T cell development in the human thymus

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ACADEMIC DISSERTATION

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To Saimi and Toivo
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ABSTRACT

The immune system should react effectively towards harmful pathogens but still tolerate own tissues. The tolerance comprises central and peripheral tolerance. Negative selection, where the cells that react harmfully towards own tissues are eliminated, is part of central tolerance. One part of peripheral tolerance is the population regulating the cells that have escaped the negative selection. Errors in tolerance cause autoimmune diseases, an example of which is APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy.

The basis of this tolerance is the development of T lymphocytes in the thymus where a safe and effective mature T cell population from the T cell precursors coming from the bone marrow is shaped. During this program most of the developing thymocytes die and only about 3% survive and form the final T cell population. To survive, thymocytes have to recognize peptides presented in the major histocompatibility complex with proper affinity with their T cell receptor, recombined from α and β chains. T cell receptors have a huge variation in sequence in their complementarity determining region 3 (CDR3), due to somatic recombination and additional editing.

In this thesis I have studied this development of T cells in the human thymus, and then more specifically the regulatory population of CD4+ T cells, which express forkhead box 3 transcription factor, FOXP3. First, I sorted cells at different stages of development and analyzed their T cell receptor repertoire and the effect of thymic positive and negative selection on that. Very few shared sequences were found between different populations, showing the huge diversity of the T cell repertoire. Additionally, the physico-chemical features of the repertoire were examined. The basic T cell receptor structure remained the same from already the most immature population before the selections. A reduction in the mean CDR3 length was seen between the CD4+CD8+ double-positive and CD4+ populations, probably reflecting the effect of selections.

As we could see a reduction in the length of CDR3, we used this as an indicator of thymic selections, to test these in APECED patients. We compared the CDR3 length of their peripheral CD4+ T cell populations to healthy controls, also sorting the recent thymic emigrants. Differences in CDR3 lengths between the patients and healthy controls were not found.

T cell receptor and cytokines are essential in the development of regulatory T cells. Most studies are done in mice and the precise steps of development are poorly known in human. Double-positive (DP) CD4+CD8+ thymocytes are thought to be the precursors of CD4 FOXP3+ regulatory T cells. We cultured thymocytes and could see that the DP cells were more prone to apoptosis, showing their precursor cell characteristics.

Furthermore, we studied the effect of interleukin 7 (IL-7) on developing thymocytes. Mature regulatory T cells express the IL-7 receptor α chain CD127
at low levels and have been thought to be resistant to IL-7 effects at the double positive stage. However, we could see that stimulation with IL-7 in the cell culture increased the percentage of FOXP3+ CD4+CD8+ double positive and CD4 single-positive thymocytes. The mechanism behind this seemed to be an inhibition of apoptosis and possibly also increased FOXP3 expression.

Our results increase the knowledge on T cell development in human but many details are yet to be revealed.


Käytimme tätä CDR3-pituuden lyhenemistä selektioiden tutkimiseen APECED-potilaisissa. Vertasimme potilassolujen CDR3-pituuksia perifeerisissä populaatioissa terveiden verrokeiden soluihin ja eristimme myös vasta kateenkorvasta lähteneet solut. T-solujen CDR3-pituuksissa potilaiden ja verrokkien välillä ei ollut eroja.

T-solureseptori ja sytkiinit ovat tärkeitä regulatoristen T-solujen kehityksessä. Maalimalla suurin osa tutkimuksista on tehty hiirimallissa, ihmiseläiset T-solujen kehitysaskeleet tunnetaan huonommin. CD4⁺CD8⁺ kaksoispositiivisten solujen ajatellaan olevan CD4⁺ FOXP3⁺ säätelijä-T-solujen
esiasteita. Viljelimme tymosyyttejä soluviljelmässä ja havaintosimme, että solujen kasvatuksen aikana suurempi osa CD4⁺CD8⁺ soluista meni apoptoosiin, viitaten niiden olevan esiasteita kypsemmälle solupopulaatioille.

Tutkimme myös interleukiini 7:n (IL-7) vaikutusta kehittyviin tymosyytteihin. Kypsät säätelijä-T-solut ilmentävät vain vähän interleukiini-7:n α-ketjua CD127 ja niiden on ajateltu olevan resistenttejä IL-7:n vaikutuksille CD4⁺CD8⁺ vaiheessa. Solujen stimulointi IL-7:llä kuitenkin lisäsi FOXP3:a ilmentävien solujen osuutta CD4⁺CD8⁺ ja CD4-positiivisissa tymosyyteissä. Tämän ilmiön mekanismeina tulivat esiin apoptoosin esto ja mahdollisesti lisääntynyt FOXP3-expressio.

Tuloksemme tuovat merkittävästi lisää tietoa ihmisten T-solujen kehityksestä, joskin monet T-solujen erilaistumiseen liittyvät tekijät ovat edelleen tuntemattomia.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIRE</td>
<td>autoimmune regulator, <em>gene/protein</em> (human)</td>
</tr>
<tr>
<td>Aire</td>
<td>autoimmune regulator, <em>gene/protein</em> (mouse)</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APECED</td>
<td>autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy</td>
</tr>
<tr>
<td>CIITA</td>
<td>class II transactivator</td>
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<tr>
<td>CCR7</td>
<td>C-C chemokine receptor 7</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity determining region</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMJ</td>
<td>corticomedullary junction</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>double-negative, CD4⁻CD8⁻</td>
</tr>
<tr>
<td>DP</td>
<td>double-positive, CD4⁺CD8⁺</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FOXP3</td>
<td>forkhead box P3, <em>gene/protein</em> (human)</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3, <em>gene/protein</em> (mouse)</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor receptor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IgSF</td>
<td>immunoglobulin superfamily</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IPEX</td>
<td>immune dysregulation, polyendocrinopathy, enteropathy, X-linked</td>
</tr>
<tr>
<td>ISP</td>
<td>immature single positive</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>iTreg</td>
<td>induced regulatory T cell</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus tyrosine kinase</td>
</tr>
<tr>
<td>LAG3</td>
<td>lymphocyte activation gene 3 protein</td>
</tr>
<tr>
<td>LAT</td>
<td>linker of activated T cells</td>
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<tr>
<td>Lck</td>
<td>lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>mDC</td>
<td>medullary dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>the mechanistic target of rapamycin</td>
</tr>
<tr>
<td>N-nucleotide</td>
<td>nontemplated nucleotide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NK-T</td>
<td>natural killer T cell</td>
</tr>
<tr>
<td>Nrp</td>
<td>neuropilin</td>
</tr>
<tr>
<td>nTreg</td>
<td>natural regulatory T cell</td>
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<tr>
<td>P-nucleotide</td>
<td>palindromic nucleotide</td>
</tr>
<tr>
<td>pDC</td>
<td>peripheral dendritic cell</td>
</tr>
<tr>
<td>PDK-1</td>
<td>phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death 1 receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol trisphosphate</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>phospholipase C-γ</td>
</tr>
<tr>
<td>pMHC</td>
<td>complex of peptide and MHC</td>
</tr>
<tr>
<td>RORγ</td>
<td>RAR-related orphan receptor gamma</td>
</tr>
<tr>
<td>RTE</td>
<td>recent thymic emigrant</td>
</tr>
<tr>
<td>S1P1</td>
<td>sphingosine 1-phosphate receptor 1</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>STAT</td>
<td>signal-transducing activator of transcription</td>
</tr>
<tr>
<td>Tconv</td>
<td>conventional T cell i.e. other than regulatory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β, in the immune system mainly isoform TGF-β1</td>
</tr>
<tr>
<td>TfH</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNCs</td>
<td>thymic nurse cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>tissue-specific antigen</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-cell specific demethylated region</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TSP</td>
<td>thymus seeding progenitor</td>
</tr>
<tr>
<td>Tssp</td>
<td>thymus-specific serine protease</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>V(D)J</td>
<td>variable-diversity-joining</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>zeta-chain-associated protein kinase 70</td>
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This thesis is based on the following original articles, which are referred to in the text by Roman numerals.


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INTRODUCTION

The thymic development of T cells creates a diverse repertoire of cells capable of defending against many pathogens. However, cells with too high autoreactive potential must be deleted. The cells with proper-affinity receptors develop and comprise the eventual T cell repertoire.

Besides infections the immune system is involved in many diseases, from rare monogenic autoimmune diseases to very common conditions such as diabetes and vascular diseases. Innate immunity acts on pathogens on first line of defense, and helps to start the adaptive immune reactions, that are more specific.

T cells are one of the main players of adaptive immunity in both defense against different pathogens and tolerance towards host’s own tissues. The ability to fulfill these duties comes from their development and education in the thymus. They must differentiate own tissues from pathogens, and this is accomplished by central tolerance: removal of cells with harmful effects towards own cellular structures in negative selection. The thymic development also creates regulatory cells that guard harmful T cell actions in the periphery, and this is one important part of peripheral tolerance.

The existence of a regulatory population was controversial for a long time. There were difficulties in defining the regulatory cells, but since Sakaguchi et al. in 1995 found CD25 as one of the markers, they have been accepted in general. The Foxp3 gene was first found in scurfy mice and then in IPEX patients (Brunkow et al., 2001, Chatila et al., 2000). FOXP3 as the key modulator of the regulatory T cell fate and function was later identified (Hori et al., 2003, Schubert et al., 2001). The knowledge of their functions and development has since increased, but how they are selected in the thymus from other T cells is presently not understood in detail.

As the development in the thymus is important for T cells and the immune system, my thesis concentrates on it. My main research goals have been analyzing the development of T cells and particularly the regulatory T cells and how their developmental path differs from that of other T cells in human. As thymus samples are hard to obtain, especially in humans, there are still many open questions. Thus, most of the data has been obtained in the mouse model. As an example of failure of immune tolerance I have studied the autoimmune disease APECED, mostly the thymic part of its pathogenesis.
REVIEW OF THE LITERATURE

1 T LYMPHOCYTES AND THEIR FUNCTIONS

The adaptive immunity comprises two main cell populations, T (thymus-derived) lymphocytes and B (bone marrow-derived) lymphocytes, of which B lymphocytes secrete humoral antibodies and T cells act through cell-to-cell contacts. T cell functions depend on the T cell receptor (TCR), a heterodimer surface protein that recognizes antigens presented in the major histocompatibility complex (MHC) by the antigen presenting cell (APC). The two chains of the TCR heterodimer are in 95% of T cells α and β, and in the remaining 5% γ and δ. This thesis concentrates on the development of αβ T cells, and from this on T cells indicates to αβ T cells.

T lymphocytes can be divided into two populations according to the expression of co-receptor of TCR, cluster of differentiation (CD) 4 or 8. CD4+ T cells recognize antigens presented in the MHCII molecule on APCs and CD8+ cells in the MHC molecule existing on almost all nucleated cells. These features delineate their somewhat different tasks in the periphery. T cell development in the thymus designates the lineage commitment to either CD4 or CD8 cells, but upon activation in the periphery their functions are further adjusted.

1.1 T cell activation

Before any function, T cells must be activated. This is mediated through an immunological synapse that forms as a result of the close interaction of a T cell with an APC and describes the site where the TCR is triggered by its antigen, the peptide–MHC complex in the APC membrane. In addition to the initial event in activation, the antigen recognition by the TCR, naïve T cells require co-stimulatory signals to differentiate into effector cells. These signals can also be inhibitory, in that case they lead to anergy or cell death. The activation process is carefully regulated, the circumstances where it can happen are restricted to hamper harmful reactions. Activation takes place in the lymph nodes or other organized lymphoid tissues, it is regulated by when and how much peptide is presented, and upregulation of co-stimulatory molecules by inflammation on APCs is needed to introduce the second signal.

Naïve T cells need three kinds of signals for activation and differentiation. Dendritic cells (DC) provide directly first two and the third is mediated by cytokines, also mainly secreted by DCs. The first signal is the TCR recognition of antigen displayed in the MHC-molecule of the APC with proper affinity. The co-receptor CD4 or CD8 also binds MHC and stabilizes the receptor complex for sufficient signaling to take place. The first signal without further
activation results in anergy. The second signal mainly comes from the co-stimulatory CD28 molecule on the T cell binding the B7 molecule (CD80 or CD86) on the activated professional APC. The different possible co-stimulatory signals or alternatively co-inhibitory signals are described in chapter 1.1.2. The third signal comes from cytokines secreted by APCs. The pathogen influences the spectrum of cytokines secreted, and thus determines what kind of effector functions T cells acquire. Activated T cells also start to secrete cytokines, to reinforce the immune response and their own differentiation. When activated, T cells proliferate and differentiate into effector cells and do not require co-stimulation to act (Das et al., 2001, Smith-Garvin et al., 2009).

1.1.1 TCR signaling pathway

The recognition of antigen by the TCR complex in proper conditions leads to T cell signaling. The precise mechanism of this first triggering event is unclear; no final consensus of how the receptor binding is translated into an activating signal has yet been reached. One suggested model is that the binding of pMHC (complex of peptide and MHC) to the TCR results in transduction of mechanical forces and TCR quaternary change to initiate TCR signalling (Li et al., 2010). Some studies also report aggregation of several TCRs, suggesting another model of activation (Smith-Garvin et al., 2009). The co-receptor CD4 or CD8 is in any case needed to mediate intracellular signaling events. The signaling pathway is described in Figure 1, in which only CD4 is shown for simplification. The final event in this cascade is the activation of transcription factors resulting in cell proliferation and differentiation.
Figure 1. **TCR signaling pathway.** Binding of pMHC (MHC not shown) to TCR and CD4 brings the CD4-associated Lck (lymphocyte-specific protein tyrosine kinase) together with the cytoplasmic regions of the CD3 chains called ITAMs (immunoreceptor tyrosine-based activation motifs). Lck phosphorylates tyrosine residues in the CD3 chains, allowing tyrosine kinase ZAP-70 (zeta-chain-associated protein kinase 70) to bind them. Lck then activates ZAP-70, which in turn phosphorylates scaffold protein LAT (linker of activated T cells). LAT binds the key signaling protein phospholipase C-γ (PLC-γ) to the cell membrane. The co-stimulation through CD28 induces its tyrosine phosphorylation, which activates PI phosphoinositol 3-kinase (PI3K) that produces phosphatidylinositol trisphosphate PIP₃. Phosphoinositide-dependent protein kinase 1 (PDK-1) then phosphorylates Akt that enhances cell survival and upregulates cell metabolism. Activated PLC-γ cleaves PIP₂ to diacylglycerol DAG and inositol triphosphate IP₃. This initiates three signaling pathways resulting in the activation of
transcription factors leading to cell proliferation and differentiation: I) IP₃ increases intracellular Ca²⁺ concentration and activates a phosphatase, calcineurin that activates a transcription factor NFAT (nuclear factor of activated T cells), II) DAG recruits protein kinase C-θ that activates CARMA, which leads to the activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), and III) DAG recruits Ras-Grp, a guanine-nucleotide exchange factor, which activates a small GTPase Ras which, in turn, activates MAP (mitogen-activated protein) kinase cascade, activating Fos, a component of the AP-1 transcription factor. Modified from Hayashi et al. and Murphy (Hayashi et al., 2007, Supporting information, Murphy, 2012)

1.1.2 Co-stimulatory and co-inhibitory signals

The repertoire of co-receptors on T cells is versatile and responses to changes in the tissue environment, their ligands and counter-receptors are found on the cells interacting with T cells. Most co-signaling molecules belong to the immunoglobulin superfamily (IgSF) or tumour necrosis factor superfamily (TNFSF). CD28 family and B7 family members B7-1 (CD80) and B7-2 (CD86) are the most important of the IgSF; they primarily interact with each other (Chen and Flies, 2013). CD28 co-stimulatory signal is required for most of the responses against antigens, without it TCR induces clonal anergy, functional inactivation, or cell death (Linsley and Ledbetter, 1993).

The CD28 homologue CTLA-4 (cytotoxic T-lymphocyte associated protein 4) binds B7 molecules with higher affinity than CD28 molecules and provides inhibitory signals (Linsley et al., 1991). CTLA-4 expression differs from CD28 expression. It is expressed in response to TCR ligation and constitutively on Tregs, whereas CD28 is expressed on resting and activated T cells. CTLA-4 is crucial for Treg function but also for conventional T cells in transmission of inhibitory signals. The majority of CTLA-4 is located in the intracellular compartments including trans-Golgi-network, lysosomes, and endosomes (Rudd et al., 2009). The transmembrane protein TCR-interacting molecule acts as chaperone for the transport of CTLA-4 to the cell surface (Valk et al., 2006). CTLA-4 has both cell-extrinsic and -intrinsic mechanisms for its function. Extrinsic mechanisms are suggested to include secretion of soluble CTLA-4, production of indoleamine 2, 3-dioxygenase (IDO), and involvement of Tregs. Probable cell-intrinsic mechanisms comprise ligand competition and association of aCTLA-4 cytoplasmic tail with signaling components that inhibit TCR signaling. Some data also indicate effects in adhesion, motility and pro-survival pathway, possibly raising TCR signaling threshold by limiting contact between T cells and APCs (Rudd et al., 2009).

The programmed death 1 receptor (PD-1) and its ligands PD-L1 and PD-L2 also mediate negative signals inhibiting T cell responses (Fife and Pauken, 2011). Upon TCR-PD-1 ligation phosphatases SHP-1 and SHP-2 are recruited. They dephosphorylate proximal signaling molecules and downregulate the
activation of the PI3K and Akt pathways (Keir et al., 2008). In addition to the inhibition T cell activation this has been suggested to lead to conversion of conventional T cells into Tregs (Xing and Hogquist, 2012).

Lymphocyte activation gene 3 protein (LAG3, CD223) is a molecule with structural homology to CD4 that functions as a co-inhibitory molecule. LAG3 interacts with MHC class II molecules, which it binds with higher affinity than CD4 (Huard et al., 1995). LAG3 negatively regulates cellular proliferation, activation, and homeostasis of T cells, and has also been reported to play a role in the Treg suppressive function (Sega et al., 2014).

1.1.3 Cytokines

The third signal guiding the T cell differentiation comes from the cytokines. All T cells secrete interleukin-2 (IL-2) upon activation that acts as a proliferative signal. The different effector populations and their signature cytokines are discussed below and are shown in Fig. 2.

1.2 T cell subsets

1.2.1 CD4+ T cells

The CD4+ T cells, also known as helper T cells (Th), activate and control other cells, by cell-to-cell interactions or by secreting cytokines during the immune response. They activate antibody secretion by B cells, enhance the responses of CD8+ T cells, and regulate macrophage function. Because their activation can lead to stimulation of many other cells and thus potentiate the immune response and start autoimmune reactions, the process is carefully regulated. The activation process was described in more detail in chapter 1.1. Additionally, regulatory T cells, a subpopulation of CD4+ T cells, work especially in monitoring reactions of other cells.

APCs in the lymph nodes presenting the cognate antigen activate CD4+ T cells into effectors that have specialized functions. During the priming, the local cytokine milieu, antigen concentration, signal strength, and co-stimulatory signals steer the functional development. After this certain transcription factors and secreted cytokines are typical for each population, the cytokines further induce the chosen differentiation lineage and inhibit others (Zhu et al., 2010). This differentiation is not permanent, as cells can in some circumstances change their differentiation lineage (Zhou et al., 2009). Immune reactions in vivo have components of several types of Th responses and it is the balance between them that designates the outcome. Mosmann and colleagues (Mosmann et al., 1986) first found Th1 and Th2 cells in mice and for a long time they were thought to be the only effector populations, but later studies have found Th17, Tfh (T
follicular helper) and induced Tregs as additional effectors. Recently, also IL-9-producing Th9 cells and IL-22-producing Th22 cells have been suggested as additional helper T cell populations (Raphael et al., 2015).

1.2.1.1 Th1 cells

Th1 cells help in responses towards intracellular pathogens by stimulating infected cells to destroy the invading pathogens. In addition, they have specific functions in autoimmune reactions. Th1 cells activate macrophages by secreting interferon-γ (IFN-γ), they also sensitize macrophages to IFN-γ effects with CD40L-CD40 interaction. Also lymphotoxin-α secreted by Th1 cells can substitute CD40L in the activation. Additionally, Th1 cells kill chronically infected cells, induce T cell proliferation, and recruit new macrophages to the site of infection (Murphy, 2012). Th1 cells are as well producing IL-2 which induces T cell proliferation, and many of them also TNF-α (tumor necrosis factor α) which activates epithelium at the site of infection (Zhu et al., 2010).

IL-12 and IFN-γ are the third signal to induce Th1 differentiation through the JAK (Janus tyrosine kinase)-STAT (signal-transducing activator of transcription) pathway. DCs and macrophages secrete IL-12 that signals trough STAT4 to initiate IFN-γ production (Thierfelder et al., 1996). Additionally, IL-18 increases IFN-γ production synergistically with IL-12 (Nakanishi et al., 2001). Activated NKT (natural killer T) cells may also secrete IFN-γ that promotes expression of IL-12 in activated macrophages and IL-12R in T cells (Murphy et al., 2000, Szabo et al., 1997). Additionally, through STAT1 IFN-γ induces expression of T-bet, a T-box transcription factor, that upregulates IFN-γ and IL-12R expression and thus creates a positive amplification loop (Lighvani et al., 2001, Szabo et al., 2000). IFN-γ also suppresses IL-4 expression which inhibits Th2 differentiation (Elser et al., 2002).

1.2.1.2 Th2 cells

Th2 cells fight infections caused by parasites, particularly helminths, by activating responses mediated by eosinophils and mast cells, and by inducing IgE antibody isotype expression by B cells. As a harmful effect, the Th2 response is also thought to be responsible for allergies.

IL-4 is the crucial cytokine in priming of the Th2 cells. Eosinophils, basophils, and mast cells are suggested to be the initial source of IL-4 in triggering of the Th2 response (Murphy, 2012). Additionally, IL-5 and IL-13 control the Th2 response (Bouchery et al., 2014). Stimulation through IL-4 receptor by IL-4 or IL-13 activates STAT6 that induces expression of transcription factor GATA3, promoting further secretion of IL-4 and IL-5 by Th2 cells (Zhu et al., 2010).
1.2.1.3 Th17 cells

The main function of Th17 cells is to protect the host against extracellular bacteria and fungi through stimulating neutrophils to clear the pathogens. Additionally, they are suggested to act in autoimmune diseases. When IL-6 and transforming growth factor β (TGF-β) are available but IL-4 and IL-12 are absent, T cells differentiate into Th17 cells and start to express the transcription factor RORγt (RAR-related orphan receptor gamma) through activation of STAT3. IL-6 induces IL-21 and IL-23R, which also promote RORγt expression (Zhou et al., 2007). After activation, Th17 cells secrete IL-17A, IL-17F, and IL-6 (Zhu et al., 2010).

1.2.1.4 Tfh cells

Follicular helper T cells are a recently found population. Their main function is to provide help to B cells in antibody production in lymphoid follicles. Tfh cells can be identified mainly by their location and expression of CXCR5 and ICOS, also expression of the transcription factor Bcl-6 is typical. IL-21 and IL-6 are suggested to induce their differentiation, and after priming they secrete IL-21 (Fazilleau et al., 2009).

Figure. 2. T helper cell populations, their signature cytokines and transcription markers. The cytokines, TCR signaling and CD28 co-stimulation upon activation by the APC lead to differentiation of the CD4+ T cell. The cytokines leading to differentiation of each subset are shown above the population as are the main transcription factors needed for their function. Activated T cells secrete cytokines or express surface molecules, which are shown underneath each cell.
1.2.1.5 Regulatory T cells

Regulatory T cells regulate other cells in immune responses. Regulatory T cells comprise two groups, the natural regulatory T cells (nTregs) that develop in the thymus, and the induced regulatory T cells (iTregs) that develop from naive conventional CD4+ T cells in the periphery. The hallmark of both natural and induced Tregs is the expression of transcription factor FOXP3. The main cytokine able to induce FOXP3 expression is TGF-β1 (Marie et al., 2005). Additionally, certain cell populations lack FOXP3 but produce immunosuppressive cytokines, and some cells express FOXP3 without clear regulatory functions. These populations are discussed briefly later, in chapter 1.2.1.5.5.

1.2.1.5.1 Identification of natural Tregs

The natural regulatory T cells were first identified in 1995 in mice when Sakaguchi and colleagues found a population with high expression of the IL-2 receptor α-chain, CD25, and a capability to suppress autoimmune reactions (Sakaguchi et al., 1995). In 2001 several groups characterized these CD4+CD25+ regulatory T cells in human (Baecher-Allan et al., 2001, Jonuleit et al., 2001, Ng et al., 2001). CD25, however, turned out to be an indefinite marker, because also activated T cells express it. Even up to 15% of CD4+ cells in the peripheral blood are CD25+, only 1-2% of these cells with the highest CD25 expression are true Tregs (Baecher-Allan et al., 2001, Sakaguchi et al., 2010). The most definitive marker for true Tregs, Foxp3, was first identified in Scurfy mice and right after that FOXP3 was found in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) patients (Bennett et al., 2001, Brunkow et al., 2001, Chatila et al., 2000, Fontenot et al., 2003, Hori et al., 2003, Roncador et al., 2005).

FOXP3 as an intracellular marker sets limitations for functional studies of regulatory T cells, and several surface markers have been identified, none of which can be used as the only marker. Low expression of CD127, the α-chain of the IL-7 receptor, is characteristic, albeit not specific, for regulatory T cells. Also conventional CD4+ T cells tend to downregulate CD127 expression after activation (Liu et al., 2006, Mazzucchelli and Durum, 2007, Seddiki et al., 2006). Expression of a homing marker CD62L, L-selectin, can be used as an additional marker to differentiate between Treg cells and recently activated conventional CD4+ T cells, they are CD25highCD127lowCD62L+ and CD62Llow, respectively. Surface molecules CD103 and glucocorticoid-induced tumor necrosis factor receptor (GITR) are also used for Treg identification (Sakaguchi et al., 2010).

Another intracellular marker that most Tregs express at high levels is CTLA-4, which is essential for Treg functions. CTLA-4 is also expressed on the
surface of activated conventional T cells, and cannot thus distinguish Tregs from other T cells (Jago et al., 2004).

![Diagram of T cell markers]

**Figure 3. The most often used markers for Treg identification.** FOXP3 and CTLA-4 are intracellular markers, the others are surface markers.

### 1.2.1.5.2. FOXP3

FOXP3, the transcription factor that is required for Treg development and function, belongs to the forkhead/winged-helix family of transcriptional regulators. The human *FOXP3* gene contains 11 exons and maps to Xp11.23 in the genome (Brunkow et al., 2001). Three different FOXP3 isoforms have been found in man, FOXP3 lacking exon 2 (FOXP3ΔEx2), FOXP3 lacking exon 7 (FOXP3ΔEx7), and the full-length FOXP3 (Allan et al., 2005, Smith et al., 2006). The role of alternative isoforms is not clear, neither is it known whether single Tregs express all of these isoforms or does each cell express distinct isoforms (Ramsdell and Ziegler, 2014).

Genetically defective *FOXP3* leads to autoimmune conditions. In human mutations in the *FOXP3* gene cause the IPEX syndrome, in which the most common symptoms diarrhea, insulin-dependent diabetes mellitus, thyroid disorders, and eczema become apparent around the first month after birth (Bennett et al., 2001). In mice, *Foxp3* mutations result in a fatal systemic autoimmunity disease named Scurfy (Brunkow et al., 2001). Forced expression of FOXP3 creates suppressive activity in both Tregs and Tconvs (conventional T cells, i.e. other T cells than Tregs), proving its function in suppression (Aarts-Riemens et al., 2008, Fontenot et al., 2003).

To mediate the suppressive function, FOXP3 acts as a transcriptional activator and repressor on a large set of target genes. Binding regions for Foxp3 have been found for over 700 genes in mice. Several target genes are defining the Treg phenotype such as *Ctla4*, *Il2ra*, *Nrp1*, and *Tnfrsf18* (GITR). The TCR signaling pathway is affected at multiple levels, targets including surface molecules, signaling components, and transcriptional regulators. In addition to functional genes, FOXP3 also regulates expression of non-coding RNA (Marson et al., 2007, Zheng et al., 2007). An effect on cytokine secretion is also seen, as
forced expression of FOXP3 is able to decrease the production of IL-2, IL-4, and IFN-γ (Bettelli et al., 2005). FOXP3 has somewhat different transcriptional programs in the thymus and periphery, in the thymus it is involved in the establishment of a FOXP3-dependent differentiation program, including also nuclear factors that control chromatin remodeling and gene expression (Zheng et al., 2007)

Although many target genes of FOXP3 binding are identified, the exact mechanism of FOXP3 function is not clear as only part of the transcription factor binding is associated with transcriptional regulation and FOXP3 has also indirect targets affecting Treg function that are regulated through other transcription factors. Thus FOXP3 has been connected to the functions of nuclear factor of activated T cells (NFAT) (Zheng et al., 2007). FOXP3 and NFAT share several target genes, for example IL2 is activated by NFAT and repressed by FOXP3 whereas they both upregulate CD25 and CIta4 (Wu et al., 2006). The mechanism behind FOXP3 effect on NFAT-dependent genes is unknown. The suggested models include competing for DNA binding, sequestering NFAT from binding to DNA, and the formation of a co-operative complex between NFAT and FOXP3 (Schubert et al., 2001, Bettelli et al., 2005, Wu et al., 2006). Surprisingly, in a recent mouse study, a deficiency of two out of three NFAT-family members (NFAT1, NFAT2, and NFAT4) did not alter the suppressor activity in vitro or in vivo, suggesting that at least high levels of NFAT activity are not required for the regulatory function after differentiation (Vaeth et al., 2012).

Another transcription factor connected to Foxp3 is NF-κB, with which Foxp3 physically associates and blocks its ability to induce the expression of the NF-κB-dependent gene, A20. In scurfy mice NFAT and NF-κB activation is increased (Bettelli et al., 2005).

1.2.1.5.3 Function of natural Tregs

Natural regulatory T cells execute their regulatory function by migrating to the site of inflammation and suppressing different types of cells including effector T cells, DCs, NKT cells, NK cells, macrophages, and B cells. Before action Tregs must be activated through TCR with their cognate antigen, but after this the suppression is not antigen-specific (Takahashi et al., 1998). However, more recent studies suggest that suppression may be more effective when the regulatory T cell and the suppressed T cell have the same antigen specificity (Corthay, 2009). Suspected mechanisms of suppression include both contact-dependent and cytokine-mediated means, so far, no universal mechanism has been found. Some controversies from studies of mice and humans exist. This can be caused by different mechanisms used depending on the site, features of the target of suppression, and the Treg activation status. Defects in mechanisms connected to TGF-β, CTLA-4, IL-2 or its receptor are known to cause fatal
autoimmune or inflammatory disease at least in mice (Sakaguchi et al., 2009, Schmidt et al., 2012).

CTLA-4 has several, mostly contact-dependent, mechanisms for suppression, however, recent studies have also revealed secretion of a soluble CTLA-4 isoform (Ward et al., 2013). In addition to Tregs it is also expressed on activated conventional T cells, in which it (i) competes with CD28 for binding to the costimulatory B7 molecule (i.e. CD80 or CD86), (ii) transduces negative signals resulting cell cycle arrest and inhibition of IL-2 secretion, and (iii) limits cell-to-cell contact of T cells and APCs (Schmidt et al., 2012). In Tregs CTLA-4 downregulates B7 expression by DCs, thus inhibiting the APC co-stimulatory function and activation of effector T cells (Oderup et al., 2006). Qureshi and colleagues found that CTLA-4 is able to capture its ligands from other cells by trans-endocytosis, providing one possible mechanism for B7 down regulation (Qureshi et al., 2011). Another CTLA-4-dependent mechanism is stimulating DCs to express IDO that catabolizes conversion of the amino acid tryptophan to kynurenine which is toxic to T cells adjacent to DCs (Sakaguchi et al., 2009, Van de Walle et al., 2011). Additionally, IDO can lead to the generation of induced Tregs (Curti et al., 2007).

Activated Tregs can kill B cells, preferentially those which present antigens, by releasing granzymes, leading to cell lysis through a perforin-dependent manner (Zhao et al., 2006). The same mechanism can be used for eliminating CD4+ and CD8+ effector T cells, monocytes, and DCs (Grossman et al., 2004). LAG3, a CD4-related activation-induced surface molecule expressed on Tregs, can induce inhibitory signals through MHC class II molecules that suppress DC function (Sakaguchi et al., 2010).

Tregs secrete immunosuppressive cytokines and downregulate other cells to secrete cytokines. IL-10 secreted by Tregs suppresses the production of IL-2, TNF-α, and IL-5, and can inhibit the functions of other cells, including DCs and macrophages, and recruit more T cells to the regulatory population (Couper et al., 2008, Roncarolo et al., 2006). Like IL-10, TGF-β plays a crucial role in the induction of Tregs, but its function as a suppressor molecule is controversial (Chen et al., 2003). Galectin-1, which is preferentially expressed on Tregs, can cause cell cycle arrest, apoptosis, and inhibition of secretion of pro-inflammatory cytokines in responder cells (Garin et al., 2007). It is not clear whether galectin-1 is secreted or whether it demands cell-to-cell contacts for its effects (Shevach, 2009). High expression of CD25 by Tregs is thought to absorb IL-2 from the use of other cells (Pandiyan et al., 2007).

A recently found cytokine that may mediate Treg immunosuppressive functions is IL-35 that can inhibit the proliferation of Tconv cells (Chaturvedi et al., 2011). Its role is unclear in man as the first studies reported that contrary to murine Tregs, human Tregs do not express IL-35. It was also suggested that Tregs induce other cells to secrete IL-35 (Bardel et al., 2008, Collison et al.,
A later study found that human Tregs do not express IL-35 constitutively but activation leads to IL-35 expression in Tregs at even higher level than in Tconvs (Chaturvedi et al., 2011). In addition, IL-35 is suggested to induce the iTreg regulatory population that uses IL-35 for suppression but does not require IL-10, TGF-β, or Foxp3 (Collison et al., 2010).

Approximately 50% of human Tregs express ectonucleoside triphosphate diphosphohydrolase-1 (CD39) which hydrolyses extracellular ATP to ADP or AMP which is further degraded to adenosine by 5'-nucleotidase (CD73). Adenosine signals are suggested to inhibit both DCs and activated T cells by increasing the levels of cyclic AMP, and induce anergy and iTreg generation (Shevach, 2009).

**Figure 4. Summary of the main suppression mechanisms of Tregs.** Tregs mediate their suppressive effects mainly by affecting APCs and conventional T cells. High CD25 expression deprives other cells from IL-2. Granzymes and perforin are toxic to adjacent cells. CTLA-4 has several suppression mechanisms including downregulation of CD80 and CD86 expression on DCs, and stimulation of DCs to express IDO that catabolizes conversion of toxic kynurenine. Tregs inhibit TCR signaling of conventional T cells by affecting NFAT, NF-κB, and calcium influx. They generate immunosuppressive adenosine, and secrete immunosuppressive cytokines IL-10, TGF-β, and IL-35. Modified from Schmidt et al. (Schmidt et al., 2012).
1.2.1.5.4 Induced Tregs

In addition to thymus-derived natural regulatory FOXP3+ T cells also naïve T CD4+ CD25− cells can be induced into functional FOXP3-expressing Tregs (iTregs) in the periphery under sub-immunogenic conditions. Transient expression of FOXP3 is also taking place in nonsuppressive conventional CD4+ T cells upon antigen stimulation (Allan et al., 2007, Gavin et al., 2006, Walker et al., 2003). In vitro FOXP3 expression can be induced in CD4+ T cells with antigen stimulation in the presence of TGF-β. These cells are also functionally suppressive but the acquired phenotype is not stable (Chen et al., 2003). The stability of different Treg populations has been disputed recently. They seem to have plasticity like other T cell populations, though the FOXP3+ cells with the epigenomic modifications typical for Tregs are thought to be stable (Sawant and Vignali, 2014).

Like activation of nTregs, induction of iTregs requires TCR signaling and IL-2. In addition, it requires TGF-β for the induction of FOXP3 expression (Fantini et al., 2004), and also other factors, such as retinoic acid and TSLP (thymic stromal lymphopoietin), have been connected to iTreg induction (Sun et al., 2007, Maldonado and von Andrian, 2010). Also the features of the APC affect the induction, as some APCs seem to be more effective in inducing Tregs.

DCs capable of inducing Tregs are called tolerogenic DCs. Several subpopulations of tolerogenic DCs with different surface and intracellular markers can be identified. The highest tolerogenic potential is suggested to be in the lymphoid-tissue resident plasmacytoid DCs and in the CD103+ DCs that express IDO and secrete retinoic acid (Maldonado and von Andrian, 2010). Coombes et al. have shown in mice that CD103+ DCs in the mesenteric lymph nodes induce Foxp3 expression in T cells, and this is mediated by TGF-β and retinoic acid, which they metabolize from vitamin A, as a necessary cofactor (Coombes et al., 2007). Besides DCs also structural cells, such as intestinal epithelial cells, and certain macrophages promote iTreg differentiation by secreting tolerogenic cytokines (Hadis et al., 2011, Maldonado and von Andrian, 2010, Probst et al., 2014). Furthermore, some cells are preferred precursors of Tregs, the conversion of the recent thymic emigrants (RTE) to Tregs is more efficient than that of other T cells (Paiva et al., 2013).

The phenotypes of nTregs and iTregs are very similar with minor differences, see summary in Table 1. Both populations secrete TGF-β and IL-10. iTregs express typical Treg cell markers such as CD25, CTLA-4, GITR and CD103. Compared to iTregs, nTregs have higher expression of PD-1 (pdcdf1), neuropilin 1 (Nrp1), Helios (Ikzf2), and CD73, however, the populations are not easy to distinguish. Helios was suggested to be specific for nTregs, but later studies have shown that it is also upregulated at substantial levels in lymphopenia or by agonist peptides, and additionally upon activation of CD4+ T cells (Akimova et
In humans, a minor population of nTregs has been found that does not express Helios (Himmel et al., 2013). At the moment Nrp1 is suggested to be a marker sufficiently specific for distinguishing between different regulatory populations (Yadav et al., 2012). Demethylation patterns, that are discussed in more detail in chapter 2.6.4, are more heterogeneous in iTregs than in nTregs in which demethylation of CpG islands in Foxp3 conserved non-coding region 2 (Treg-specific demethylation region or TSDR) is an important feature and is thought to reflect stable, constitutive Foxp3 expression (Huehn et al., 2009).

As the main functions of nTregs are in maintaining immune homeostasis and autoimmune reactions, iTregs function more locally, in local immune suppression, oral tolerance, fetal tolerance, and mucosal tolerance. Their suppressive capacity seems to be somewhat lower than that of nTregs. TCR repertoire of iTregs seems to have little, less than 10%, overlap with the nTreg repertoire and the Tconv repertoire, suggesting that iTregs are a possible self-antigen specific population among conventional T cells (Yadav et al., 2013).

Table 1. Treg subsets.

<table>
<thead>
<tr>
<th>Treg subset</th>
<th>Natural Treg (nTreg)</th>
<th>Induced Treg (iTreg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main function</td>
<td>Immune homeostasis, autoimmune reactions</td>
<td>Local suppression, oral tolerance, mucosal tolerance</td>
</tr>
<tr>
<td>Origin</td>
<td>Thymus</td>
<td>Conventional T cell activation in the periphery</td>
</tr>
<tr>
<td>Activation</td>
<td>Antigen with IL-2</td>
<td>Antigen presented by tolerogenic APC with TGF-β, IL-2, retinoic acid?, TSLP?</td>
</tr>
<tr>
<td>Secreted cytokine</td>
<td>TGF-β, IL-10</td>
<td>TGF-β, IL-10</td>
</tr>
<tr>
<td>Markers</td>
<td>CD25, CTLA-4, GITR, CD103, PD-1$^{\text{high}}$, Nrp1$^{\text{high}}$, Helios, CD73</td>
<td>CD25, CTLA-4, GITR, CD103</td>
</tr>
<tr>
<td>Demethylation status</td>
<td>Stable TSDR</td>
<td>Heterogenous patterns</td>
</tr>
</tbody>
</table>
**1.2.1.5.5 Other regulatory cell populations and nonregulatory FOXP3+ cells**

Regulatory cell populations comprise also other populations besides CD4+ FOXP3+ natural and induced regulatory T cells. Many of these cells lack FOXP3, whereas FOXP3 can also be expressed in cells with no regulatory capacity.

Th3 cells are a suppressive T cell population lacking FOXP3. They have been found predominantly in the mucosal system, and characterized by their production of especially TGF-β, but also IL-4 and IL-10 (Das et al., 2001). Their TGF-β secretion is suggested to induce Foxp3-expressing Tregs that would be their suppression mechanism (Carrier et al., 2007).

Tr1 cells also lack FOXP3 expression, but they are able to suppress immune and autoimmune responses by secreting high levels of IL-10 and TGF-β. In addition, they secrete low levels of IL-4 and IL-17. They can be identified by their expression of CD49d and LAG-3. Tr1 cells release granzyme B and perforin to kill myeloid cells and they may also inhibit T cell responses via cell-contact dependent mechanisms such as CTLA-4 and PD-1. They have been found in various tissues during autoimmune inflammation, and they seem to play a role in transplantation tolerance (Pot et al., 2011, Roncarolo et al., 2014).

NKT cells are a unique, innate immunity resembling subset of T cells that express a restricted repertoire of αβ T cell receptors but also NK cell markers such as CD161 and CD94 (Godfrey and Kronenberg, 2004). They have an autoreactive potential and they may also have regulatory functions (Balato et al., 2009). TCR of NKT cells does not recognize peptides presented by MHC class I or II, instead they recognize glycolipids presented by Cd1, which is a non-classical antigen presenting molecule. Upon activation they release Th1-type cytokines including IFN-γ and TNF-α, but also Th2-type cytokines IL-4 and IL-3, and they also have cytotoxic activity (Smyth and Godfrey, 2000). The development of NKT cells, details of which are poorly known, also takes place in the thymus. The lineage divergence is thought to take place before full expression of TCR (Berzins et al., 2004).

FOXP3+ T cells also contain a minor population which are not regulatory cells. FOXP3 expression can be transiently upregulated in CD4+ T cells without a Treg phenotype and function, as previously mentioned. The nonregulatory phenotype can be induced when TGF-β is absent upon activation (Allan et al., 2007, Gavin et al., 2006, Tran et al., 2007). Regulatory roles have also been proposed for γδ T cells (Wesch et al., 2014).

**1.2.2 CD8+T cells**

CD8+ T cells, cytotoxic T cells when activated, are mainly killer cells that eliminate dysfunctional and malignant cells, or cells infected with virus or with
some other intracellular pathogen. Like CD4+ lymphocytes they develop in the thymus as naïve cells and become activated upon antigen recognition.

Activation of CD8+ T cells can happen in two ways. Firstly, mature DCs with high intrinsic co-stimulatory activity can activate them in some viral infections without the help of CD4+ effector cells, after which CD8+ T cells produce IL-2 that stimulates their proliferation and differentiation. The second, and the more common option is that CD4+ effector cells likewise recognize the antigen presented by the APCs and leading to increased levels of co-stimulatory activity on APCs. Additionally, CD4+ cells produce IL-2 that induces CD8+ T cell proliferation (Murphy, 2012).

Cytotoxic cells can mediate their effects through cell-to-cell contacts or secretion of cytokines. Infected cells mark themselves as targets by expressing viral peptides on their surface in MHC which cytotoxic T cells bind followed by killing of the infected cells. Cytotoxic T cells can insert perforins into the target cell membrane and produce pores through which granzymes enter, activate caspase enzymes with proteolysis and mediate apoptosis of the target cell (Peters et al., 1991). Alternatively, they can bind the Fas molecule on the target cell using Fas ligand, activate caspases, and induce apoptosis (Bossi and Griffiths, 1999). In addition, CD8+ T cells can produce cytokines, including IFN-γ, TNF-α and lymphotoxin-α, to reinforce antiviral defences (Betts et al., 2004, Das et al., 2001).

The CD8+ population also includes regulatory cells expressing FOXP3 (Suzuki et al., 2012). Thymic CD8+FOXP3+ cells express also CD25, GITR, and CTLA-4 and inhibit autologous CD25- T cells through contact-dependent mechanisms (Cosmi et al., 2003). In the periphery CD8+ Tregs can be identified by their expression of CD103 (Uss et al., 2006), but their definite functions are not clear.

1.3 T cell receptor and major histocompatibility complex

The initial event for triggering T cell activation and the above described actions is the recognition of the cognate antigen by TCR that forms the basis of antigen-specific adaptive immunity. TCR can recognize antigens only when they are presented in the MHC molecule and this pMHC interaction with TCR creates a functional immunological synapse, the synapse being the interface between the APC and the T cell. The TCR features that control specificity for the MHC seem to be evolutionary conserved (Scott-Browne et al., 2011).

1.3.1 T cell receptor structure

The αβ T cell receptor, a heterodimer composed of disulfide-linked α and β chains, resembles the antigen binding fragment of the immunoglobulin
molecule but it only has one binding site and is expressed on the cell membrane. The α and β chains are composed of variable (V) and constant (C) domains, followed by a transmembrane segment and cytoplasmic tail (Mazza et al., 1998). The antigen-binding site of the TCR in the variable region comprises of six loops, three in both α and β chains. They are called complementarity determining regions (CDR1, 2 and 3), of which CDR1 and CDR2 are germline-derived, and CDR3 somatically recombinated randomly and imprecisely from V, D and J (joining) in Vβ and V and J segments in Vα, with the addition of P- (palindromic) and N-(nontemplated) nucleotides to the joining regions, a mechanism that creates a highly diverse region (Fig. 5). The recombination events are described in more detail in the T cell development section, in 2.3. Primarily CDR1 and CDR2 contact the helices on the MHC molecule and CDR3 contacts the displayed peptide (Birnbaum et al., 2012), but all CDR loops have been shown to contact both the peptide and the MHC to some extent (Burrows et al., 2010). The Vα domain contacts mainly the amino-terminal half of the bound peptide and the Vβ domain the carboxy-terminal half of the peptide (Hennecke and Wiley, 2001).

![Figure 5. The complementarity determining regions of the Vβ chain of TCR. High diversity of CDR3 is generated with somatic recombination of V, D, and J segments, and addition of N- and P-nucleotides to the ligation sites.](image)

The X-ray crystallography has revealed the three-dimensional structure of TCR. TCRαβ has a relatively flat ligand-binding surface, in some receptors with a linear cavity, for pMHC interaction to facilitate binding to usually linear peptides presented in the MHC. The Cβ domain bends much more acutely towards the Vβ domain, compared to the Cα domain position to Vα domain. A 12-residue long rigid FG loop protrudes out of Cβ domain, and forms a canopy and a side-wall of a cavity created by asymmetrically disposed TCR Cα and Cβ ectodomains (Wang and Reinherz, 2012). Mouse studies have shown that the loss of this FG loop attenuates negative selection at the DP (double positive, CD4+CD8+) stage in the thymus, without blocking positive selection, and affects peptide-mediated activation of mature T cells (Sasada et al., 2002, Touma et al., 2006). When pMHC interacts with the TCRαβ, the FG loop aids in amplifying and exerting the force on CD3εγ (Reinherz, 2015). As a whole, the
TCRαβ-pMHCI interaction seems to be diagonal, with variability in tilt, twist, and shift, whereas, the TCRαβ approach onto pMHCII is more orthogonal, although some TCR-MHC complexes may also have different docking topologies (Wang and Reinherz, 2012).

TCR αβ heterodimer is non-covalently associated with the signal-transducing CD3 subunits (CD3εγ, CD3εδ, and CD3ζζ). Each subunit comprises an extracellular Ig (immunoglobulin)-like domain followed by a short stalk region called connecting peptide, a transmembrane helix, and a cytoplasmic tail. The interaction between TCRαβ and pMHC ligand initiates a cascade of downstream signalling events via the ITAMs in the cytoplasmic tail of the CD3 subunits (Wang and Reinherz, 2012). Of the CD3 subunits the CD3ε seems to have a critical role in pre-TCR signaling, as in knockout mice that lack CD3ε, thymic development is blocked at the early double negative CD4−CD8− stage (DeJarnette et al., 1998).

**Figure 6. TCR complex-MHC interaction.** The antigen is bound to MHCII consisting of α and β chains. TCR that has α and β chains, both having constant and variable regions, is associated with CD3 molecules that are needed for signal transduction. The co-receptor CD4 interacts with the proximal region of MHCII and brings Lck in contact with the cytoplasmic tail of CD4. TM, transmembrane. Modified from Wang and Reinherz. (Wang and Reinherz, 2012).


1.3.2 Co-receptors

The co-receptors CD4 and CD8 are crucial for TCR functions. They do not bind the antigen, but rather contact a conserved membrane-proximal region of the MHC, delivering the tyrosine kinase Lck into the area of TCR-pMHC interaction so that exposed ITAMs on CD3 tails can be phosphorylated on tyrosine residues to allow Zap-70 recruitment and assembly of the downstream signalling components (Wang and Reinherz, 2012). Another function may be to stabilize the interaction between the TCR and pMHC to increase duration of the interaction that gives enough time to mediate an intracellular signal (Das et al., 2001).

The CD8 transmembrane co-receptor is encoded by $\text{CD8}\alpha$ and $\text{CD8}\beta$ genes. Each chain consists of a single Ig-like domain followed by a stalk region, a transmembrane helix, and a short cytoplasmic tail. The isoform CD8$\alpha\beta$ is mostly found on the surface of cytotoxic T cells, and thymocytes, whereas the CD8$\alpha\alpha$ isoform is expressed on γδ T cells, some NK cells, and some intraepithelial lymphocytes (Gangadharan and Cheroutre, 2004). CD4 comprises four Ig-like domains in tandem with a short stalk region and a transmembrane helix (Li and Mariuzza, 2013).

1.3.3 Major histocompatibility complex

The major histocompatibility complex region was first discovered in connection with transplantations, as it had an essential role in determining of the allograft outcome. Thereafter its role in antigen presentation has been revealed. It is located in the chromosome 6 in humans and contains more than 200 genes. The MHC genes can be divided into three classes from which class I and II are essential for lymphocytes, class III is connected with innate immunity and complement factors, encoding for example complement proteins C4, C2, and factor B, and also cytokines TNF-α and lymphotoxin (Murphy, 2012). The MHC class I and II proteins in human are called the human leukocyte antigen (HLA) because of their role in antigen presentation (Floess et al., 2007). Humans express six different class I and six different class II genes. The highly polymorphic loci in the genome for class I genes are HLA A, B, and C; for the class II genes they are HLA DP, DQ, and DR. Many autoimmune diseases have been linked with HLA molecules, people with certain alleles have an increased risk for autoimmunity; while some alleles may have a protective effect against autoimmune manifestations (Sollid et al., 2014).

The MHCI molecule consists of two non-covalently associated polypeptide chains: an $\alpha$-chain encoded in the MHC and a non-polymorphic $\beta2$-microglobulin encoded on a different chromosome. The $\alpha$-chain forms three domains, $\alpha1$, $\alpha2$, and $\alpha3$, from which $\alpha1$ and $\alpha2$ form the peptide-binding cleft.
Binding of the peptide, 8 to 10 amino acids long in the case of MHCI, is stabilized by contacts in the amino and carboxy termini of the peptide and invariant sites of MHC at each end of the cleft (Rudolph et al., 2006).

The MHCII molecules consists of non-covalently associated α and β chains, which both span the membrane and are encoded in the MHC. The four MHCII domains are α1, α2, β1, and β2 (Fig. 5). The ends of the peptide binding cleft formed by the MHCII are more open than in MHCI allowing longer peptides, around 13 to 17 amino acids, to bind. This binding stabilizes the MHC molecule (Rudolph et al., 2006). There are different docking modes for the pMHC-TCR complex, but one rule for the contact is the same: the Vβ domain contacts the α1 helix and the Vα domain contacts the β1 helix in the case of MHCII and the α2 helix in the case of MHCI. The TCRαβ/pMHCII complexes are more orthogonal compared to the complexes formed by MHCI that have a more variable docking topology (Wang and Reinherz, 2012).

Antigens displayed in the MHCI that is expressed on virtually all nucleated cells, come from viruses or certain bacteria or self-proteins in uninfected cells, and originate in the APC cytosol. These intracellular proteins are degraded by proteasomes, and transporters associated with antigen processing-1 and -2 (TAP-1 and -2) on endoplasmic reticulum (ER) membrane help the peptides to enter ER where they bind MHCI with the help of chaperones calnexin and calreticulin, and other molecules. Thereafter the peptide-MHCI complex is transported to the cell surface (Germain and Margulies, 1993). The presentation of an antigen from a pathogen in MHCI indicates intracellular infection and leads to destruction of the infected cell usually by the cytotoxic T cell. Some DCs can obtain exogenous antigens from infected cells and they load these peptides to MHCI; this phenomenon is called cross-presentation. This is beneficial for the host in microbial infections where APCs are not infected and also in response against tumors (Joffre et al., 2012, Platzer et al., 2014).

MHCI is expressed on DCs, macrophages and B cells, and in some mesenchymal cells and they present peptides derived from self-proteins, or from pathogens and microbiota. They are internalized via cell-surface receptors, end up in an endosome where acidification activates proteases to degrade the peptide. Vesicles containing MHCII molecules produced in the ER fuse with these endosomal vesicles and MHCII binds the peptide (Germain and Margulies, 1993). A MHC class-II associated invariant chain (Ii) controls MHCII movement, inhibits peptide binding in ER with its short fragment CLIP and facilitates export (Romagnoli et al., 1993). Another molecule, MHC class II-like molecule HLA-DM stabilizes empty MHC class II molecules, and catalyzes the release of the CLIP-fragment from MHCII and binding of other peptides (Das et al., 2001). The MHCII expression on cells is regulated by the class II transactivator (CIITA) (Holling et al., 2004).
2 T CELL DEVELOPMENT IN THE THYMUS AND CENTRAL TOLERANCE

The development of T cells takes place in the supportive microenvironment of the thymus, into which precursors migrate from the bone marrow. The thymus in the anterior superior mediastinum is built up from endodermal layer during embryonic development.

2.1 The structure of the thymus and cell types

The thymus has two lobes which can be further divided into lobules, each containing outer cortical and inner medullary regions. Thymocytes migrate through the thymus, from the corticomedullary junction (CMJ) to the cortex and then to the medulla, during their development, and certain events take place in different regions of the thymus with specific cell types supporting these functions. Developing thymocytes can be divided into different populations according to the expression of different surface molecules. The CD4⁻CD8⁻ double negative (DN) population is the most immature one, the maturing population is the CD4⁺CD8⁻ DP population, and the CD4⁺ and CD8⁺ single positive (SP) are the most mature populations. These are discussed in more detail below.

Stromal cells of the thymus include cortical and medullary epithelial cells, fibroblasts, endothelial cells, DCs, and macrophages (Anderson et al., 2006), of which especially the epithelial cells influence the thymocyte development. In addition to thymocytes, also B cells, macrophages and DCs migrate into the thymus from the bone marrow. Thymic DCs can be subdivided into three groups, resident classical DCs, migratory classical DC, and migratory plasmacytoid DCs. These populations are somewhat different with respect to their origin, responsiveness to chemokines, and location in the thymus (Klein et al., 2009).

The cortex contains cortical epithelial cells (cTECs), immature thymocytes, and macrophages. Cortical epithelial cells are arranged in a three-dimensional structure that supports close interactions with DN and DP thymocytes. Furthermore, individual cTECs can form multicellular complexes that encompass up to 20 thymocytes; these cells are called thymic nurse cells (TNCs) (Klein et al., 2014).

The medulla contains more mature, single-positive thymocytes than the cortex. Also some mature SP cells exist in the medulla that have recirculated from the periphery (Hale and Fink, 2008). The medulla has its own medullary thymic epithelial cells (mTECs) that are capable of expressing tissue-specific antigens (TSAs). In addition the medulla has specialized hematopoietic APCs (Klein et al., 2014). Hassal’s corpuscles are poorly known groups of epithelial cells within the thymic medulla that seem to activate DCs with TSLP to express
high levels of CD80 and CD86 and induce Treg development (Watanabe et al., 2005).

The CMJ is rich in vasculature and serves as the trafficking point for coming and going thymocytes (Nitta et al., 2011, Lind et al., 2001). After the thymic development, mature T cells express the S1P1 (sphingosine 1-phosphate receptor 1) that mediates their export from the thymus (Kurobe et al., 2006).

Figure 7. A. Cells in the thymus. The double-negative (DN) thymocyte precursors enter the thymus via blood vessels at the CMJ. They migrate to the cortex where they become double-positive (DP) thymocytes. From the cortex the cells migrate to the medulla and become single-positive (SP). Typical cell types and structures at each location are shown. B. Hematoxylin and eosin stained human thymus section. A Hassal's corpuscle is marked with an asterisk. The picture is used with the permission of the copyright holder Eliisa Kekäläinen.

2.2 T cell precursors and early events in the thymocyte development

Thymocytes develop from hematopoietic stem cells (HSC) via common lymphocyte precursors (CLP) originating from the bone marrow. Thymus seeding progenitors (TSP) are CD34+CD45RA+CD7+ cells that have T, B and NK cell precursor activities (Blom and Spits, 2006). The most immature population in the thymus, early thymic progenitors (ETP), are CD34+CD38low cells that lack expression of recombination-activating gene 1 (RAG1), which is required for TCR rearrangements, and CD1A, cytoplasmic CD3, CD2 and CD7, which are all
present in committed T cell precursors in the thymus (Blom et al., 1997). After commitment to the T cell lineage developing cells express CD34, CD1a, and CD7 (Patel et al., 2012). Additionally, they express IL-7Rα; IL-7 is important for human T cell development already from lymphoid precursors, however, IL-7Rα is not needed for T cell lineage decision (Hong et al., 2012). As mentioned earlier, the most immature thymocytes are DN for CD4 and CD8 expression but they become CD4+CD8+ DP through the stage of immature single positive (ISP) CD4+ cells. CD4 is expressed first, then CD8β, and thereafter CD8α (Blom and Spits, 2006).

The transcription factor Notch is required for induction and maintenance of T cell specification. Conditional deletion of Notch in mice results in inhibited T cell development and its relevance has also been shown in humans (Blom and Spits, 2006). Sustained Notch signaling guides thymocytes to the T cell lineage and suppresses B cell development. The Notch genes encode several receptors, from Notch1 to Notch4, which have many potential ligands and they can activate a broad range of different target genes. The expression of Notch receptors differs during T cell development. TSPs express both Notch1 and Notch2, but not Notch3, however, uncommitted CD34+CD1a- human postnatal thymocytes express also Notch3, and this expression persists till the DP stage. Notch expression is shut down in mature CD4 and CD8 SP cells. Notch ligands are expressed on cTECs and mTECs, both cells having slightly different ligand expression profiles (Taghon et al., 2012).

2.3 T cell receptor development

The first TCR loci rearrangements, in the order δ>γ>β>α, take place during the early stages in T cell development. Here only the development of the αβ receptor is discussed in more detail. The rearrangement of TCR is shown in Fig. 8 and the timing of TCR developmental events in Fig. 9.

Two recombination activating genes, RAG1 and RAG2 are responsible for the recombination process. Their task is to initiate recombination by introducing a single-strand nick in DNA, precisely at the border between the heptamer of the recombination signal sequence and the coding segment (Fugmann et al., 2000, Halapi et al., 1999). At the joining of V, D, and J exons, the junctional region can be modified to increase antigen receptor diversity. An asymmetric hairpin opening results in one DNA strand being longer than the other, and P-nucleotides according to the longer strand are inserted to the shorter strand. Terminal deoxynucleotidyl transferase (TdT) and DNA polymerase μ further increase the diversity by catalyzing the addition of N-nucleotides (Nishana and Raghavan, 2012). This modification is not random as the coding end sequence motifs influence the process (Nadel and Feeney, 1997).
The β recombination takes place before the α recombination. First, Dβ segments rearrange to Jβ segments, then the Vβ to DJβ rearrangement takes place. After transcription and splicing the Cβ region is also combined (Fig. 8). Thymocytes with productive TCRβ V-DJ rearrangements have been found already in the DN CD34⁺CD1a⁺ population, and with a less sensitive method in the D4 ISP population, but thymocytes without TCRβ can still be found in the CD4 ISP and in the early double-positive population. After upregulation of CD4, most cells are β-selected, meaning that after a productive β-chain rearrangement, the β chain is expressed with invariant pre-T-α and the CD3 complex, involved in proximal signal transduction, on the surface (Blom and Spits, 2006, van Oers et al., 1995). Without functional β-chain the pre-T-cell receptor with pre-T-α is not produced and the cell will die, unless it is rescued by further rearrangements as two clusters of Dβ and Jβ are upstream of the two Cβ genes. The β-selection leads to signals causing phosphorylation and degradation of RAG2, ending β-chain gene arrangement and ensuring allelic exclusion of the β locus (Nishana and Raghavan, 2012). Thymocytes begin to proliferate until RAG1 and RAG2 are re-expressed and the recombination of the α-locus begins (Ji et al., 2010).

The α-chain consists only of V, J, and C regions, of which the V and J regions are first rearranged and then combined with the C region (Fig. 8). Repeated attempts to produce a functional α-chain is possible. Rearrangements continue until the cell with paired α- and β-chains receives a positive selection signal. The first population in which in-frame α-recombinations, and thus TCR surface expression, are seen is the DP CD3low population (Yassai and Gorski, 2000). The α-chain rearrangements can occur in both chromosomes and T cells may have two different α-chains (Das et al., 2001). Approximately 30% of conventional T cells express two different TCRs. This is even more common in regulatory T cells, since 50 to 99% of them express two T cell receptors (Tuovinen et al., 2006). When the TCR is recombined and expressed on the cell surface the cells are susceptible to further selection events.
Figure 8. T-cell receptor α- and β-chain rearrangement. Both α- and β-chains are recombined from discrete segments during T cell development. For the α-chain, Vα segment rearranges to Jα segment and these create a VJα exon that is transcribed and spliced to join Cα. The resulting mRNA is translated to the TCR α-chain protein. For the β-chain Vβ, Dβ, and Jβ segments are rearranged, first Dβ to Jβ, and then this DJβ to Vβ. Resulting VDJβ region is transcribed and spliced to join Cβ, and the mRNA is then translated to the TCR β-chain protein. The α- and β-chains pair to yield the TCR heterodimer. There are several J segments but all are not shown for simplification. Modified from Murphy (Murphy, 2012).
2.4 TCR repertoire

The great diversity of the TCR repertoire creates the ability to recognize a wide variety of pathogens. The thymic development, with somatic recombination from several gene segments and adding nucleotides, results in an estimated of $10^{15}$ possible sequence combinations (Birnbaum et al., 2012). The human naïve T cell pool consists of estimated $10^{11}$ to $10^{12}$ cells, which indicates that the theoretical whole diversity is not represented in the T cell profile of an individual (Vrisekoop et al., 2014, Qi et al., 2014).

The T cell repertoire can be examined according to the length profile of the CDR3 regions or the sequences, the latter becoming more common due to new high-throughput sequencing techniques. The attempts to sequence the whole T cell Vβ repertoire have resulted in 1-5 million unique TCR Vβ sequences. However, one blood sample is unable to represent all the unique sequences (Arstila et al., 1999, Robins et al., 2009, Warren et al., 2011). Comparisons made between two individuals show 1 to 10% shared sequences (Warren et al., 2011, Robins et al., 2010). The use of the Vβ gene segments is
very variable, a 2500-fold difference has been found between the most and the least used gene segments (Freeman et al., 2009).

The thymic development and T cell selection also shape the TCR repertoire. CDR3 length shortens during thymic selections, described below, and positive selection has been suggested to favor survival of shorter CDR3 regions, possibly because of a more effective TCR-pMHC interaction, or negative selection may eliminate longer sequences if they show too high affinity (Yassai et al., 2002). It has been shown in mice that the pairing of α- and β-chains happens between chains with relatively similar loop sizes, but this has not been shown to influence the mean CDR3 length (Hughes et al., 2003).

2.5 Central tolerance

The development of T cell repertoire that is effective but not harmful to own tissues requires strict control of the developing thymocytes. This is accomplished with central tolerance, which means the selective events in the thymus that delete autoreactive T cells. Possible shortcomings are compensated with peripheral tolerance.

2.5.1 Positive selection

After TCR recombination the DP thymocytes undergo positive selection, the recognition of self-peptides in MHC molecule with TCR. The specificity of antigen recognition is determined by previous V(D)J recombination events. Thymocytes recognizing the self-pMHC complex with affinity sufficient to induce positive selection but not too high to lead to elimination in negative selection, will survive, proliferate and develop further. In addition to the TCR-pMHC interaction, the TCR signaling event is also affected by the co-receptor CD4 or CD8, and the recognition must result in a stable binding for signaling events to occur (Germain, 2002).

The developed TCR only recognizes antigens displayed in the MHC, and this is known as MHC restriction. Two models have been suggested to be the basis for this event, and probably both in fact influence the process. The germline model proposes that because of the co-evolution of TCR and MHC molecules, MHC restriction is intrinsic to the TCR structure, and the sequences lacking MHC reactivity have been eliminated. The selection model proposes that MHC restriction is not intrinsic, but rather is regulated by the CD4 and CD8 co-receptors that promote TCR signaling through co-receptor binding to MHC during the positive selection (Rangarajan and Mariuzza, 2014). The recognition of MHC depends on conserved CDR1 and CDR2 residues, encoded within the V gene segment. The somatically recombined CDR3 loops, encoded in the VDJ region of the β-chain and the VJ region in the α-chain, can fine-tune the affinity
Thymocytes unable to recognize pMHC with their TCR will die by neglect which happens in up to over 90% of the thymocytes (Germain, 2002).

Thymocytes before positive selection are mainly DP TCRαβlow, and after positive selection events they become DP TCRαβhigh and also express CD3 at high levels (Vandekerckhove et al., 1994). CD69, a C-type lectin-like signaling receptor, is expressed on the surface of cells undergoing or undergone a positive selection (Sancho et al., 2005). A study with transgenic mouse suggested the role of CD69 to be in the inhibition of the export of mature single positive thymocytes to the periphery (Feng et al., 2002).

Positive selection takes place in the cortex, and it is largely mediated by cTECs. These cells have a unique pMHC-ligandome, the set of peptides displayed in the MHC, that are not found in other parts of the thymus (Klein et al., 2009). Recent findings suggest that positive selection requires unique MHC-associated peptides generated with certain intracellular proteolytic enzymes. β5t-containing thymoproteasome is important for the selection of CD4+CD8+ repertoire in mice (Nitta et al., 2010). For CD4+CD8- T cells, on the other hand, lysosomal proteases thymus-specific serine protease (Tssp) and cathepsin L are necessary for optimal positive selection (Gommeaux et al., 2009, Honey et al., 2002). In addition, cTECs are capable of macroautophagy, a mechanism helping unconventional loading of peptides onto MHCII through an endogenous route (Nedjic et al., 2009). Studies in TCR-transgenic mice have shown that the positively selecting peptides are either low-affinity antagonists or weak agonists, whereas negatively selecting peptides are primarily high-affinity agonists (Klein et al., 2009).

Recent studies have found a novel gene and a protein needed especially for positive selection in the thymus, Themis (thymocyte-expressed molecule involved in selection) (Fu et al., 2009, Johnson et al., 2009). Themis expression is up-regulated in the late DN and eminently in DP thymocytes, mainly in the cortex, and it persists until it is down-regulated after positive selection. In mature T cells it is expressed in low amounts (Gascoigne and Palmer, 2011, Fu et al., 2009). The function of Themis seems to be in the early TCR signalling cascade, where it reduces signal strength in response to low-affinity but not to high-affinity MHC peptides. Themis acts through controlling actions of the phosphatase SHP-1, which reduces TCR signalling and selection thresholds, and in addition helps to discriminate between agonist and lower-affinity antagonist ligands (Fu et al., 2013).


**2.5.2 CD4 versus CD8 lineage choice**

Simultaneously with positive selection, also the selection for CD4 or CD8 co-receptor takes place, and the transcription of the non-selected co-receptor locus is silenced (Sawada et al., 1994). The co-receptor binds MHC in the TCR-pMHC interaction, and probably facilitates optimal signaling, through action of Lck and ZAP-70 (Fig. 6.) (Bosselut et al., 1999, Germain, 2002).

Several models have been suggested to be behind the CD4-CD8 lineage decision. The instruction model suggested that biochemically distinct intracellular signals are generated upon TCR and CD8 molecule interaction with the MHCI ligand compared to the interaction of TCR and CD4 with the MHCII molecule. These different signals then induce the lineage commitment, and the expression of the receptor not involved in signal generation is lost (Borgulya et al., 1991, Robey et al., 1991). The selection model proposed that DP thymocytes are already randomly committed to a lineage, or upon positive selection make a stochastic lineage choice irrespective of their TCR specificity. Because this results in some cells having a receptor that is not optimal for their TCR-pMHC interactions, elimination of these cells takes place later. Cells that express the wrong co-receptor will not get life-sustaining signal and die, while the cells with the right co-receptor survive and develop further (Chan et al., 1993).

Mouse studies have found caveats in both of these theories, and new theories have been developed. In the default theory DP cells develop into CD4 cells by default, even without TCR signaling, but TCR engagement with MHCI can change their path (Suzuki et al., 1997). At the moment the kinetic signaling model seems to be the most probable explanation. It suggests that once a DP thymocyte receives a selection signal, it downregulates CD8 but not CD4. If the TCR of a thymocyte recognizes MHCII, signaling persists but if it recognizes MHCI, signaling through TCR is terminated. The length of the signaling is thus the decisive factor for the co-receptor choice (Singer et al., 2008).

After the TCR signaling, cytokines also affect the lineage choice. Mostly after positive selection the cells become responsive to cytokines. IL-7 can promote Runx3 expression and CD8 lineage choice (Park et al., 2010). In addition, IL-15 has been shown to induce CD8+ T cell development (McCaughtry et al., 2012).

**2.5.3 Negative selection**

After positive selection CD4 and CD8 thymocytes migrate to the medullary region. Mouse studies have shown that TCR engagement of immature cortical DP thymocytes increases CCR7 (C-C chemokine receptor 7) surface expression, and CCR7 ligands, for example CCL19 and CCL21 are produced by mTECs; these interactions guide the thymocytes towards the medulla. In CCR7-deficient mice,
the export of thymocytes straight from the cortex to the circulation is possible, but they show autoimmune reactions as a sign of incomplete negative selection (Ueno et al., 2004).

As T cells must tolerate own tissues throughout the body, expression of a diverse set of peptides from different tissues is required to take place in the thymus. This far autoimmune regulator gene AIRE is the only known factor to mediate the promiscuous expression of tissue-restricted antigens in mTECs needed for negative selecion (Kisand et al., 2010, Anderson et al., 2002, Liston et al., 2003). In addition, AIRE seems to modulate the differentiation of mTECs and regulate the expression of intrathymic chemokines guiding thymocytes and DCs. Defects in the AIRE gene lead to a rare autoimmune disease called APECED (Laan et al., 2013) which is discussed later in this thesis. In the thymic medulla AIRE is expressed mainly by mTECs, but also by DCs. All AIRE-expressing cells express class II HLA in high levels, and 20% of them also express co-stimulatory molecules CD80 or CD86, and the CD40 antigen (Heino et al., 1999). The role of AIRE in the central tolerance must, however, cover also other mechanisms in addition to mediating the expression of tissue-restricted antigens, as Aire-deficient mice develop autoimmune reactions also to Aire-independent antigens (Kuroda et al., 2005, Niki et al., 2006). The precise mechanisms of this still remain unknown.

In addition to the AIRE-dependent mechanisms, peripheral self-antigens are represented by DCs that have migrated from the periphery into the thymus. Because DCs are more effective in antigen presentation, there is also a mechanism that transfers antigens, and even intact MHC molecules, from mTECs to DCs for presentation (Gallegos and Bevan, 2004). This enables the presentation of AIRE-dependent antigens by DCs. Negative selection events take place mostly in the medulla, but it seems to happen to some extent also in the cortex. In the cortex, the cells responsible for negative selection seem to be DCs instead of cTECs (Klein et al., 2014).

The determining factor in the regulation of negative selection is the affinity of the TCR for the pMHC, since a too high affinity leads to clonal deletion by apoptosis or anergy. However, no clear-cut border exists for the affinity, and increased affinity may also result in the development into Treg (Fig. 10). *In vitro* studies have shown that negative selection signals result in a strong transient Ca++ influx and Erk signaling (Daniels et al., 2006). Also a greater recruitment of TCR signaling components like Lck and Zap70 may cause the negatively selecting signal (Mallaun et al., 2010), and the CD28 co-stimulation is needed for clonal deletion (Pobezinsky et al., 2012).
2.6 Regulatory T cell lineage commitment

The commitment to the regulatory T cell lineage occurs during thymic development beside conventional T cell development. Increased receptor affinity is needed for the development of regulatory T cells but is not sufficient for lineage induction, and at least the cytokines and cells secreting them further guide this development. In addition to TCR signaling, also other signals are important for Treg development, including CD28 costimulation, and transcription factors NFAT, AP-1, NF-κB, and Foxo1/3 (Konkel et al., 2014). The currently leading theory proposes a two-step model in which the high TCR affinity induces CD25 expression and permits cytokine signaling that leads to FOXP3 expression (Lio and Hsieh, 2008). How the FOXP3 expression is conducted and when, is not precisely known. Altogether the timing of the lineage commitment events has been controversial.

2.6.1 T cell receptor affinity

One identification marker of regulatory T cells is T cell receptor affinity below the threshold leading to negative selection which is, however, higher when compared to conventional T cells. The first found indication of increased TCR strength in Tregs was the high expression level of CD25, CD5, and CTLA-4, which are induced upon TCR stimulation (Josefowicz et al., 2012). Functional support for this came from studies where increased frequencies of Tregs were found in mice with defects in the negative feedback of TCR stimulation (Carter et al., 2005). It is suggested that most of the differences in the repertoires of Tregs and conventional T cells comes from the events and survival in negative selection.
FOXP3-expressing T cells have a survival advantage compared to FOXP3-negative T cells with equivalent TCR signaling. Expression of many pro-survival molecules is connected to FOXP3 (Josefowicz et al., 2012).

The connection of TCR signaling and early FOXP3 expression is not fully clear. FOXP3 expression is seen in DN cells without detectable TCR (Tuovinen et al., 2008a). Another study, however, suggests that early TCR expression can be detected in these pre-DP cells expressing FOXP3 (Nunes-Cabaco et al., 2010). In any case, a great majority of the FOXP3+ thymocytes are DP CD3high or CD4 SP and they express TCRs (Nunes-Cabaco et al., 2010). These cells are already positively selected and they undergo negative selection.

However, the same self-peptide can select both Tregs and Tconvs with the same TCR, and it has also previously been shown that TCR repertoires of Tregs and conventional T cells overlap (Wojciech et al., 2014). These findings indicate that in addition to the TCR affinity also other factors in TCR signaling play a role in the commitment to the Treg lineage. Suggested factors are for example encounter of ligand at different stages of development or on different APCs, duration of the TCR-ligand interaction, and avidity of the interaction, which here means the number of engaged TCRs (Bains et al., 2013, Klein et al., 2014, Wojciech et al., 2014). A computational study by Bains et al. suggests two possible models for selective affinity. In the first one they propose that T cells can continuously re-assess the fate decisions on the basis of multiple summed TCR signals from TCR-pMHC interactions. In the other model they suggest that TCR sensitivity is modulated during development, and the same pMHC may result in different outcomes at different stages of development (Bains et al., 2013).

There is evidence that TCR sensitivity to signaling in fact fluctuates during the development. Most evidence supports increased sensitivity during maturation through subcellular localization of signaling molecules such as Lck, inhibition of ERK activation, increased expression of SHP-1, upregulation of the negative regulator CD5, and increased expression of ZAP-70 (Azzam et al., 2001, Bains et al., 2013, Stephen et al., 2009). A microRNA miR-181a that enhances sensitivity to TCR stimulation is, however, reduced during thymic development (Li et al., 2007).

CTLA-4 has been shown to be important in TCR threshold regulation and its overexpression is able to skew also the T cell repertoire of conventional T cells into auto-reactive T cells (Verhagen et al., 2013, Yamaguchi et al., 2013).

2.6.2 FOXP3 in Treg development

The expression of FOXP3 is important for Treg development and function but some studies have also suggested that FOXP3 is not required for initial Treg lineage commitment in the thymus, but rather it stabilizes the T cell phenotype.
First FOXP3-expressing cells are found in the DN population (Liu et al., 2014).

Induction of FOXP3 expression is controlled via several signaling pathways. These include TCR, IL-2, STAT, Smad, TGF-β, PI3K, Akt, mTOR (the mechanistic target of rapamycin) and Notch (Ohkura and Sakaguchi, 2010).

Antigen presentation by cTECs or mTECs lead to induction of FOXP3 expression in developing Tregs (Aschenbrenner et al., 2007, Liston et al., 2008). TCR signaling, co-receptor molecules, and cytokine receptors are needed for active FOXP3 expression (Huehn et al., 2009). The cytokine effects are discussed in more detail in 2.6.3. In short, before FOXP3 expression takes place high affinity TCR interactions induce CD25 expression, then IL-2 or other γ-chain cytokine signaling events leads to FOXP3 expression. The precise mechanism seems to be that the CD28 co-stimulation induces Foxp3 expression, probably through NF-κB activation and binding of c-Rel to noncoding sequence elements in the Foxp3 gene (Zheng et al., 2010).

Recently the role of micro RNAs also has been studied in Treg development and functions. Of these especially the role of miR-155 in Treg development has been studied in the mouse model. Foxp3 binds an intron within the DNA sequence that encodes miR-155 precursor mRNA and it was suggested that miRNA-155 expression regulated by Foxp3 plays a role in Treg differentiation and function (Lu et al., 2009, Zheng et al., 2007). Lu et al. found that SOCS1 is the direct target of miR-155 and that miR-155 upregulated IL-2 expression by decreasing the suppression by SOCS1. MiR-155 knockout mice had reduced numbers of Tregs but with normal functions, suggesting a role for miR-155 in T cell development (Lu et al., 2009).
Figure 11. Factors controlling FOXP3 expression in developing thymocytes and mature Tregs. Modified from Klein et al. (Klein and Jovanovic, 2011). In the beginning of Treg development TCR/CD28 signal may mediate enhanced Foxp3 promoter accessibility and via PKCθ result in the binding of c-Rel to CNS3 which opens the Foxp3 locus. Transcription factors downstream of TCR/CD28 signaling (NFAT, AP-1, CREB, c-Rel) enhance responsiveness to cytokines through upregulation of IL-2R subunits. At a later stage in Treg development TSDR becomes demethylated, the mechanisms of which are not yet known. The TGF-β sensor is not considered essential for Treg development in the thymus.

2.6.3 Cytokines in the thymus

The thymic niche consists of stromal cells, cytokines, and other mediators that regulate the T cell development in the thymus. Different regions in the thymus form unique microenvironments for thymocyte development. Studies from mice have shown that the thymic niche regulates Tregs development, as the developing Tregs with the same antigen specificity compete for the limited niche for Treg development (Bautista et al., 2009, Leung et al., 2009).

In addition to TCR signals, also cytokines are able to induce FOXP3 expression. Cytokine-mediated signals are crucial for FOXP3 expression as there is a complete lack of Tregs in mice deficient for the common cytokine-receptor γ-chain (Fontenot et al., 2005b). Of the cytokines using the receptor with common γ-chain IL-2 is known to be essential for Treg development. However, it seems that other common γ-chain cytokines such as IL-7 and IL-15 can at least...
partly compensate for its loss (Vang et al., 2008). Mice deficient both in IL-2Rα and IL-7Rα have the phenotype seen in γc-deficient mice with no Foxp3+ thymocytes (Bayer et al., 2008). The signaling cascade following IL-2 stimulation involving JAK1, JAK3, and STAT5 results in FOXP3 expression (Huehn et al., 2009). In Stat5 knockout mice the numbers of Tregs and thymocytes are greatly reduced (Yao et al., 2005).

In addition to common γ-chain cytokines, also TGF-β is important for Tregs in maintaining the FOXP3 expression and homeostasis in the periphery. Its role in the thymus, however, is still controversial (Marie et al., 2005). A recent mouse study suggests that apoptosis of thymocytes leads to TGF-β secretion that promotes Treg development by inducing Foxp3 expression (Konkel et al., 2014). Another mouse study found that TGF-β protected thymocytes from negative selection and inhibited Treg apoptosis, possibly through the action of Bcl-2 family proteins (Ouyang et al., 2010).

2.6.3.1 IL-7

IL-7 is required especially for the early development of all T cells at the DN stage, but also for the later stages and peripheral homeostasis. The IL-7R signaling is thought to coincide the TCRβ-selection of the DN thymocytes (Hong et al., 2012). The IL-7 or IL-7R α-chain deficiency results in a dramatic loss of thymocytes and mature T cells in mice. IL-7 promotes cell survival in the thymus and periphery by up-regulating the expression Bcl-2 family antiapoptotic genes. In fact, forced Bcl-2 expression in the thymus can rescue T cell development in IL-7R −/− mice (Maraskovsky et al., 1997).

In the thymus IL-7 is produced by thymic epithelial cells, mostly the CD45-negative non-hematopoietic cells (Hong et al., 2012). The expression is concentrated in the cortico-medullary junction and medulla but is also seen in some cells in the cortex. These places are most likely those where IL-7 signaling is needed in the T cell development (Alves et al., 2009, Zamisch et al., 2005). The developing thymocytes can also affect IL-7 expression by cross-talking with TECs. This was shown in a mouse study where the signals delivered by DP thymocytes resulted in a decrease of IL-7 expression by TECs (Alves et al., 2010).

The IL-7 receptor consists of an α subunit associated with the common γ-chain of the IL2R-family. After IL-7 binds to the receptor, Jak family kinases Jak1 and Jak3 associate with the receptor subunits and are activated via cross-phosphorylation. They in turn phosphorylate STAT5 docking sites on the receptor. STAT5 is recruited to the receptor and activated via phosphorylation by the Jak kinases. This results in STAT5 translocation to the nucleus and initiation of mRNA transcription. One of the important effects is the aforementioned upregulation of the anti-apoptotic factor Bcl-2. IL-7R engagement also activates the PI3K pathway that results in Akt activation. IL-7
signaling can be modulated by the suppressors of cytokine signaling proteins (SOCS-3) through association or inhibition of Jak-1 and the subsequent inhibition of STAT5 phosphorylation (Hong et al., 2012).

The role of IL-7 for Treg development has been unclear. The effects of IL-7 are regulated predominantly by modulating the receptor levels and mature Tregs express only low levels of IL-7R α-chain, CD127 (Malchow et al., 2016, Mazzucchelli and Durum, 2007). Lack of IL-7 during Treg commitment in mice decreases the amount of Tregs but does not prevent the full development (Bayer et al., 2008). Some controversial findings come also from the stage where IL-7 affects thymocyte development. The importance of IL-7 for early DN stages and then later for SP stages is clear but it has been shown in mice that DP thymocytes are not responsive to IL-7 between β-selection and positive selection (Van De Wiele et al., 2004). A recent study in human cells, however, shows that DP thymocytes are responsive to IL-7 as measured by STAT5 phosphorylation (Nunes-Cabaço et al., 2011).

### 2.6.4 Epigenetic factors

The Treg development is solidified by stable expression of FOXP3. Epigenetic modifications like DNA methylation at CpG motifs, i.e. cytosine and guanine triphosphate oligonucleotides with phosphodiester links, and histone-methylation and –acetylation are important in the gene expression control as they alter the higher order chromation structure and determine the accessibility of DNA to transcription factors (Klein and Jovanovic, 2011). There is accumulating evidence supporting the view that FOXP3 epigenetic regulation is critically involved in Treg development and maintenance (Huehn et al., 2009). Distinct regions of the Foxp3 locus have different DNA methylation patterns and histone modification in Tregs and conventional T cells. There are three highly conserved non-coding regions in the FOXP3 locus that regulate FOXP3 transcription and they are a target for the epigenetic modifications: FOXP3 promoter, TGFβ sensor, and Treg-cell-specific demethylated region, see Fig. 11 (Huehn et al., 2009).

The FOXP3 promoter is activated by TCR signaling through binding of NFAT and AP1 (Mantel et al., 2006). The CpG motifs in the FOXP3 promoter of Tregs are almost completely methylated while they are only weakly methylated in resting conventional T cells, and FOXP3 promoter has stronger association with acetylated histones in Tregs than in Tconvs (Kim and Leonard, 2007).

The TGFβ sensor has binding sites for NFAT and SMADs. The chromatin in the TGFβ sensor region is in an accessible state in cells that express FOXP3, also in induced Tregs, and it was found that Tconvs transiently express FOXP3. While TGFβ induces chromatin remodeling in the TCRβ sensor region, it may also increase the accessibility to the FOXP3 promoter (Huehn et al., 2009).
The most prominent differences in the methylation pattern are found in the CpG-rich TSDR. It is fully demethylated in Tregs and methylated in Tconv, and the enhancer activity of TSDR decreases after methylation. TSDR seems to act by stabilizing the FOXP3 expression. Thus cells that are transiently expressing FOXP3 have a methylated TSDR (Huehn et al., 2009, Polansky et al., 2008). A recent study shows that Treg precursors, here the CD4+ CD25\textsuperscript{high} thymocytes, are fully methylated in the Foxp3 TSDR, but after anti-CD3/anti-CD-28 stimulation and IL-2 become demethylated and acquire the Treg epigenome and Foxp3 expression (Toker et al., 2013).

In addition to FOXP3, also other genes important for Treg function have TSDRs, including \textit{Ctla4}, \textit{Il2ra}, \textit{Tnfrsf18} (gene of GITR), and \textit{Ikzf2} (gene of Helios). With these many Treg-specific epigenetic modifications, Tregs can be better identified by their Treg-specific epigenome than only FOXP3 expression (Brunkow et al., 2001).

\subsection*{2.6.5 Timing of commitment}

The timing of Treg cell commitment in the thymus has been controversial, and no definitive consensus has yet been reached. Human and mouse studies have somewhat different findings, mouse studies being more conflicting.

Commitment in the CD4 SP stage, from Foxp3\textsuperscript{-} precursor cells, is supported by findings that most of the Foxp3\textsuperscript{+} cells are found to reside in the medulla (Fontenot et al., 2003, Fontenot et al., 2005a). There are, however, DP Foxp3\textsuperscript{+} cells also in the cortex that are suggested to be Treg precursors or an independent population (Lee and Hsieh, 2009, Liston et al., 2008). They are suggested to represent even one fourth of all Foxp3\textsuperscript{+} thymocytes, and express high levels of CD69, a marker for positive selection, and CCR7, usually guiding thymocytes towards the medulla (Liston et al., 2008). These findings could indicate commitment already in the cortex. There is also evidence that commitment to the regulatory lineage might precede the Foxp3 expression, so the lineage commitment cannot be solely defined by Foxp3 expression (Gavin et al., 2007, Lin et al., 2007).

Most human studies favor commitment at the DP stage. DP FOXP3\textsuperscript{+} thymocytes show markers of immaturity, such as susceptibility to apoptosis and some expression of RAG-2, but also markers of being positively selected such as CD3, CD69, and CD5 (Lee and Hsieh, 2009, Tuovinen et al., 2008b). A statistical multiple linear regression analysis of the relationship between DP FOXP3\textsuperscript{+} cells and CD4 SP FOXP3\textsuperscript{+} cells indicates that the DP FOXP3\textsuperscript{+} cells are precursors of CD4 SP FOXP3\textsuperscript{+} cells (Nunes-Cabaço et al., 2011). Additionally a recent computational analysis of Treg commitment also suggests that Treg development begins already from immature DP thymocytes in the cortex (Bains et al., 2013). A study by Lee et al. suggests that many DP FOXP3\textsuperscript{+} may in fact
represent doublets of CD4 FOXP3⁺ and CD8 cells and thus bias some studies (Lee and Hsieh, 2009).

3 PERIPHERAL TOLERANCE

The elimination of auto-reactive cells by negative selection is not flawless, and additional mechanisms are needed to maintain tolerance. The natural Tregs from the thymus and induced Tregs act in active suppression. Beside the active suppression mechanisms there are also mechanisms with respect to encountering the antigen, to keep the harmful processes towards own tissues at bay. These include anergy, ignorance, and deletion of self-reactive T cells. Also the balance between different Th responses helps to maintain tolerance.

3.1 Tregs in periphery

Both the natural and induced Tregs are responsible for peripheral dominant tolerance. Most of the cells are suggested to be nTregs (Gratz et al., 2013). Their function, however, is mostly similar and their differentiation in the periphery is not simple, and they probably work together. Both leave the thymus as naïve cells and respond to antigen recognition by proliferation, increase in suppressive activity, and migration to the tissue where the antigen resides. Additionally, some cells from both populations survive as memory cells after activation (Gratz et al., 2013). Before activation the cells can be considered as a resting population, as they express lower levels of CTLA-4. This type of cells are not actively cycling, and they do not function as potential suppressors as activated Tregs (Miyara et al., 2009).

The maintenance of Tregs seems to be mostly depending on IL-2, which promotes their survival and functional competence, including FOXP3 and CTLA-4 expression (Barron et al., 2010, Furtado et al., 2002). Tregs do not produce IL-2 and they are thus reliant on its secretion by other cells (Benoist and Mathis, 2012). Mice deficient for components of IL-2 signaling pathway have reduced numbers of functionally suppressive Tregs, and this leads to autoimmune manifestations (Barron et al., 2010, Sadlack et al., 1993).

In addition to the total lack of functional Tregs in IPEX, several human diseases, including autoimmune reactions, chronic infections, and cancer, are connected to Treg imbalance, affecting also patient survival (Asano et al., 1996, Mougiakakos et al., 2010). It has also been found that they may play a role in acute infection, and that also the steady state of Tregs may correlate with the disease outcome (Koivula et al., 2014). Tregs act in immune responses regulating the magnitude and duration of the response.

Immunotolerance in the gut is important in dealing with the microbiota and food antigens. Oral tolerance is a mechanism that induces tolerant response
to orally ingested antigens, by at least one means being Treg conversion (Faria and Weiner, 2005). Another physiological condition that seems to need Treg actions is pregnancy, as increased numbers of activated Tregs have been found at the uteroplacental interface (Loewendorf et al., 2014).

### 3.1.1 Conversion of Tregs

The conditions that lead to the conversion of CD4+ FOXP3- cells into peripheral Tregs include chronic or suboptimal stimulation by an agonist peptide, oral administration of an agonist peptide, homeostatic signals when transferred into lymphopenic host, and response to helminthic infections. Also activation by commensal pathogen antigens in the colon leads to a regulatory response (Benoist and Mathis, 2012). However, it is also suggested that CD4+CD25+Foxp3+ T cells can differentiate into Th17 cells in the presence of IL-6 and absence of TGF-β and they thus can have dual effects in the immune response (Xu et al., 2007).

### 3.2 Other mechanisms of peripheral tolerance

The peripheral tolerance mechanisms include, in addition to the active suppression by Tregs, also passive mechanisms to maintain tolerance. Anergy results from impaired T cell activation, either lacking a co-stimulatory signal or mediating an inhibitory signal upon antigen presentation. Several mechanisms may promote anergy by inhibiting the mTOR pathway. mTOR belongs to the PI3K family and it mediates the effects of several environmental factors (Powell and Delgoffe, 2010).

Ignorance covers mechanisms that limit antigen presentation of certain tissues to T cells. This can be achieved by hiding tissues behind a barrier impermeable for circulating cells, such as the blood-brain barrier. This, however, is not absolute since some cells do access the central nervous system. Other immunologically privileged sites include eyes, testicles, and placenta (Murphy, 2012).

Deletion of self-reactive cells can happen through Fas- or Bim-mediated apoptosis (Xing and Hogquist, 2012). Fas is expressed on T cells and the Fas-ligand is expressed in T cells after activation and IL-2 stimulation, causing activation-induced cell death. Fas-induced apoptosis is important in eliminating T cells after repeated antigen stimulation (Strasser and Pellegrini, 2004). Bim-mediated apoptosis is needed for terminating immune response in acute infections (Pellegrini et al., 2003).
Mutations in the autoimmune regulator –gene AIRE cause a rare autosomal disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also known as autoimmune polyglandular syndrome type 1 (APS1). In APECED more than 70 possible mutations have been described (De Martino et al., 2013) in the human AIRE gene that is mapped to chromosome 21q22.3 (Aaltonen et al., 1997). APECED is usually recessively inherited but some sporadic dominant patterns have also been found (Cetani et al., 2001). In some areas, including Finland, Sardinia, and Iran, founder mutations are suspected to cause increased frequency of APECED (Ahonen et al., 1990, Mathis and Benoist, 2009, Rosatelli et al., 1998, Zlotogora and Shapiro, 1992)

As AIRE has an essential role in central tolerance driving the expression of TSAs in the thymus, its defects lead to T cell dysfunction and failure of tolerance, causing destruction of target tissues by cellular and antibody-mediated mechanisms (Arstila and Jarva, 2013, De Martino et al., 2013). AIRE dysfunction leads to impaired negative selection and escape of autoreactive T cells to the periphery. These cells are most probably responsible for the processes leading to the clinical manifestations. Acquired AIRE-deficiency also leads to autoimmune manifestations, often seen in thymoma patients (Scarpino et al., 2007). Most of the findings of the function of AIRE come from Aire knock-out mice. The results, however, are not totally applicable to the human disease. Also the specific mechanisms how mutations of AIRE cause the disease APECED are not thoroughly known, and AIRE is also suspected to have some function in the periphery.

4.1 Clinical manifestations

The target tissues in APECED include multiple endocrine organs. The most frequent three findings are Addison’s disease, hypoparathyroidism and mucocutaneous candidiasis, found in 70% of the patients. Two of these symptoms are needed for the diagnosis (Kisand et al., 2011). Other possible targets include the gonads, stomach, small intestine, liver, thyroid, pancreatic islands, nails, teeth, melanocytes, hair follicles and conjunctiva, resulting in for example ovarian failure, hepatitis, thyroiditis, type 1 diabetes or alopecia (Wolff et al., 2013, Mathis and Benoist, 2009). Gastrointestinal symptoms, such as constipation and diarrhea are also common (Arstila and Jarva, 2013). The clinical manifestations vary from patient to patient but usually the symptoms cause substantial problems. The symptoms begin usually in the childhood or early adulthood, most often before the age of ten (Kisand et al., 2011). In addition to AIRE, some studies have found evidence that additional genetic loci, mostly the HLA, affect certain disease manifestations (Halonen et al., 2002)
The most typical autoantibodies found in APECED patients are antibodies neutralizing cytokines, especially type I interferons, IFN-ω and IFN-α, and cytokines related to the Th17 response (Kisand et al., 2011). Neutralizing antibodies against IFN-ω are found in 100% and against IL-22 in over 90% of the patients (Wolff et al., 2013). Anti-IFN-ω antibodies are the most specific antibody for APECED, as they have not been found in other diseases or physical conditions than APECED or thymoma (Kisand et al., 2011). Organ-specific autoantibodies against the target tissues can be found in 8–66% of the patients (Kisand et al., 2011, Arstila and Jarva, 2013). They are usually related to the autoimmune manifestations of these organs, and they may appear later than cytokine-neutralizing antibodies, which usually precede the onset of the disease (Wolff et al., 2013). Neutralizing autoantibodies against IFN-ω and IFN-α thus offer higher diagnostic value and have been included in the diagnostic criteria of APECED (Arstila and Jarva, 2013). There are also gene tests available for diagnostics to specify the AIRE mutation.

4.2 AIRE and thymus in APECED

The highest expression level of AIRE is found in the thymus, in mTECs and DCs (Heino et al., 1999). AIRE is also expressed in low levels in other tissues apart from the thymus, in lymph nodes, fetal liver, and spleen, in DCs and specific Aire-expressing cells (Heino et al., 1999). Peripheral AIRE has been suggested to play a role in peripheral tolerance, but this has not been proven (Heino et al., 1999, Metzger and Anderson, 2011). The AIRE protein is most often localized in the cell nucleus and consists of specific domains including the aminoterminal homogeneously staining region (HSR) domain, the nuclear localization signal, the Sp100, AIRE1, nucP41/75, DEAF1 (SAND) domain, two plant homeodomain type zinc fingers, and four LXXLL motifs (Peterson et al., 2008).

The models trying to explain AIRE function in the thymus can be divided into two, the molecular biology models and the cell biology models (Danso-Abeam et al., 2011). Both further comprise several theories. In the classical transcription model TSAs are the direct target genes of the AIRE activity, and thus defects in AIRE lead to their impaired expression (Anderson et al., 2002). This is supported by the findings that AIRE contains DNA binding domains, it is able to bind DNA via these domains, and it has the capacity to activate transcription (Kumar et al., 2001, Pitkänen et al., 2000). AIRE seems to act as a coactivator in a large transcriptional complex, regulating transcriptional processes (Johnnidis et al., 2005). In this function it has a large set of interaction partners, which can be divided into four main classes: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing factors. Knockdown of these partners reduces the expression of AIRE-dependent genes (Abramson et al., 2010). Further support for this model comes from mouse data.
that suggests that transgenes using target gene promoters are also dependent on Aire (Liston et al., 2004). However, there are also caveats in this theory. Although AIRE can bind DNA, it does not act like a regular transcription factor, and its targets lack shared promoter regions (Anderson et al., 2002, Danso-Abeam et al., 2011). Also the precise molecular mechanism of AIRE action is unclear. There are also TSAs that are upregulated independently of AIRE, and AIRE also regulates the expression of non-TSA genes in mTECs, either positively or negatively (Derbinski et al., 2005, Johnnidis et al., 2005, Sato et al., 2004). AIRE’s influence on the TSA expression is mostly quantitative, as many TSAs are expressed at some level also in the absence of AIRE (Mathis and Benoist, 2009).

Another transcription model is the model depicting AIRE as a random transcriptional activator. In this theory AIRE would affect gene expression by modification of chromatin structure, or general accessibility to DNA (Abramson et al., 2010). Aire-dependent TSAs are found in chromosomal clusters that could support this theory (Derbinski et al., 2005). Presentation of AIRE-induced TSAs can also happen by DCs with cross-presentation mechanism.

In addition of upregulating TSA expression, AIRE has also a role in mTEC differentiation which is needed for optimal processing and expression of antigens and the intrathymic thymocyte migration shown in mice (Anderson et al., 2002, Laan et al., 2009). Another model for AIRE function is consequently the maturation model which suggests the main role of AIRE to be in the development of mTECs (Matsumoto, 2011, Matsumoto, 2013). AIRE seems to have effects on induction of the apoptosis in end-stage terminally differentiated mTECs and in mTEC differentiation. Studies suggest a role especially in the terminal maturation, as cells lacking AIRE remain premature (De Martino et al., 2013, Gallo et al., 2013). In this model, lack of AIRE would result in diminished expression of TSAs since AIRE-expressing cells do not develop or die. Defects in Aire lead to changes in the thymic morphology in mice, including concentrated distribution of mTECs to the medulla and lack of Hassal’s corpuscle structures (Yano et al., 2008). AIRE has also been found to control the expression of transcription factors associated with developmental plasticity of progenitor cells in the thymic organogenesis regulated by Nanog, Oct4, and Sox2 on mTECs (Gillard et al., 2007).

Failures on these suspected mechanisms of AIRE function are also thought to be behind the pathogenesis of APECED. Defects in AIRE lead to impaired thymic development of T cells and escape of autoreactive cells, and the effect is suspected to be mostly on the negative selection (Chan and Anderson, 2015). Additionally, AIRE has a role in Treg development. Some studies suggest that Aire-expressing mTECs are behind the generation of TSA-specific Foxp3+ Tregs, these Aire-expressing mTECs can also serve as APCs and thus enhance the selection of Tregs (Aschenbrenner et al., 2007, De Martino et al., 2013). A recent study in mice suggests that AIRE may also play a role in the generation of
unique Treg subsets during the perinatal period, and these Tregs seem to be more efficient suppressors (Yang et al., 2015). The detected defects in Treg and other T cell populations are described in the following section.

The great variety of APECED manifestations irrespectively of the AIRE genotype indicates that several factors influence the disease phenotype (De Martino et al., 2013).

### 4.3 T cell dysfunction

The autoimmune manifestations found in APECED are mainly considered T cell-mediated and abnormalities in several T cell populations have been found. The number of Tregs is reduced in APECED patients (Kekäläinen et al., 2007, Ryan et al., 2005). Also the expression of FOXP3 in a single-cell level in Tregs is reduced and they also have defective suppressive function in vitro (Kekäläinen et al., 2007). In mouse studies with new sequencing methods differences in the Treg repertoires of Aire-deficient and Aire-sufficient mouse were found, affecting especially the TCRs with low frequency (Perry et al., 2014). The most prominent defects are found in the CD45RO+ Treg populations that are the activated population mostly responsible for cell regulatory functions (Laakso et al., 2011). Increased frequency of CD4+CD25+ cells was found in some studies, however, in a later study no difference in CD4+ activated or memory cells was found between the patients and controls (Wolff et al., 2010). A reduced response to IL-17F and IL-22 has also been found (Kisand et al., 2010)

APECED patients have cell populations that bear markers typical of naïve cells such as CD45RA and CCR7 that show signs of functional activation, for example CD8+ cells expressing perforin (Laakso et al., 2011). This is true also for CD31+ cells, the recent thymic emigrants, suggesting that their activation takes place in the thymus (Laakso et al., 2011). The thymus has been suggested to act as a tertiary lymphoid tissue (Laakso et al., 2011). Certain lymphoid tissue inducer cells have been suggested to work in the activation process, as they secrete IL-22. This type of cells are the first ones in the thymus inducing AIRE expression in immature mTECs, and bear markers typical of APCs (Kim et al., 2009, Rossi et al., 2007).
AIMS OF THE STUDY

The development of T cells in the thymus is the basis of effective host defense mechanisms but also of tolerance in the immune system. My aim was to study this development in human. The more specific aims were:

I To analyze the effect of positive and negative selection on the repertoire of developing thymocytes

II To study the role of AIRE in the thymic selections

III To specify developmental events in the CD4⁺CD8⁺ DP and CD4⁺ SP thymocyte populations and to time the commitment to the regulatory T cell lineage

IV To compare the precursors of regulatory and conventional T cells for TCR signaling, response to cytokines, especially IL-7, and apoptosis susceptibility
SUMMARY OF MATERIALS AND METHODS

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

1 SAMPLES

1.1 Thymus samples (I-IV)

Thymic tissue was obtained from children undergoing corrective cardiac surgery. The number of samples and the mean age of patients and controls in each article are shown in Table 2.

1.2 Blood samples (II)

Peripheral blood samples were taken from healthy volunteers and APECED patients. The cord blood samples used have been previously published (Talvensaari et al., 2002).

Table 2. Summary of samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number</th>
<th>Mean age (years)</th>
<th>Range (years)</th>
<th>Female (%)</th>
</tr>
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<tbody>
<tr>
<td>Thymic tissue (I)</td>
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<td>0.0-4.9</td>
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<tr>
<td>Thymic tissue (II)</td>
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<td>0.3-0.3</td>
<td>0</td>
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<tr>
<td>Thymic tissue (III)</td>
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<td>0.6</td>
<td>0.0-3.0</td>
<td>22</td>
</tr>
<tr>
<td>Thymic tissue (IV)</td>
<td>42</td>
<td>1.0</td>
<td>0.0-10.8</td>
<td>48</td>
</tr>
<tr>
<td>Blood (APECED patients) (II)</td>
<td>5</td>
<td>44.8</td>
<td>32-56</td>
<td>80</td>
</tr>
<tr>
<td>Blood (healthy controls) (II)</td>
<td>8</td>
<td>42.8</td>
<td>29-55</td>
<td>63</td>
</tr>
</tbody>
</table>

2 CELL ISOLATION AND SEPARATION

2.1 Cell isolation (I-IV)

Thymocytes were released from the thymic tissue samples by mechanical homogenization. Peripheral blood mononuclear cells were extracted using Ficoll-Paque (GE Healthcare) gradient centrifugation.
2.2 Immunomagnetic cell separation (II-IV)

Selected subsets were isolated using immunomagnetic cell separation with mAb and magnetic beads (Dynal, Oslo, Norway) or ready-made antibody-coated Dynabeads according to the manufacturer’s instructions. The purity of the isolated cells was tested with flow cytometry. It was variable depending on the subset, but was typically >80%. Isolated cells were either directly lysed for RNA extraction or, if necessary, detached using the DETACHaBEAD reagent (Dynal). CD8 lymphocytes and CD8+CD45RA- and CD8+CD45RO- subpopulations were separated with beads as previously described (Laakso et al., 2011) (II). CD4 lymphocytes were separated using CD4 antibody-coated Dynabeads (Invitrogen, Carlsbad, USA).

3 CELL CULTURE (III- IV)

Thymocytes were cultured in a 12-well or 24-well plate overnight. Cells were cultured in culture media containing 1 ml RPMI medium (Life Technologies, Paisley, UK), supplemented with heat-inactivated human AB serum (Finnish Red Cross Blood Service or from a volunteer’s blood sample), HEPES, L-glutamine, 2-mercaptoethanol, streptomycin, and penicillin (supplements from Sigma Aldrich, St. Louis, MO, USA).

Soluble CD3 antibodies were used at a concentration of 2 μg/ml. In cultures with plate-bound mAb anti-CD3 was incubated in the culture well for 1h, after which unbound mAb was washed away. Control cells were kept at +4°C overnight (III).

The cytokine IL-7 (Immunotools, Friesoythe, Germany) was used at a final concentration of 10 ng/ml. Stimulation with anti-CD3 mAb was done in solution. The cells were incubated for 10 minutes on ice with 1 μg anti-CD3/5 × 10^6 cells, followed by cross-linking with 4 μg goat-anti-mouse IgG (both purchased from Immunotools) (IV).
4 FLOW CYTOMETRY AND SORTING (I-IV)

The antibodies used in the experiments were direct fluorochrome conjugates. They were purchased from BD Biosciences (Becton Dickinson, San Jose, USA), eBioScience (San Diego, USA), Immunotools, and Santa Cruz Biotechnology (Dallas, USA). Monoclonal antibodies used in the analyses are summarized in Table 3.

Following the surface staining the cells were fixed, permeabilized, and stained for the intracellular molecules CTLA-4, FOXP3 and Ki-67 using a FOXP3 staining kit from eBioscience, according to the manufacturer's protocol. The specificity of FOXP3, CD25, and CTLA-4 detection was verified using fluorochrome and isotype-matched control antibodies: FITC-conjugated mouse IgG1 and PE-Cy5 mouse IgG2 (BD Biosciences) and PE-Cy7 mouse IgG1 (eBioscience).

Flow cytometry was performed using the FACScan instrument (Becton Dickinson), Cyan ADP instrument (Beckman Coulter), or FACSARia instrument (Becton Dickinson). The data analysis was done with Cellquest, FACSDiva, Summit 4.3 and FlowJo programs. Fluorescence compensation settings were optimized using BD Bioscience CompBeads or unstained cells.

The thymocyte populations at different stages of development can be identified by their expression of surface markers. The cells subject to positive selection are DP thymocytes expressing a surface TCR, and thus also CD3 that is connected to TCR expression. The negative selection occurs at any stage after surface TCR expression, in DP or SP cells. The sorted populations were the DP CD3 negative cells representing cells before positive and negative selection, DP CD3 low cells, DP CD3 high cells, and CD4 SP CD3 high cells. These populations are subject to selections, CD4 SP cells are thought to be already positively and negatively selected. Cell sorting (I-II) was done using the FACSARia instrument (Becton Dickinson), and the purity of the isolated populations was verified with FACSARia or FACScan instrument (Becton Dickinson). The purity was at least 89% in all cell populations.
Table 3. Summary of monoclonal antibodies

<table>
<thead>
<tr>
<th>Specificity-Fluorochrome</th>
<th>Manufacturer</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-FITC</td>
<td>BD Biosciences</td>
<td>I, II</td>
</tr>
<tr>
<td>CD3-PE</td>
<td>Immunotools, Friesoythe, Germany</td>
<td>I, IV</td>
</tr>
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<td>CD4-Alexa Fluor700</td>
<td>BD Biosciences</td>
<td>IV</td>
</tr>
<tr>
<td>CD4-APC-Cy7</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>CD4-biotin</td>
<td>BD Biosciences</td>
<td>IV</td>
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<tr>
<td>CD4-PE-Cy5</td>
<td>BD Biosciences</td>
<td>I, II</td>
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5 DETECTION OF APOPTOTIC CELLS (III-IV)

Apoptosis detection was done after an overnight culture using the apoptosis staining kit with Annexin and PI (Becton Dickinson) according to manufacturer's instructions.
6 PCR AND SEQUENCING (I-IV)

6.1 Quantitative PCR

Isolated cells were lysed with TriPure Isolation Reagent (Roche, Basel Switzerland) and total RNA was isolated with RNeasy MiniKit columns (Qiagen, Crawley, UK). First-strand cDNA was synthesized using oligo-dT-primer (Sigma) and AMV-reverse transcriptase (Finnzymes, Helsinki, Finland). The equivalency and quality of the cDNA samples was tested by quantitative PCR amplification of the β-actin gene, using a commercial primer-probe set (Applied Biosystems, Foster City, USA).

Quantitative PCR was done with Taqman Universal Master Mix (Applied Biosystems) and the iCycler iQ instrument (Bio-Rad, Hercules, USA). Primer-probe sets for TCR Cα and TCR Cβ were purchased from Applied Biosystems as assays-by-design sets; the sequences have been previously published (Tuovinen et al., 2006). Other primer-probe sets, Bcl-2, BIM, and FOXP3 were commercially available assays also from Applied Biosystems. All assays except Bcl-2 were intron-spanning. To prevent contamination by genomic DNA in the Bcl-2 assay decontamination reactions were done with deoxyribonuclease (Sigma) after RNA isolation. The quantitative PCR data was normalized against β-actin expression levels and shown on a relative scale, where comparisons indicate the difference in PCR cycles during the exponential phase of the reaction.

6.2 TCR repertoire analysis

The spectratyping profiles show the amount of each CDR3 length found in the population and indicate polyclonality. Spectratyping was done with a previously described method (Pannetier et al., 1995, Currier and Robinson, 2001). Shortly, the cDNA was used as a template for PCR with TCR V gene and C gene specific primers, run for 40 cycles, followed by a 15-cycle run-off reaction using an internal, FAM-labeled C or J gene primer. The primer sequences have been previously published (Genevee et al., 1992, Puisieux et al., 1994). The primers were purchased from Sigma-Aldrich. The amplicons were analyzed by capillary electrophoresis with the ABI-PRISM 3730 DNA analyzer (Applied Biosystems).

Areas of individual peaks in the CDR3 length profile were expressed as percent of the combined areas of all the peaks in the profile. Comparison of the profiles was done by extracting the relative peak areas of one profile from the other. The absolute values of differences were summed and divided by two. The values were thus put within the range of 0 – 100 %, with 100% indicating that the repertoires had no overlap. Comparison of spectratyping profiles to a naïve polyclonal repertoire was done as previously described (Laakso et al., 2011).

The mean length of the CDR3 loop was analyzed by calculating from the
Immunoscope profiles of Vβ-Cβ data. The profiles of the CDR3 lengths were calculated from the sequences. The CDR3 region was defined to encompass residues 95-106 (Chothia et al., 1988).

6.3 Sequencing

First, total cellular RNA was used to prepare 50μl of first-strand cDNA and then 2 μl was used for the PCR, two separate reactions were pooled before analysis. The size and quality of the PCR-products were determined with Bioanalyzer 2100 (Agilent Technologies, USA). The DNA concentrations were measured with Qubit fluorometer (Invitrogen). The purified and quantified PCR-products were used in the emulsion PCR process necessary prior to 454 pyrosequencing (Margulies et al., 2005). The sequencing was conducted at Institute of Biotechnology (Helsinki, Finland) with 454-GS FLX –protocol offering read length of ~250 bp (454 Life Sciences, Roche Diagnostics, USA). Productive 454 sequence reads were divided into eight sample specific data sets using identifying tags (6 bp sequence) before forward primer (5’- TCC CTC ACT GTG ACA TCG GCC CA -3’). The linkers and tags added to the 5’-end of the forward primer had the following sequences: 5’-GCC TCC CTC GCG CCA TCA GAG CAG C, 5’-GCC TCC CTC GCG CCA TCA GTA CAG C, 5’-GCC TCC CTC GCG CCA TCA GTA GCT A, and 5’-GCC TCC CTC GCG CCA TCA GTC TGT A. The sequence of the reverse primer was 5’-GCC TTG CCA GCC CGC TCA GCC TGG CCC GAA GAA CTG CTC A. Reads in length of more than 70 bps were included in the analysis and translated into the corresponding amino acid sequences in correct frame.

6.4 Physico-chemical profiling

CDR3 sequence sets were aligned according to their central residue to study and compare the physico-chemical tendency of their neighboring residues. Index values for hydrophobicity, acidity and molecular mass were then assigned to each position in each sequence. Hydrophobicity was evaluated according to the Kyte and Doolittle scale (Kyte and Doolittle, 1982) with negative values for non-hydrophobic and positive for hydrophobic residues. Acidity was evaluated according to the isoelectric point (pI) of residues, low pI being associated to acid and high to basic residues. Molecular weight of residues was analysed in a range of 75-192 Da, with a 13 Da step. Low molecular weight corresponds to small and high weight to large residues.

Curves describing mean global characteristics were determined for each subset analyzed by plotting mean hydrophobicity, acidity or molecular weight values against locations in the CDR3 loops (Fig. 6). Sequences originating from subsets of interest were superposed according to their central residue and
pooled. Positions of the superposed sequences are then compared between two pools for each physico-chemical property with

\[ E_i = \frac{|m(P_i^{\text{Pool1}}) - m(P_i^{\text{Pool2}})|}{\sqrt{\frac{v(P_i^{\text{Pool1}})}{n_i^{\text{Pool1}}} + \frac{v(P_i^{\text{Pool2}})}{n_i^{\text{Pool2}}}}} \]

where \( m(P_i) \) is the mean value obtained for the property \( P \) at position \( i \), \( v(P_i) \) is the variance and \( n_i \) the number of sequences. The difference between the two T cell populations for the property \( P \) at position \( i \) is significative for \( E_i > 1.96 \) with type I error at 5%.

### 6.5 Methylation analysis

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). Bisulfite conversion was performed with Qiagen Epitect, according to the manufacturer’s instructions. The demethylation status of the TSDR region (Treg-specific demethylated region) was quantified by real-time PCR as previously described with minor modifications (Wieczorek et al., 2009). PCR was performed with Bio-Rad iCycler.

### 7 STATISTICAL ANALYSIS

Statistical analysis was done by using SPSS and GraphPad Prism software. The results are presented as arithmetic means and standard deviations. The normality of the data sets was tested with Shapiro-Wilk or Kolmogorov-Smirnov normality test. P-values for differences were calculated using Student’s two-tailed paired T-test. P-value <0.05 was considered significant.

### 8 ETHICAL CONSIDERATIONS

The pediatric Ethics Committee of the Helsinki University Hospital approved the study, and informed consent was obtained from the parents of the children, patients, and the healthy volunteers.
RESULTS

1 TCR REPERTOIRE DEVELOPMENT (I)

1.1 Thymic T cell subsets and selections

To study the TCR repertoire development we sorted thymocyte populations at different developmental stages. The populations chosen were DP CD3 negative (DP<sub>neg</sub>), DP CD3 low (DP<sub>low</sub>), DP CD3 high (DP<sub>high</sub>), and the CD4 SP CD3 high (CD4) population, as these are the populations subjected to selective events. The T cell subsets in the thymus were identified first according to their CD4 and CD8, and then CD3 expression.

The expression of CD3 can be used to time different stages because of its close interaction with the T cell receptor. The most immature DN thymocytes do not have a functional TCR and CD3 surface expression. At the DP stage, the most immature thymocytes are CD3 negative but they have already undergone β-selection as we could see from the normal and polyclonal spectratyping profile. The expression of the TCR Cβ mRNA was also high already in this population, compared to the TCR Cα mRNA, which had lower expression in the DP<sub>neg</sub> population. Cα spectratyping profiles also showed polyclonal rearrangements in the DP<sub>low</sub> population and later (I, Fig. 2 A&B). The α-chain is recombined and CD3 expression increases after TCR expression begins. The positive selection takes place after the expression of TCR from the DP<sub>low</sub> population onwards. Negative selection can take place after TCR expression, earliest at the DP<sub>low</sub> stage but also at the SP stage. Markers indicating selective events, CD5 and CD69, increased as cells matured (I, Fig. 2 C). Most of the positive selection events likely took place at the DP<sub>low</sub> stage, because the biggest relative increase was found in the transition from DP<sub>low</sub> to DP<sub>high</sub> cells, however, the increase was still continuing to the CD4 population.

1.2 TCR length

The somatic recombination creates α and β CDR3 regions of certain length. We wanted to study whether the selective events in positive and negative selection shape the length repertoire of the CDR3. The average length of each population was assessed by spectratyping, where we used 3 Vβ genes with 13 Jβ genes.

We could see a reduction in the average CDR3 length between the DP<sub>low</sub> and DP<sub>high</sub> stages, indicating mostly the influence of positive selection. Further decrease, however, was seen also between DP<sub>high</sub> and CD4 stages, possibly reflecting an effect also from a negative selection.

We compared the spectratyping profiles from different populations to the naïve cord blood repertoire. The most immature, DP<sub>neg</sub>, population was
closest to the naïve repertoire when the spectratyping profiles were compared, with 19.6% difference, while the mature CD4 population differed the most, with 26.7% (I, Fig. 3). This showed that divergence from the naïve repertoire occurs upon selection events, with decrease of polyclonality.

1.3 Sequence diversity

The spectratyping profiles show only the distribution of CDR3 lengths, not how many different CDR3 each peak contains. To specify more precisely the effects of selections on the repertoire, we sequenced the cell populations. We wanted to compare the sequences found from each population, to find out whether some sequences are specifically lost during selections. Surprisingly, only a few sequences were shared between the populations, namely two between DP<sub>neg</sub> and DP<sub>low</sub>, and two between DP<sub>hi</sub> and CD4 of 1217 unique sequences in two thymuses. We also analyzed how many new sequences appeared after each round of examination, and it seemed that the samples were sequenced close to saturation. The limiting factor was thus probably the number of sorted cells (I, Fig. 4 D).

1.4 Physico-chemical features

As the sequences were very variable, we wanted to study if the structure of the CDR3 loop differed notably between the cell clones. A recently developed method by Baussand and Gorochov (I) was utilized for the populations sorted from two thymuses. This visualized the CDR3 similarity concerning hydrophobicity, acidity, and molecular mass in positions around the central residue (I, Fig. 5). CDR3β residues are typically polarized, N-terminal residues being more hydrophobic than the C-terminal ones, and the N-terminal half has higher hydrophobicity index. There is also a tendency to select for negatively charged residues in the C-terminal half of TCR. Molecular weight profiles are more complex, but in short small residues were used in the tip of the loop, and bigger ones in the C-terminus. Further quantitative analysis of the sequence pools compared the mean CDR3β molecular weight, mean sequence length, and mean global charge differences at each CDR3 position using the Pepstats software (Rice et al., 2000). Various differences between subsets from the same thymus were found but the differences did not build up progressively through developmental stages. In addition, significant changes at a particular CDR3 position were never found in both thymus samples. In conclusion, this approach did not reveal any progressive and constant CDR3β physicochemical modification pattern from the analyzed thymuses.
2 AIRE AND THYMIC SELECTIONS (II)

After having studied the effect of thymic selection on the developing thymocytes, we next moved to APECED as an example of failure of this development. Shortening of the CDR3 region in the Vβ is seen during the normal thymic development. In APECED autoreactive T cells escape thymus and this is thought to result from defects in AIRE that is responsible for promiscuous expression of peripheral antigens mainly in the negative selection. Additionally, defects in AIRE are suggested to affect the development of thymic epithelial cells and thymic structure (Passos et al., 2015).

2.1 Positive and negative selection and TCR length

We found an average 1.9 base pair shortening between the nonselected CD3neg and the CD4 population (Fig. 12), that has also been previously reported from both mice and humans (Yassai et al., 2002, Yassai and Gorski, 2000, Matsutani et al., 2011). The mean shortening between the DPlow and DPhigh populations was 1.5±0.6 base pairs (bp) and between the DPhigh and CD4+ SP populations 0.6±0.5 bp (II, Fig. 1).

The CDR3 region mostly interacts with the presented peptide, whereas CDR1 and CDR2 are connected to the MHC of the pMHC complex. As the shortening spans the thymocytes most probably subjected to negative selection, we used CDR3 length analysis as a surrogate of functioning negative selection in APECED patients.

2.2 TCR length in APECED patients

First, we used immunomagnetic separation to isolate CD4+ lymphocytes from 4 patients and 4 controls. 15 Vβ-specific primers were used for spectratyping and measurement of the average CDR3 length. Spectratyping profiles showed skewed TCR repertoires in APECED patients with oligoclonal expansions (II, Fig. 2). To quantify this, we compared the repertoire with the average cord blood repertoire. The patients showed deviation from the naïve repertoire by 26±7.7% and the controls by 20.0±6.8% (p<0.001). This difference, however, did not affect the average CDR3 length, which was 26.5±1.6 bp in patients and 26.6±1.6 bp in controls (Fig. 12).

Secondly, we isolated CD8+ lymphocytes from 4 patients and 4 controls. We had not studied this process previously but the same kind of shortening in the thymus has been reported from human CD8+ cells (Yassai and Gorski, 2000). There was no difference in the length of CDR3 between peripheral CD4 and CD8 cells in our setting, the lengths were 26.6±1.6 and 26.7±1.3 bp, respectively, confirming the earlier reported shortening also in CD8 cells. The repertoires of
CD8⁺ lymphocytes were more skewed with oligoclonal expansions as previously reported (Laakso et al., 2011). The patients had on average longer CDR3 length (27.4±1.9 bp) but this was not statistically significant, and there was considerable variation (Fig. 12) (II, Fig. 4).

It would also be possible that some smaller population would be subjected to impaired selections, and would thus not be seen in our bulk analysis. CD8⁺ CD45RO RA⁺ cells in APECED patients have been shown to contain highly abnormal, activated cells. Thus, to more precisely analyze the CDR3β length in CD8⁺ cells, we isolated CD8⁺ CD45RO RA⁺ and CD8⁺ CD4RO⁻RA⁻ cells. In the CD45RO⁺ population the mean length was 27.7±0.8 bp in the patient group and 27.9±1.3 bp in the control group whereas in the CD45RA⁺ cells the same lengths were 28.3±0.5 bp and 28.1±1.2 bp, not showing significant difference (II, Fig. 5).

Our analysis did not indicate that there would be such a failure in the selection in the absence of AIRE that would allow escape of cells with longer CDR3β.

Fig. 12. The average CDR3 length in the studied populations. Error bar shows standard deviations of the means. The differences in CDR3 length between patients and controls were not significant. **, p<0.0001.

3 DP T CELLS AS PRECURSORS OF SP CD4 TREGS (III)

From the study of the whole T cell population and T cell repertoire we moved to a more precise analysis of the development of regulatory T cells. There is conflicting data from the timing of the commitment of T cells to the regulatory
lineage in the thymus, and results from humans and mice are somewhat different. We wanted to study if the DP FOXP3 thymocytes showed features of precursor cells, such as higher susceptibility to apoptosis, in order to assess their relation to the CD4 thymocytes.

3.1 Cell culture leads to a decrease of CD25+ and FOXP3+ cells in the DP population but not in the CD4 SP population

To assess the precursor features of DP thymocytes, we cultured thymocytes overnight with growth factor deprivation and CD3 stimulation that mimics TCR signaling. As mentioned above, increased susceptibility to apoptosis is a known feature of immature precursor cells. We tested this with both CD25 and FOXP3 as a marker of Treg cells.

Overnight culture did not result in a significant change of CD25 expression in CD4 SP cells, but in the DP population the frequency of CD25+ thymocytes decreased to <50% from the original after overnight culture. Stimulation with soluble anti-CD3 increased CD25 expression within CD4 cells but not in DP cells (III, Fig. 1).

CD25 is expressed also in activated cells and that was possibly at least a partial reason for the CD25 increase after CD3 stimulation. Thus, FOXP3 is a more reliable marker of Treg cells. Next, we analyzed FOXP3 expression after overnight incubation. The frequency of FOXP3-expressing DP thymocytes decreased by 50%, whereas overnight culture resulted in an increase in FOXP3+ cells in the CD4 SP population from 8.4% to 11.6% (Fig. 13). The average decrease in the DP FOXP3+ population was 34%, and the increase in the CD4 SP population was 64%. Stimulation with soluble anti-CD3 mAb had similar effects, as the FOXP3-expressing cells increased among CD4 cells and decreased in the DP population (III, Fig. 2).

Our data show that the DP and CD4 populations responded differently to the culture and TCR stimulation. The expression of CD25 and FOXP3 was different in these conditions, but it had the same trends.
Fig. 13. FOXP3 expression in DP and CD4+ cells in the thymus. The proportion of FOXP3+ cells in DP and CD4 populations is shown with and without overnight culture. *p<0.05.

3.2 Overnight culture increases thymocyte apoptosis

As there was a decrease in the DP FOXP3+/CD25+ population, which we considered to be precursor cells, we studied apoptosis as the possible mechanism behind the change to assess the susceptibility of cells to apoptosis. Apoptosis was enhanced by growth factor deprivation and TCR stimulation that are both known to trigger apoptosis of immature thymocytes. For apoptosis detection we used annexin and propidium iodide (PI). Annexin reveals the early apoptotic cells and PI the late apoptotic cells, which have already an eroded cell membrane (van Engeland et al., 1998).

Unfortunately, FOXP3 staining cannot be used together with these reagents, as FOXP3 staining requires permeabilization of the cells that leads to exposure of annexin and PI targets, and CD25 was used instead. To see how well CD25 expression correlated with FOXP3 expression we isolated the CD25+ and CD25- populations from the DP and CD4 cells and measured the level of FOXP3 mRNA with quantitative PCR. The level of FOXP3 mRNA was over 100-fold higher in CD25+ populations when compared to CD25- populations (III, Fig. 4). This indicates that CD25 could be used as a surrogate marker for FOXP3+ cells.

We detected on average <6% apoptotic cells among the freshly isolated thymocytes. After overnight culture the frequency of apoptotic cells was on average 23%, both annexin+ and PI+ cells included. In the DP population the increase was slightly more prominent than in CD4 cells, but this difference was not significant (III, Fig. 3). Thus, we could show that apoptosis increased in the culture, providing a possible mechanism for the loss of DP CD25+ cells.
3.3 Expression of survival and apoptosis markers

Increased apoptosis was found in both DP and SP populations. To further compare the populations, we isolated the CD25+ and CD25- populations from DP and CD4 cells and measured Bcl-2 and BIM expression. Bcl-2 and BIM are regulators of thymocyte survival with opposite effects, Bcl-2 promotes survival and BIM promotes apoptosis. We could see that the CD25+ populations had a higher expression of both Bcl-2 and BIM, although there was a considerable variation between individuals and not all of the differences were statistically significant (III, Fig. 4). After this we measured the effect of overnight culture to the expression of these genes in thymocytes. These thymocytes mostly represent CD4 positive cells and as with flow cytometry, we could see increased FOXP3 expression after an overnight culture. However, this was not statistically significant. After an overnight culture we could see a significant decrease in the expression of Bcl-2 (III, Fig. 5), indicating that it was not a crucial factor for survival. The expression of BIM was undetectable, probably because of its role in apoptosis.

4 TCR SIGNALING AND IL-7 IN TREG DEVELOPMENT (IV)

The DP and CD4 FOXP3+/CD25+ cells responded differently to growth factor deprivation. Next we studied the effects of IL-7, which represents a common γ-chain cytokine, on DP and CD4 cells. IL-2 is an essential cytokine for all T cells, and IL-7 is also important for early T cell development and survival in the periphery. However, the data of the role of IL-7 in the thymus for different Treg precursors have been conflicting.

4.1 CD127 in Tregs and thymus

The IL-7 receptor consists of a common γ-chain, CD132 and an α-chain, CD127, and the cytokine effects are regulated by receptor expression, mainly by expression of CD127 (Hong et al., 2012). Because mature Tregs express CD127 only at low levels, the significance of IL-7 for Tregs also in the thymus has been somewhat unclear.

We could see that CD127 was differentially expressed in the FOXP3+ and FOXP3- thymocytes. We measured CD127 in DN, DP CD3neg, DP CD3low, DP CD3high, and CD4 CD3high populations. The expression of CD3 was not similar in the FOXP3+ and FOXP3- populations. In the FOXP3+ population it was heterogeneous, and in the FOXP3+ cells most cells were CD3high (Fig. 14). In FOXP3+ cells, the mean CD127 fluorescence intensity (MFI) peaked in the DN population, whereas in the FOXP3- cells it peaked in the DP CD3high population. CD127 MFI decreased in the FOXP3+ cells gradually as they developed, the
The sharpest reduction was seen in the transition from DP CD3\textsubscript{high} to CD4 cells. There was a significant difference in CD127 MFI in the CD3\textsubscript{high} and CD4 populations between FOXP3\textsuperscript{+} and FOXP3\textsuperscript{-} populations, FOXP3\textsuperscript{-} cells having higher CD127 MFI (IV, Fig. 1). Altogether, CD127 MFI started to diverge after the DP CD3\textsubscript{low} stage, from which the selection mainly begins.

Figure 14. CD3 and FOXP3 expression in DP cells. Double-positive thymocytes were gated in the CD4/CD8 plot. CD3 and FOXP3 expression of a representative sample are shown. The majority of FOXP3\textsuperscript{+} cells express high levels of CD3.

4.2 IL-7 effects in developing Tregs

Mouse studies have suggested that thymocytes are unresponsive to IL-7 between β-selection and positive selection (Van De Wiele et al., 2004). In human, however, it has been shown that DP FOXP3\textsuperscript{+} thymocytes respond to IL-7 stimulation as the binding of IL-7 to its receptor leads to phosphorylation of STAT5, and its activation levels were comparable to the ones of CD4 SP FOXP3\textsuperscript{+} cells. In the DP FOXP3\textsuperscript{-} population STAT5 phosphorylation after IL-7 stimulation occurred at a lower level but in the CD4 SP population the FOXP3\textsuperscript{-} cells had the same level of P-STAT5 as their FOXP3\textsuperscript{+} counterparts. A similar IL-7 effect was also seen in the DP CD25\textsuperscript{+} and CD25\textsuperscript{neg} and CD25\textsuperscript{low} populations, the latter populations being less responsive to IL-7 stimulation as measured by STAT5 phosphorylation (Nunes-Cabaço et al., 2011).

To analyze IL-7 effects in the developing Tregs, we cultured thymocytes overnight with and without added IL-7. DP FOXP3\textsuperscript{+} thymocytes downregulated their CD127 expression after IL-7 stimulation, by 40.0 ± 25.0% (p<0.05), as did CD4 FOXP3\textsuperscript{+} cells, by 68.8 ± 8.5% (p<0.0003). This showed that IL-7 had an effect on them as CD127 downregulation is a known consequence of IL-7 stimulation.

We could see after an overnight culture with IL-7 that IL-7 promoted the development of Tregs at the CD4\textsuperscript{+}CD8\textsuperscript{+} DP stage. DP thymocytes were gated, and the frequency of FOXP3\textsuperscript{+} cells was analyzed with flow cytometry. First, the frequency of FOXP3\textsuperscript{+} DP cells increased from 0.2% to 0.4% after IL-7 stimulation.
The MFI of FOXP3 expression in DP cells did not significantly differ from that of FOXP3+ CD4+ SP cells (FOXP3 MFI 27.3 vs. 30.6, p = 0.06). Secondly, CTLA-4 expression in DP cells also increased, from 0.2% to 0.3% (p<0.03). Intracellular CTLA-4 expression in DP FOXP3+ thymocytes also increased, from 20.9% to 31.7%, whereas in FOXP3- thymocytes CTLA-4 expression was only 0.2% with no increase after IL-7 stimulation (IV, Fig. 3).

In addition to the increased frequency of FOXP3+ cells, IL-7 also promoted Treg-cell associated phenotype. This was seen as an increase in FOXP3 MFI in FOXP3+ DP cells from 27.3 to 35.2 (p < 0.02) (IV, Fig. 2D), and also increased CTLA-4 MFI in the DP cells from 35.5 to 53.9 (p < 0.003) (IV, Fig. 3B).

Similar effects were seen in CD4 cells. The frequency of CD4+ SP FOXP3+ cells increased from 5.2% to 9.2% (p < 0.0002). Additionally, FOXP3 MFI in CD4+ SP cells increased from 31 to 44 (p < 0.0009), and intracellular CTLA-4 levels increased from MFI 33 to 47 (p < 0.01) (IV, Fig. 7).

4.3 IL-7 protects developing Tregs from apoptosis but does not affect TCR signaling, or T cell proliferation or methylation status

IL-7 is known to modulate TCR signaling in developing thymocytes. It has been suggested that TCR signaling makes thymocytes more sensitive to IL-7 (Hennion-Tschelzoff et al., 2013). We measured CD69 expression after α-CD3 mAb stimulation. CD69 is connected with the activation of T cells but it is also connected to positive T cell selection. We could see that in DP thymocytes CD69 MFI increased from 216 to 232 but in the DP FOXP3+ population CD69 expression was already high (CD69 MFI 585) and further increase by anti-CD3 stimulation was not seen (data not shown). With IL-7 preincubation, α-CD3 mAb increased FOXP3 expression but this was not higher than with IL-7 alone.

IL-7 is a growth factor, and thus increased FOXP3 expression could be due to increased proliferation of FOXP3+ cells. We measured the expression of Ki-67, a known proliferation marker in the DP FOXP3+ cells with and without added IL-7. There was no significant increase of Ki-67+ cells in the FOXP3+ population after IL-7 stimulation (IL-7- 6.1% ± 2.5%, and IL-7+ 4.7% ± 1.8%, NS) (IV, Fig. 4B), showing that IL-7 did not increase the proliferation of DP FOXP3+ cells. Also in the DP FOXP3+ population there was no significant change in the Ki-67 expression (IL-7- 0.5% ± 0.4%, and IL-7+ 0.4% ± 0.3%).

Epigenetic changes are known to stabilize Treg phenotype. To test whether IL-7 had an effect on FOXP3 demethylation, we measured the relative proportion of demethylated and methylated TSDR DNA after IL-7 stimulation by real-time quantitative PCR method described by Wierczorek et al. (Wierczorek et al., 2009). We found no difference in the methylation status of TSDR after IL-7 stimulation (∆Ct value 7.4 ± 1.0 in the absence and 7.3 ± 1.0 in the presence of IL-7) (IV, Fig. 4C). These results do not support the role of IL-7 in regulating
FOXP3 demethylation in the thymus. However, we used nonsorted thymocytes which might conceal subtle changes in a small population. Unfortunately, the isolation of IL-7-treated CD25+ DP cells in sufficient numbers for analysis was not successful.

As IL-7 is known as a T cell survival factor, we also analyzed the frequency of apoptotic cells after culture with and without added IL-7. We used annexin V/PI staining for apoptosis detection. FOXP3 staining requires permeabilization and thus it cannot be used with this apoptosis detection kit. Instead, we used CD25 which is also connected with the regulatory phenotype and FOXP3 expression. Tuovinen et al. detected a very high expression of FOXP3 mRNA in DP CD25+ cells, almost two orders of magnitude higher than in DP thymocytes in general (Tuovinen et al., 2008b). Additionally, in the original publication III we could see that the level of FOXP3 mRNA in CD25+ cells was over 100-fold higher in CD25+ than CD25- populations, as measured in both DP and CD4 thymocytes (III, Fig. 4). Like the frequency of FOXP3+ DP cells, also the frequency of CD25+ DP cells increased when IL-7 was added, from 0.7% to 0.9% (p<0.03) (IV, Fig. 5). DP CD25+ cells were more susceptible to apoptosis than their CD25- counterparts (57.6% vs 18.3%, p<0.00002). IL-7 saved cells from apoptosis, in the DP CD25+ population the frequency decreased to 42.1% (p <0.03) and in the DP CD25- population to 11.5% (p < 0.03) (IV, Fig. 6). CD4 cells were less prone to apoptosis, with 10.2% apoptotic cells in the CD25+ population, and 11.8% in the CD25- population, and 3.2% and 6.4% with added IL-7, respectively (IV, Fig. 7G).

In conclusion, we could see an increased frequency of FOXP3+ cells in DP and SP populations with IL-7 stimulation which seemed to be because of decreased apoptosis and increased frequency of Tregs.
DISCUSSION

1 SELECTIONS SHAPING THE T CELL REPERTOIRE

The positive and negative selection are based on TCR-pMHC interactions. CDR3, which is the most variable region of T cell receptor molecules, is mostly responsible for the selective interactions. (Khailaie et al., 2014) The diversity of T cell repertoire is established in the thymus through random and imprecise recombination of TCR segments, after which the positive and negative selection eliminate 95% of these cells. The overall diversity of TCRs is difficult to define as one blood sample cannot present the whole TCR repertoire of estimated $10^{12}$ T cells and theoretically the possible $10^{15}$ different sequence combinations (Birnbaum et al., 2012, Qi et al., 2014). The repertoire is established in the first decades of life and persists via homeostatic proliferation of naïve T cells, though the richness modestly declines with age (Qi et al., 2014).

The qualitative and quantitative effect of thymic selections on the TCR repertoire is not known in detail. To analyze the effect of massive elimination of T cells under positive and negative selection, we studied the T cell receptor repertoire through Vβ CDR3 regions in thymocyte populations at different stages of development. This gave a picture of the TCR repertoire at each specific time point. The TCR repertoire was very diverse in sequences, even adjacent populations shared very few similar sequences. The overall diversity in spectratyping profiles, however, decreased, due to the elimination of sequences in the selections. The physicochemical profile of TCR remained similar when hydrophobicity, acidity, and molecular mass in positions around the central residues were examined. Also further quantitative analysis of the sequence pools that compared the mean CDR3β molecular weight, mean sequence length, and mean global charge differences at each CDR3 position using pepstats software did not reveal any progressive and constant modification patterns.

It was somewhat surprising that so few shared sequences were found. This, however, must reflect the huge diversity of the sequences, and also the fact that 90 to 95% of thymocytes die during development. The number of sequences was limited, which is affected by the sample size, flow cytometry sorting, PCR amplification, and the sequencing method. Each sample had originally 1.4 million cells, and the sequencing resulted in a maximum of 1167 sequences, though our examination of new appearing sequences showed that the sample was sequenced close to saturation. New deep and ultra-deep sequencing methods could help to find more overall and unique sequences.

In accordance with the observations of Robins et al. (Robins et al., 2009), our sequencing analysis could capture the CDR3 length repertoire at least as carefully as the spectratyping method, comparing the number of peaks from the CDR3 lengths of sequencing and the spectratyping.
Robins and colleagues found that the most frequent sequences were the closest to the germline. As there were so few shared sequences, we could not analyze at which step certain sequences were lost.

A caveat in the physico-chemical profiling method was the sensitivity. Because no consistent differences were found between the thymic populations, our sample size or the method may not be sensitive enough to reveal minor differences of the CDR3. However, when compared to the peripheral population, the most mature CD4 population showed most similarity, proving that the method could find differences.

2 ROLE OF AIRE IN THE THYMIC DEVELOPMENT

Developing thymocytes migrate to the medulla where they interact with mTECS, and they express a variety of tissue restricted antigens under the control of AIRE. In addition to TSAs, AIRE also controls the expression of miRNAs which affect the organization of thymic architecture and the posttranscriptional control of TSAs (Passos et al., 2015). Thus, the main suspected mechanisms behind the pathogenesis of APECED are the impaired expression of TSAs in the absence of AIRE and defects in the development of thymic epithelial cells. Because of its expression in the medulla, AIRE facilitates especially the negative selection (Liston et al., 2003). We found no difference in the CDR3 lengths between the APECED patients and healthy controls. The timing of this shortening coincided with positive and negative selection. Both the effects on TECs and TSA expression could affect the selections and could thus lead to defects in shortening of CDR3 in selections. The effect on thymic architecture could affect the development in many stages, not only in the negative selection.

The shortening of CDR3 length during thymic development has not been definitively connected with positive or negative selection, although the effect on positive selection is primarily suggested. Shorter CDR3 regions are thought to increase positive selection in mice, at least when they lack TdT (Gilfillan et al., 1994). It has been suggested that shorter CDR3 enables a more efficient interaction of TCR and pMHC (Yassai et al, 2002). Longer CDR3 could also increase affinity towards pMHC and would thus lead to negative selection (Yassai and Gorski, 2000).

There could also be some small population with a difference in the CDR3 length besides the ones that we analyzed. Albeit the CD8+ CD45RA+RO- population is abnormal in APECED patients, containing activated perforin+ effector/memory cells (Laakso et al., 2011), there was no significant difference in the CDR3 length between the patients and controls. The next population to look at could be the regulatory CD4+ FOXP3+ population, or CD8+ FOXP3+ cells. There is, however, conflicting evidence on the effects of AIRE on Treg selection. Aire-deficient mice have normal numbers of Tregs (Anderson et al., 2005), and
another mouse study of TCR repertoire suggests that Aire has only little effect on Treg selection (Daniely et al., 2010). On the contrary, another mouse study shows that Aire is required for Treg selection of two TCR transgenic lines specific for endogenous prostate-specific antigen (Malchow et al., 2013). Additionally, a recent study suggests that Aire enforces immune tolerance by directing autoreactive T cell specificities into the Foxp3+ lineage (Malchow et al., 2016). All these studies are from mice and it has been shown earlier that AIRE-deficiency in human and mice do not lead to exactly similar diseases.

The CDR3 shortening most likely is connected with the thymic selections. As no difference between patients and controls in CDR3 length was found, our results suggest that other mechanisms of AIRE function besides TSA expression in selections also affect the pathogenesis of APECED.

3 FACTORS INDUCING LINEAGE SELECTION

Regulatory T cells develop concomitantly with conventional T cells in the thymus and the development has many common features. It is not clear when the deviation of their developmental paths takes place, and what is the final differentiation factor. Substantial evidence prove the importance of FOXP3 in Treg development. Defects in FOXP3 result in autoimmune manifestations (Bennett et al., 2001) and its forced expression in conventional T cells is able to confer suppressive activity (Hori et al., 2003, Fontenot et al., 2003). However, many studies also show that FOXP3 per se does not indicate stable suppressive function (Allan et al., 2007, Miyara et al., 2009). In the thymic development, it seems to be expressed after the deviation of development into Treg lineage by other factors.

TCR is essential for the regulatory T cell development. The direct evidence of this phenomenon comes from mouse studies. Tregs do not develop in RAG -/- mice (Pachelczyk and Kern, 2008). Additionally, in vitro and in vivo experiments show that stimulation with the cognate antigen is needed to activate the suppressor function (Klein et al., 2003, Takahashi et al., 1998). In humans the direct role of TCR in Treg development is not possible to show, but many studies suggest this through other methods. Treg development in humans is associated with markers of positive selection, for example enhanced expression of CD69 and CD27 (Cupedo et al., 2005). Additionally, binding sites for TCR activated NFAT and AP1 are found in the human FOXP3 promoter and these factors are directly activated by TCR stimulation (Fig. 11.)(Mantel et al., 2006). A deficiency of ZAP70 in humans is associated with reduced numbers of thymic FOXP3+ Treg cells. Also reduced numbers of AIRE+ mTECS, impaired terminal differentiation of mTECS, and depletion of medullary DCs are seen in ZAP70 deficient patients (Poliani et al., 2013).
The repertoire of Tregs is suggested to be more self-reactive compared to that of conventional T cells. Mouse studies have shown that low-affinity TCR interactions do not lead to Treg development (Jordan et al., 2001). However, recent studies show that the TCR repertoires of Tregs and conventional T cells also overlap (Wojciech et al., 2014). TCR stimulation can induce FOXP3 expression but FOXP3 expression could also be stochastic and then may protect cells with high-affinity receptors from negative selection (Pacholczyk and Kern, 2008). FOXP3 has also been suggested to induce apoptosis that could be saved by cytokines (Tai et al., 2013). Some additional features in Tregs may resist negative selection such as increased CTLA-4 expression, which can also take place independent of FOXP3 (Walker, 2013).

There are also several additional pathways beside TCR signaling that affect the Treg differentiation and proliferation including JAK3/STAT5, Notch, CD80/86, ICOS/ISOSL, CD40/CD40L, TSLP, and the common γ chain cytokines including IL-2 and IL-15 (Martin-Gayo et al., 2010, Nazzal et al., 2014, Nunes-Cabaço et al., 2011, Watanabe et al., 2005). TSLP effect comes through DCs, Hassals corpuscles secrete TSLP which activates myeloid DCs to induce Tregs from CD4+ CD25- thymocytes (Watanabe et al., 2005). Plasmacytoid DCs in the human thymus are reported to promote CD4+CD25+FOXP3+ Treg development from DP CD69highTCRhigh thymocytes when activated with CD40L and IL-3. Positively selected DP thymocytes show enhanced expression of CD40L on TCR engagement and thus they provide the activation signal to plasmacytoid DCs (Martin-Gayo et al., 2010). MTECs have been shown to promote the survival and proliferation of Tregs in the thymus in an ICOSL-dependent manner. In this process CD4 cells are needed as a source of IL-2 (Nazzal et al., 2014). These mechanisms show that many thymic cells are needed to support T cell development. In addition to the already mentioned cell types, B lymphocytes and macrophages also have a role in T cell development since they secrete IL-15 (Caramalho et al., 2015b).

There are three suggested models for Treg development based on mouse experiments: a TCR-instructive model where Tregs are selected depending on their higher TCR-reactivity towards self-peptides, a two-phase model where the T cell receptor recognition is the first step and cytokines are responsible for the second step, and a stochastic model where the Treg fate is decided early during the development, as these cells are then more resistant to negative selection (Hsieh et al., 2012). The two-step model is favoured by findings that thymocytes that differentiate to regulatory T cells first express CD25 before FOXP3 expression through TCR stimulation takes place. This is followed by FOXP3 expression, which is induced after cytokine stimulation by IL-2 or IL-15 (Lio and Hsieh, 2008, Vang et al., 2008). At this stage TCR stimulation is not needed anymore for the induction of FOXP3 expression.
(Caramalho et al., 2015b). FOXP3 expression is associated with positive selection (Caramalho et al., 2015a).

Of the common γ chain cytokines, the effect of IL-2 and IL-15 on Treg development is better shown. Our results indicate that IL-7 has a role in the induction of FOXP3 expression, though IL-2 and IL-15 may be the primary stimulatory cytokines. A recent study shows that the role of IL-2 and IL-15 are independent of each other (Caramalho et al., 2015b). In addition of promoting the development of Treg phenotype, IL-7 also protects developing Tregs from apoptosis. In the article III we did not find a direct answer to the question of what explains the higher susceptibility of DP FOXP3+ cells to apoptosis compared to the CD4 FOXP3+ cells. According to our results, increased expression of BIM was not the explanation, neither was there an increased expression of Bcl-2 in CD4 cells. Both DP and CD4+CD25+ populations had increased expression of BIM and Bcl-2. A recent study in mice the role of Foxp3 in promoting apoptosis through the Puma pathway could offer one explanation (Tai et al., 2013). These authors found that Foxp3 is a potential pro-apoptotic transcription factor that is lethal to developing thymocytes unless they receive survival signals from the common γ-chain cytokines, which promote FOXP3 expression in developing thymocytes as well as they also promote cell survival. Tai et al. found that the Foxp3 transgene expression promoted the expression of the pro-apoptotic protein Puma and phosphorylation of Bim but no increased expression of Bim protein was seen. Thus, in our study, the deprivation of common γ-chain cytokines secreted by TECs could result in apoptosis of CD25+ cells that are mostly FOXP3+. Analysis of Bim phosphorylation is a possible future target of research method as we only measured Bim expression. A clear caveat in the article III was that apoptosis was not de facto measured in the CD25+/CD25- populations due to the limited number of colors in the flow cytometric device that we used. In the article IV, however, apoptosis in DP CD25+ and CD25- populations was measured, and as we could assume, DP CD25+ cells were more prone to apoptosis than their CD25- counterparts or CD4 CD25+ cells.

It has been observed in mice that Bcl-2 expression is downregulated in the DP population. Bcl-2 is highly expressed in the DN population, downregulated in the DP CD3low population, and again expressed after positive selection in the DP CD3high population, and this was not dependent on TCR stimulation (Gratiot-Deans et al., 1994). This observation resembles the timing of IL-7 effects reported from mice (Van De Wiele et al., 2004), and IL-7 is known to upregulate Bcl-2. In our study (III) we did not find a difference in Bcl-2 expression between DP CD25+ and CD4 CD25+ populations. However, DP FOXP3+ and thus also most of the CD25+ thymocytes seem to be already positively selected. Cytokines were mostly not available in our setting, and this
could also have an effect on our results. In our culture, the effect of IL-2 can not
be excluded as some T cells in the thymus can produce IL-2 (Malek, 2008).

Additionally, recent studies have revealed the significance of epigenetic
regulation of Treg-specific genes in the development of regulatory T cells. This
regulation is induced by TCR stimulation and takes place before FOXP3
expression (Morikawa and Sakaguchi, 2014). Also cytokines, at least IL-2, may
affect this regulation (Toker et al., 2013). In our study, we did not see increased
TSDR demethylation in the Foxp3 locus after IL-7 stimulation, but we only
studied bulk thymocytes.

4 TIMING OF COMMITMENT TO THE REGULATORY T CELL LINEAGE

In addition to the factors inducing the Treg lineage choice, also the timing of
commitment is somewhat unclear.

T cell progenitors are found in the human thymus during the 8th week
after gestation and mature T cells at 12th and 13th gestational weeks (Haynes
and Heinly, 1995). In humans already fetal Tregs express FOXP3, CTLA-4, and
GITR, and they are able to suppress T cell proliferation (Cupedo et al., 2005,
Darrasse-Jeze et al., 2005).

The first cells expressing FOXP3 in humans are found in the DN
population with negative surface staining of the TCR (Tuovinen et al., 2008a).
Another study, however, questioned this finding because they had found that
the majority of these cells are CD3/TCRαβlow (Battaglia et al., 2008). Nunes-
Cabaco et al. found FOXP3 mRNA in only few CD4+ CD8+ CD3- cells but more in
cells from the CD4 ISP population onwards, although at low levels in the CD4 ISP
and DP CD3- populations. Using immunostaining some CD1a+ thymocytes
expressing FOXP3 were found in the cortex, but the majority of FOXP3+ cells
were found in the corticomediulary junction and medulla (Nunes-Cabaco et al.,
2010). According to these results it seems that some cells might express FOXP3
without detectable surface TCR, although this FOXP3 expression may still be
TCR-dependent since productive rearrangement, TCRβ and TCRα mRNA
expression have been shown to take place before the DP stage (Nunes-Cabaco
et al., 2010). Cytokines could play a role in inducing FOXP3 expression at this
stage. Indeed, we could see a significant increase in the expression of FOXP3
after IL-7 stimulation in overnight culture in DN cells (results not shown).
Nevertheless, the contribution of these DN FOXP3-expressing cells to the
mature Treg population is unknown.

In the double-positive thymocyte population there are definitely
thymocytes expressing CD25 and FOXP3. Additionally, they express other
markers related to regulatory functions and they have a suppressive function
(Cupedo et al., 2005, Darrasse-Jeze et al., 2005, Nunes-Cabaço et al., 2011).
These cells express markers connected with some degree of immaturity, for
example RAG-2, but these cells also express markers of positive selection such as high levels of CD3 and CD27 (Caramalho et al., 2015a, Tuovinen et al., 2008b). It is controversial whether these DP FOXP3+ thymocytes function as precursors for CD4+ FOXP3+ thymocytes. Our results (III-IV) showed that DP CD25+ thymocytes are more prone to apoptosis than their CD4+ counterparts, indicating a precursor phenotype of DP CD25+ thymocytes. Also linear regression models indicate that DP FOXP3+ cells contribute to the mature CD4+ Treg population (Nunes-Cabaço et al., 2011). The expression of FOXP3 in the DP population was low, mostly below 1% of the DP thymocytes expressed FOXP3 in our studies. Despite low expression, more thymocytes may already be addressed to the regulatory lineage, they do not yet express FOXP3. In murine models the expression of Foxp3 is mostly seen in the CD4 population, not in the DP population (Lee and Hsieh, 2009). We could see that IL-7 had an effect on FOXP3+ DP thymocytes which showed a mature, already positively selected phenotype. It has been suggested that DP cells between β selection and positive selection are resistant to IL-7 effects (Van De Wiele et al., 2004). However, we could see differential CD127 expression also between different DP populations, the differences being small but significant (IV). DP FOXP3+ cells have also been shown to phosphorylate STAT5 in response to IL-7 stimulation (Nunes-Cabaço et al., 2011). Most of the effect may still be after positive selection as previously speculated.

CD4+ Tregs with efficient regulatory functions are the main population of FOXP3+ human thymocytes (Darrasse-Jeze et al., 2005). In our studies concerning CD4+ population in the thymus approximately 4-8% of cells express FOXP3 while in the periphery Tregs represent about 5 to 12% of the CD4+ cell populations (Darrasse-Jeze et al., 2005). It is controversial whether also peripheral CD4+ Tregs recirculate back to the thymus and thus contribute to the CD4+ Treg population.

Our results support the commitment also at the DP stage as those cells were more susceptible to apoptosis and were also prone to IL-7 effects.
Fig. 15. Development of human CD4 Tregs in the thymus. Modified from Caramalho et al. (Caramalho et al., 2015a). FOXP3 expression is seen already in the DN population, but their development to DP FOXP3 cells is not certain. TCR interactions (not shown here) and cytokines promote FOXP3 expression. The expression of CD25 usually precedes FOXP3 expression. The influence of the common γ-chain cytokines IL-2 and IL-15 on Treg development is known, our studies and some earlier studies additionally suggest that IL-7 has a role in Treg development in the DP stage. The development of Tregs may happen through cortical DP or medullary CD4 cells. The cell types involved are shown. cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; pDC, peripheral dendritic cell; mDC, medullary dendritic cell.
CONCLUDING REMARKS

This thesis concentrates on the development of T cells in the human thymus. Most published data come from mice and as the mouse data is not always applicable to humans, studies with human cells are needed. Of the T cells, the regulatory population and the differences in their development compared to conventional T cells were studied in more detail.

Our results show that the T cell repertoire in the human thymus is very diverse at the sequence level but the basic structure of the T cell receptor remains the same when physico-chemical features are considered. We could see a reduction in the mean CDR3 length, mostly between the DP CD3\textsubscript{low} and DP CD3\textsubscript{high} population, indicating mostly the effect of positive selection. However, as some shortening was seen in the stage from DP\textsubscript{high} cells to CD4 cells, also the negative selection is possible. For the function of the T cell receptor, the similar structure is to some point needed, and thus this can be a somewhat expected result. On the other hand, it is surprising that so few shared sequences were found, but this reflects the huge diversity of the human T cell repertoire.

**AIRE** – the autoimmune regulator – is a gene that is defective in an autoimmune disease called APECED. AIRE regulates the presentation of tissue antigens to developing thymocytes, and thus regulates the thymic selection. As we could see a shortening of CDR3 length at the point of thymic selection, we compared the CDR3 lengths of APECED patients to healthy controls. Our results did not show any significant difference in the length of CDR3 between these groups. Also AIRE-independent peptides influence the development of thymocytes and as earlier speculated additional mechanisms must be behind the actions of AIRE.

The point where the developmental path of regulatory T cells deviates from that of conventional T cells is not clear. FOXP3 expression can be seen in some DN thymocytes but its expression occurs in the DP population and in our studies, in approximately 4-8% of the CD4 thymocytes. In the DP population FOXP3 expression is seen in less than 1% of the cells, and it is controversial whether these cells are the precursors of CD4 FOXP3\textsuperscript{+} regulatory T cells or whether they represent an independent population. Human studies mostly favor the former possibility. We saw that DP CD25\textsuperscript{+} cells were more prone to apoptosis in overnight culture, strengthening the view that they are phenotypically precursor cells.

Cytokines are an important factor in the thymic development in addition to TCR interactions. We could see that IL-7, a cytokine to which mature Tregs are not very responsive due to their low expression of IL-7 receptor \(\alpha\)-chain, stimulated the development of FOXP3\textsuperscript{+} DP and CD4 thymocytes. As a similar effect was seen already in the DP population, this strengthens the view that they are developing into CD4 Tregs.
The thymic development of T cells creates the basis of regulation of adaptive immunity. As new insights of regulatory T cell manipulation are sought also for therapeutic use, it is vital to understand the details of T cell development in humans.
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