytes might up-regulate FOXP3 only after entering the SP stage, in which case these TCRs are missing from the FOXP3⁺ DP repertoire. Perhaps there is also down-regulation of FOXP3 expression during the development in the absence of some critical signals demanded for maintenance of permanent FOXP3 expression. This could explain some of the inconsistencies we see in the TCR repertoires. In addition, some of the FOXP3⁺ DP cells may differentiate into CD8⁺ FOXP3⁺ SP cells (Cosmi et al. 2003), accounting for part of the differences in the repertoires.

Our results demonstrate that FOXP3 is expressed in all developmental stages of T cell development. Consequently these results are suggestive of a linear developmental pathway for Treg cells. Thus one can propose that the development of Treg cells and other T cells is presumably broadly similar, DN cells evolving into DP cells and maturing through selection steps to CD4⁺ and CD8⁺ SP cells. There was an increasing gradient of CD5 intensity, demonstrating that the developing FOXP3⁺ cells get increasing amounts of TCR-mediated signals as they mature.

However, there was no correlation in numbers between FOXP3⁺ DN, DP and SP cells. Up-regulation of FOXP3 is probably not the final definitive point in the Treg cell lineage commitment. Thus not all thymocytes that up-regulate FOXP3 mature into Treg cells and proceed to the periphery. Since the FOXP3⁺ DP cells still show
signs of immaturity one can suggest that the final commitment occurs probably not until during the SP stage and the negative selection. In addition, to the linear developmental pathway, up-regulation of FOXP3 might occur at every stage along the developmental pathway, since the FOXP3+ cells are represented in all developmental stages.

2.2 CD8+ T cells expressing FOXP3
Our data, as well as other recent publications have shown that there is clearly FOXP3 expression in the CD8+ SP thymocyte population (Cosmi et al. 2003, Fontenot et al. 2005). These cells also have intermediate expression of CD25 (Cosmi et al. 2003, Cupedo et al. 2005). However, in contrast to earlier reports our results showed that the FOXP3 expression level in CD8+ SP thymocytes was significantly lower than either in the CD4+ SP or DP thymocytes.

In a recent report it was shown that the amount of FOXP3+CD8+ cells in peripheral blood is negligible, however a population of FOXP3+CD8+ cells in human tonsils was discovered (Siegmund et al. 2009). These cells had the Treg phenotype with the exception that they were predominantly CD25+ or CD25low. FOXP3 expression in CD8+ cells can be induced and maintained also in the periphery. These peripherally induced CD8+ FOXP3+ cells are also capable of suppressing CD4+ cells. 

A recent study showed that in vitro induced CD8+CD25+ cells expressed approximately one-ninth of the FOXP3 level of CD4+CD25+ cells from the same culture (Bisikiriska et al. 2005). The significance of FOXP3 expression in CD8+ cells is unclear and the meaning of the lower FOXP3 expression level is another peculiarity.

As the high quantity of FOXP3 is so clearly considered a prerequisite for true Treg cells, it must be contemplated whether the FOXP3 expression in CD8+ cells is an indication of a regulatory population or rather just an activation-induced phenomenon. Distinct from CD8+ thymocytes, the FOXP3 expression seems to remain continuously high in DN, DP and CD4SP cells. Perhaps the low FOXP3 expression seen in the CD8+ thymocytes is a transient phenomenon similar to the activation-induced up-regulation of FOXP3 in the periphery. Alternatively, the signals associated with MHC class I restriction and selection to the CD8+ lineage may be different, leading to a lower expression of FOXP3. On the other hand, our results demonstrated that FOXP3 is up-regulated already before the CD4/CD8 lineage decision. Thus it may be that the CD8 lineage decision affects FOXP3 transcription, decreasing or halting it.

2.3 Significance of TCR in Treg cell development
In the light of our results, TCR is not a prerequisite for FOXP3 expression. However, it is important to note that our results do not refute a pivotal role of TCR in human Treg cell development. Our results show that the FOXP3+ DP and SP cells have already up-regulated CD5, CD25 and CD69 expression. This indicates that these cells have already received TCR-mediated signals. Although TCR is not necessarily important in the up-regulation of FOXP3, its affinity and specificity have inevitably great importance in the thymic selections and it defines the specificity and function of a Treg cell.
2.4 Negative selection of Treg cells

Treg cells are shown in many instances as self-specific (Apostolou et al. 2002, Hsieh et al. 2004, Romagnoli et al. 2002, Hsieh et al. 2006, Jordan et al. 2001, Cozzo et al. 2005). If they, indeed, are self-specific how do they pass through the negative selection? In several studies it has been shown that the TCR signaling of Treg cells is defective. Cells are not unable to signal, but have altered their signaling properties, probably due to FOXP3 expression (Campbell and Ziegler 2007). Thus, if FOXP3 is up-regulated before the selection steps it could have a role in adjusting the strength of TCR-derived signaling during selection and help to avoid deletion. It is noteworthy to mention that although FOXP3 may render these cells more “stable”, it, without a doubt, does not prevent the possibility to negatively select these cells.

Another possibility to evade negative selection is suggested by the data of cells expressing two TCRs. The significance of dual-specific cells, if any, has been obscure, yet several reports have suggested an autoreactive role for dual-specific cells (Elliott and Altmann 1995, Sarukhan et al. 1998, Zal et al. 1996). When dual-specific T cells were first discovered, it was indeed thought that although bearing probably an autoreactive TCR, these cells could evade negative selection and be positively selected through the other receptor. The intracellular competition of two α chains for pairing with a single β chain decreases the surface expression of both TCRs, thus allowing the cell to down-modulate the autoreactive TCR and escape deletion (Zal et al. 1996).

2.5 Treg cells express two different TCRs

Our results clearly demonstrate that the number of dual TCR specific cells is greatly enriched among human Treg cells. As our data is based on the surface expression of two TCRs, both α chains are verifiably paired with a β chain. Also, the TCR complexes of dual-specific Tregs were effectively internalized upon stimulation referring that both receptors are able to signal competently. We can not show that both receptors participate in the selection or the responses in the periphery, but in the light of our results we may assume that both TCRs of dual specific Tregs are functional. However, due to the availability of only a few Vα specific mAbs, the high frequency of dual-specific cells we suggest is based on estimates and the actual prevalence may differ, nonetheless the relative difference between Treg cells and other T cells should not be affected.

Our data show that cells with two TCRs are significantly more common among CD25+ thymocytes already in the thymus and a high frequency of dual-specific Treg cells is seen also in the periphery. This means that the commitment of the dual TCR specific Treg cells occurs in the thymus and dual-specificity possibly has a role in directing these cells to the regulatory lineage, probably during the intrathymic selection. Of course some dual-specific cells may also up-regulate FOXP3 in the periphery after activation similarly to the conventional T cells with single TCR specificity and thus complement in part the dual-specific Treg compartment.

Some studies in mice have also reported a role for dual-specificity in the development of Treg cells. Conclusions have been made based on studies with transgenic mouse strains bearing an autoreactive TCR. It has been noted that Treg cell development is induced more efficiently in mouse models, where endogenous TCR rearrangements are possible. The presence of a secondary TCR indeed seems to provide an opportunity for the transgenic TCR to escape deletion and direct the
developing thymocyte into the Treg cell lineage (Van de Keere and Tonegawa 1998, Itoh et al. 1999, Kawahata et al. 2002, Hori et al. 2002). Tregs predominantly express endogenous TCR α chains with a transgenic β chain, as endogenous α chains possibly contribute to the positive selection of thymocytes to Tregs. RAG-deficiency blocks gene arrangements in the endogenous TCR α locus and in general abrogates the development of Tregs in TCR transgenic mice (Itoh et al. 1999). This was true even when the transgenic TCR was derived from a Treg cell (DiPaolo and Shevach 2009). On the other hand, analyses of mice unable to express two different Vα chains have shown that Treg cells can develop and function normally even when dual-specificity is prevented (Suto et al. 2002). Thus in mice, Treg development may have different requirements and the expression of two TCRs is not an absolute prerequisite for Treg cell development.

In conclusion, one explanation to the essential problem that how Treg precursors expressing autoreactive TCRs are not deleted, while other thymocytes with such TCRs are directly negatively selected, is offered by the existence of two TCR specificities. Our results imply that the presence of a second α chain could give dual-specific thymocytes, not only a chance to be positively selected, but an advantage to diverge to the Treg cell lineage instead of negative selection.

2.6 Consequences of dual-specificity on Treg cells

The possibility of two different functional Vα chains in Treg cells diversifies the human repertoire in all likelihood. The repertoires of both the selected and the secondary receptors are likely versatile on the dual specific cells (Kasow et al. 2004). This is supported by our results since the isolated CD25+ Vα2 or Vα12 cells had polyclonal α and β chain repertoires. It has been demonstrated that the dual receptor T cells can extend the repertoire for foreign antigens in non-Treg cells. In a recent study the transgenic TCR could not be selected in the thymus, so only the co-expression of a second TCR led to efficient intrathymic selection. These dual TCR cells were present at low frequencies in the naïve repertoire. However, when the non-selected TCR responded to foreign antigens presented by the self-MHC, these cells dominated the antigenic response through clonal expansion (He et al. 2002). Thus dual TCR cells can widen the functional repertoire through the second receptor.

Treg cell development has been reported to be dependent on the interaction with self-antigens in the thymus. However, other results have clearly shown that the Treg cells can respond to foreign antigens in the periphery. Several reports have shown Treg cells to have a role in infectious disease pathogenesis, for example in parasite or viral infections (Bluestone and Abbas 2003, Belkaid et al. 2006). Thus it seems plausible that natural Tregs have TCR specificities both for self and foreign. Our data offer a logical explanation for the discrepancy of Treg TCR specificities, where Treg cells can be selected through a self-specific TCR and the cells still recognize exogenous antigens through the other receptor. The repertoires of the dual-specific cells may consequently include specificities to both self and foreign. Although at least part of the Treg cells may have been selected through the autoreactive TCR, in the periphery, activation of the dual cells may occur through either of the receptors and the effect depends on the antigen encountered. Activation through the self-specific receptor might prevent autoimmunity or promote tolerance, while recognition of a foreign antigen would elicit control of pathological immune response.
Figure 10. Selection of dual-specific T cells

T cells expressing a TCR with high affinity to self antigens are deleted during the T cell development. Transgenic mouse models have shown that the expression of a second α chain can cause intracellular competition between the two α chains for pairing with the β chain. In addition, the two TCRs compete for the same intracellular signaling molecules. This may lead to down-regulation of an autoreactive TCR. Negative selection is avoided and the cell is positively selected through the other TCR to the Treg cell lineage. In the mature T cell the expression of the high affinity receptor is probably low but still sufficient to activate the cell. Alternatively the effect of the two TCRs could be synergistic. As a single TCR neither of the TCRs would be positively selected, but when signals are transmitted through both TCRs they promote selection. The fact that Treg cell population is enriched in cells expressing two TCRs suggests that dual specificity has an active role in directing cells to the Treg cell lineage.

2.7 AIRE and Treg cells

At least some of the self-antigens, important to thymic selection, are controlled by AIRE. However analysis of Treg cells in Aire−/− mice has shown them to develop and function normally (Liston et al. 2003, Kuroda et al. 2005, Anderson et al. 2005). It is possible that autoreactive T cells are held in check by the properly functioning Tregs in Aire−/− mice, thus keeping the clinical picture of these Aire knock-out mice mild.

Our data show that in APECED patients the Treg cell population is impaired, thus in humans AIRE may contribute to the development of Treg cells. The function of Treg cells in APECED patients was defective and they had an impaired capacity to suppress polyclonal responses. In addition, the level of FOXP3 expression, both mRNA and protein, was significantly lower in APECED patients. These data are in good agreement with the results suggesting that the regulatory capabilities correlate
directly with the level of FOXP3 expression (Allan et al. 2007, Wang et al. 2007).

The primary cell type in the thymus expressing AIRE, and consequently PTAs, are mTECs (Björses et al. 1999, Heino et al. 1999). The variety of peripheral antigens governed by AIRE is probably also the source of antigens that are involved in the selection of Treg cells. A recent report in mice demonstrated that Aire⁺ mTECs can directly generate antigen-specific Treg cells (Aschenbrenner et al. 2007). Our data showed that AIRE⁺ mTECs are enriched around Hassall’s corpuscles in the thymic medulla. Hassall’s corpuscles secrete TSLP inducing maturation of DCs, which in turn support the Treg cell development in humans (Watanabe et al. 2005). It is possible that these medullary DCs collect mTEC-derived antigens from apoptotic AIRE⁺ cells surrounding them and thereafter induces differentiation of regulatory T cells specific to these AIRE-controlled PTAs.

Interestingly, in mice, Hassall’s corpuscles are poorly developed (Farr et al. 2002). Additionally it was recently reported that in Aire-deficient mice, there is nearly an absence of Hassall’s corpuscle-like structures in the medulla (Yano et al. 2008). Nonetheless, Treg cells in Aire⁻ mice function normally. Thus it seems that there may be some mechanistic differences in the Treg cell development between mice and men. This may explain why mouse Treg cells are normal even in the absence of Aire.

Nevertheless, the mechanism by which AIRE-deficiency leads to a defect in Tregs is unclear. One possibility is that due to the deficiency of functional AIRE, the loss of selecting antigens impairs the Treg cell development. Our results show differences in the Treg TCR repertoire between APECED patients and controls. TCR repertoires in patients were less skewed and resembled an average naïve repertoire more than that of the controls. This may result from defective selection of the Treg TCR repertoire. However, antigen-specific failure would be expected to appear as narrow holes in an otherwise normal repertoire and this evident difference in the clonal distribution evokes an impression of a broader perturbation. The naïve-like TCR repertoire in APECED patients might also be due to defective Treg cell activation, reflecting diminished clonal expansion. The defective suppression by Tregs of APECED patients also infers that the perturbation is systemic and not restricted to AIRE-specific antigens. Indeed, compatible with our results, it has been reported that the antigen presentation might be disturbed more generally in AIRE-deficiency, not just concerning antigens under control of the AIRE (Anderson et al. 2005, Johnnidis et al. 2005).

In a recent report it was demonstrated that there is AIRE-regulated antigen presentation also in the periphery, leading to the deletion of autoreactive T cells (Gardner et al. 2008). If such peripheral expression of AIRE exists also in humans, it is missing from APECED patients. Thymic development of Treg cells seems to be impaired in APECED patients. However, it is not known whether there are disturbances in the peripheral induction of Treg cells.

In conclusion, our results suggest that a Treg cell defect is involved in the pathogenesis of APECED. In mice, Aire-deficiency does not lead to a defective function of Treg cells and Aire knock-out mice remain remarkably healthy. On the contrary in humans the AIRE-mutation causes a malfunction of the Treg cells and APECED patients suffer from various disease manifestations.
Our results show that FOXP3 is expressed already at the DN stage of the thymic T cell development and we suggest that Treg cells follow a linear developmental pathway, broadly similar to that of conventional T cells. Thus we conclude that the differentiation of Treg cells begins already at the DN stage and the cells mature through the DP stage to become CD4+ FOXP3+ Treg cells. Treg cells and conventional T cells develop from common precursor cells and the original up-regulation of FOXP3 expression may be stochastic or induced by unknown signals in the thymic microenvironment. The immature FOXP3+ Treg precursors go through thymic selections and the final lineage commitment occurs during the selections. Moreover, conventional T cells may probably convert to the Treg lineage during their development.

A subset of CD8+FOXP3+ thymocytes also exists. Whether they diverge from FOXP3+ DP thymocytes or represent an independent subset which up-regulates FOXP3 upon additional signals is not known.

AIRE contributes to the negative selection of conventional T cells. The clear defect in Treg cells of the APECED patients suggests that AIRE may also influence the development and/or selection of Treg cells in humans.
CONCLUDING REMARKS

The results of this thesis shed light on the development of Treg cells in humans and at the same time demonstrate differences between mice and men.

My data support the view that the commitment to the Treg cell lineage in the thymus is an early event. My results demonstrate that there clearly is a population of DN thymocytes already expressing FOXP3 in the absence of TCR. This model of early commitment also alludes that up-regulation of FOXP3 is not dependent on TCR-mediated signals. In addition to FOXP3, these cells already share features of Treg cells with their more mature thymic CD4⁺CD25⁺ counterparts, which are considered functional Treg cells. A similar FOXP3⁺ population is also seen in the CD25⁺ DP thymocyte subset. These cells have already rearranged their TCR, however they still show signs of immaturity. Hence it may be concluded that the differentiation to Treg cells likely begins already during the DN stage, with the precursors evolving through the DP stage to become mature CD4⁺ Treg cells. Yet the final decision to mature to Treg cells depends on the TCR and occurs during selection.

There is also clear enrichment of dual-specific cells among Treg cells in humans. My data demonstrate that the presence of a second TCR influences the fate of the dual-specific cell in a non-transgenic setting. That is, I suggest that dual-specificity does play an active role in directing cells to the regulatory lineage. Treg cell specificities are unknown, yet preference for self has been suggested. The dual-specificity of Treg cells offers an explanation firstly to the question of how cells bearing self-specific TCR can avoid negative selection and secondly, to the disagreement concerning the TCR repertoire of these cells. In addition, this is the first demonstration of dual-specificity having an influence on the normal immune system.

AIRE plays an essential role in the selection of T cells, as it contributes to the deletion of autoreactive T cells. My results of impaired function of Treg cells in APECED patients suggest that the absence of AIRE seems to disturb the development of Treg cells on some level as well. Both AIRE and Treg cells are central in the maintenance of immunological tolerance. Therefore, it seems natural that AIRE may influence the development of natural Treg cells in the thymus. The reduced function of Treg cells in APECED patients probably reflects the decreased expression of FOXP3. In addition the more naïve-like repertoire of Treg cells suggests altered selection and generation of Treg cells in the thymus and possibly also in the periphery. Contrary to humans, there is no recognized defect of Treg cells in Aire-deficient mice and respectively the autoimmune manifestations of these mice are mild. Thus the comparison of mice and humans shows the significance of Treg cells in tolerance.

Because of their therapeutic promise, Treg cells are currently the focus of intense investigation. However, before these hopes can be realized, Treg cell biology must be understood in sufficient detail. The results described in my thesis elucidate the developmental pathway of human Treg cells and their importance in autoimmune diseases. The results also provide the basis for an in-depth analysis of the early stages of Treg cell development and the significance of TCRs in lineage commitment. Detailed characterization of Treg cell defect in APECED patients will also clarify the role of AIRE gene in human Treg cell development.
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