TCR diversity and the development of regulatory T cells in the human thymus

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ACADEMIC DISSERTATION

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Contents

ABSTRACT

A diverse T cell receptor (TCR) repertoire is essential for the adaptive immune system. T cells have to recognise and react to a huge variety of potential pathogens and at the same time maintain tolerance to self-antigens. TCR repertoire is established in the thymus during T cell development. Previous estimates of TCR diversity have studied the mature repertoire in the peripheral blood, identifying 1-3 x 10^6 unique TCRβ and 0.5 x 10^6 TCRα sequences, but the human intrathymic diversity has not been previously measured. In this thesis I provide the first estimate of the total TCR diversity in the human thymus. We have used high-throughput TCR sequencing and detected up to 10.3 x 10^6 unique TCRβ sequences and 3.7 x 10^6 TCRα sequences, which sets a new lower limit for TCR diversity. Computational estimators preseq, DivE and Chao2 produced 40 to 70 x 10^6 unique TCRβ sequences and 60 to 100 x 10^6 TCRα sequences in the human thymus.

Somatic recombination produces a diverse thymic TCR repertoire that includes also the thymocytes instructed to develop into natural regulatory T cells. They are characterised by stable expression of the forkhead/winged-helix transcription factor FOXP3 and demethylation of Treg-specific-demethylated region (TSDR). Regulatory T cells develop as a separate cell lineage in the thymus and commitment occurs already at the CD4+CD8+ double positive (DP) stage. TCR-mediated signalling instructs the selection to the regulatory lineage and cytokines promote Treg development. We have used high-throughput TCR sequencing and methylation analysis of TSDR to track the timing of Treg commitment. Comparing the common TCR sequences showed that regulatory DP CD25+ and CD4SP CD25+ populations were clearly closer to each other than any other population, indicating that they are part of the common clonal and developmental pathway. The methylation analysis of TSDR in the DP and CD4+ single positive (SP) regulatory populations showed no difference between them. Thus a stable Treg phenotype can already be detected at the double positive stage.

In this thesis I have studied the role of common γ chain cytokines in regulatory T cell development in the human thymus. Since thymic commitment of human Tregs begins at the double positive stage I have studied the effects and mechanisms of interleukin-2, interleukin-7 and interleukin-15 at the

DP phase. Previous studies in murine models have established a two-step model. According to these studies, TCR signalling upregulates interleukin-2 receptor (IL-2R) and increases the responsiveness of developing Tregs to common γ chain cytokines that induce the expression of FOXP3. IL-7 has been considered to have a minor role in Treg development since mature peripheral Tregs express low levels of interleukin-7 receptor (IL-7R). We show that during thymic development DP FOXP3+ thymocytes upregulate IL-7R and are responsive to IL-7. At the DP stage TCR-signalling dictates Treg maturation during positive selection and IL-15 enhances Treg survival. After positive selection IL-2 and IL-7 increase the expression of FOXP3 and anti-apoptotic protein BCL-2 (B cell lymphoma 2) and promote Treg phenotype. DP FOXP3+ cells are highly susceptible to apoptosis but all three cytokines enhance their survival. Suppressing apoptosis is a key mechanism in promoting Treg development at the DP and CD4SP stage in the human thymus.

TIIVISTELMÄ

Laaja ja monimuotoinen T-solureseptorien repertuaari on välttämätön osa adaptiivista immuunipuolustusta. T-solujen täytyy tunnistaa valtava kirjo erilaisia vieraita patogeeneja, mutta samaan aikaan ylläpitää toleranssia ja jättää omat kudokset rauhaan. T-solujen repertuaari syntyy kateenkorvassa. Aiemmat arviot T-solureseptorien monimuotoisuudesta perustuvat kypsiin T-soluihin ihmisen verenkierrossa. Näissä on tunnistettu 1-3 x 10^6 uniikkia T-solureseptorin beeta-ketjun sekvenssiä ja 0.5 x 10^6 alfa-ketjun sekvenssiä. Ihmisen kateenkorvan T-solujen repertuaarin laajuutta ei ole aiemmin tutkittu. Tässä väitöskirjatyössä on ensimmäisen kerran mitattu ja arvioitu ihmisen kateenkorvan T-solureseptorien diversiteettia. Sekvensoimme 10.3 x 10^6 uniikkia T-solureseptorin beeta-ketjua ja 3.7 x 10^6 alfa-ketjua. Nämä tulokset asettavat uuden alarajan reseptorien laajuudelle ja monimuotoisuudelle ihmisessä. Käytimme lisäksi matemaattisia estimaattoreita preseq, DivE ja Chao2, jotka arvioivat koko kateenkorvan diversiteetiksi 40-70 x 10^6 uniikkia beeta-ketjua ja 60-100 x 10^6 uniikkia alfa-ketjua.

Kateenkorvan somaattisen rekombinaation koneisto tuottaa laajan T-solureseptorirepertuaarin, joka sisältää myös luonnollisiksi säätelijä-T-soluiksi kehittyvät tymosyytit. Luonnolliset säätelijä-T-solut ilmentävät transkriptiotekijä FOXP3:a ja niiden geeneissä TSDR-alue on pysyvästi demetyloitunut eli siitä puuttuvat geenin hiljentävät epigeneettiset merkit. Säätelijä-T-solut kehittyvät omana solulinjanaan kateenkorvassa ja valikoituvat kehitykseen jo CD4+CD8+ tuplapositiivisessa vaiheessa. T-solureseptorin kautta välittyvät signaalit ohjaavat solujen valikoitumista säätelijä-linjalle ja sytokiinit edistävät myös säätelijäsolujen kehitystä. Tässä työssä olemme käyttäneet T-solureseptorien repertuaarin sekvensointia sekä TSDR-alueen metylaatioanalyysia selvittääksemme säätelijä-T-solulinjalle valikoitumisen ajoitusta. Vertailimme kateenkorvassa CD25+ populaatioiden T-solurepertuaaria CD4+CD8+ tuplapositiivisessa sekä CD4-positiivisessa kehitysvaiheessa. Yhteisiä sekvenssejä vertaamalla totesimme, että nämä molemmat CD25+ ryhmät olivat repertuaariltaan selvästi lähimpinä toisiaan. Tämä osoittaa niiden olevan osa samaa säätelijä-T-solujen kehityskaarta. Populaatioissa ei ollut myöskään mitään eroa metylaatiostatuksessa. Vakaa säätelijä-T-solun fenotyyppi voidaan havaita jo tuplapositiivisessa kehitysvaiheessa.

Olen tässä väitöskirjatyössä tutkinut sytokiinien roolia säätelijä-T-solujen kehityksessä ihmisen kateenkorvassa. Nämä solut valikoituvat kehityslinjalleen jo tuplapositiivisessa vaiheessa, joten olen keskittynyt interleukiini-2:n, interleukiini-7:n ja interleukiini-15:n vaikutuksiin ja mekanismeihin tässä kehitysvaiheessa. Aiemmat tutkimukset hiirimalleissa ovat luoneet perustan kahden-askeleen-mallille: T-solureseptorin kautta välittyvä signalointi lisää CD25-molekyylin määrää solujen pinnalla ja näin herkistää niitä sytokiinien vaikutukselle. Sytokiinit käynnistävät FOXP3:n ilmentymisen. Interleukiini-7:lla on aiemmin oletettu olevan vain pieni rooli säätelijä-T-solujen kehityksessä, sillä verenkierron kypsät solut eivät ilmennä sen reseptoria pinnallaan. Tässä tutkimuksessa osoitamme, että kehityksen aikana tuplapositiivisessa vaiheessa FOXP3+ tymosyyteissa reseptorin ilmentyminen solujen pinnalla lisääntyy ja näin solut pystyvät reagoimaan sytokiinin IL-7 signalointiin. Tuplapositiivisessa kehitysvaiheessa T-solureseptorin kautta välittyvät signaalit ohjaavat säätelijä-T-solujen valikoitumista positiivisessa selektiossa, ja IL-15 edistää solujen selviytymistä. Positiivisen selektion jälkeen IL-2 ja IL-7 lisäävät FOXP3:n ja anti-apoptoottisen BCL-2:n ilmentymistä sekä edistävät säätelijäsolun fenotyyppiä. Tuplapositiiviset FOXP3-solut ovat hyvin alttiita solukuolemalle, mutta kaikki kolme sytokiinia edistävät niiden selviytymistä. Ohjelmoidun solukuoleman ehkäiseminen onkin tärkein mekanismi, jolla sytokiinit edistävät säätelijä-T-solujen kehitystä ihmisen kateenkorvassa.

ABBREVIATIONS

AIRE	autoimmune regulator gene/protein (human)
AP-1	activator protein 1
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
BCL	B cell lymphoma
CCL	chemokine (C-C motif) ligand
CCR7	C-C chemokine receptor 7
CD	cluster of differentiation
CDR	complementarity determining region
CMJ	corticomedullary junction
CNS	conserved non-coding sequence
CXCR5	C-X-C chemokine receptor type 5
cTEC	cortical thymic epithelial cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DGE	digital expression matrices
DN	double-negative, CD4- CD8-
DP	double-positive CD4+ CD8+
ETP	early thymic progenitor
FOXP3	forkhead box P3, gene/protein (human)
Foxp3	forkhead box P3, gene/protein (mouse)
GITR	glucocorticoid-induced tumor necrosis factor receptor
GFP	green fluorescent protein
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
ICOS	inducible T-cell costimulatory
ICOS-L	inducible T-cell costimulatory ligand
IDO	indoleamine 2,3-dioxygenase
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ISP	immature single positive
ITAM	immunoreceptor tyrosine-based activation motif

iTreg	induced regulatory T cell
JAK	Janus tyrosine kinase
LAG-3	lymphocyte activation gene 3 protein
Lck	lymphocyte-specific protein tyrosine kinase
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cell
mTOR	mechanistic target of rapamycin
N-nucleotide	nontemplated nucleotide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NGS	next generation sequencing
NKT	natural killer T cell
Nrp	neuropilin
nTreg	natural regulatory T cell
P-nucleotide	palindromic nucleotide
pDC	plasmacytoid dendritic cell
PD-1	programmed death 1 receptor
PI3K	phosphatidylinositol 3-kinase
pMHC	complex of peptide and MHC
RAG1	recombination activating gene 1
RTE	recent thymic emigrant
S1P1	sphingosine 1-phosphate receptor 1
SOCS1	suppressor of cytokine signalling 1
SP	single-positive
STAT	signal-transducing activator of transcription
SCID-X1	severe combined immunodeficiency X-linked
Tconv	conventional T cell
TCR	T cell receptor
T-bet	T-box transcription factor
TdT	terminal deoxynucleotidyl transferase
Tet	ten-eleven-translocation
TGF-β	transforming growth factor β
Tfh	T follicular helper T cell
Th	T helper cell
TNC	thymic nurse cell

TREC	TCR excision circles
Treg	regulatory T cell
TRA	tissue-restricted antigen
TSA	tissue-specific antigen
TSDR	Treg-cell specific demethylated region
TSLP	thymic stromal lymphopoietin
TSP	thymus seeding progenitor
UMI	unique molecular identifier
VDJ	variable-diversity-joining
ZAP-70	zeta-chain associated protein kinase 70

ORIGINAL PUBLICATIONS

I Vanhanen R, Heikkilä N, Aggarwal K, Hamm D, Tarkkila H, Pätilä T, Jokiranta TS, Saramäki J, Arstila TP. T cell receptor diversity in the human thymus. *Mol Immunol*. 2016 Aug; 76:116-22.

II Vanhanen R, Leskinen K, Mattila I, Saavalainen P, Arstila TP. Human regulatory T cell commitment occurs at the CD4+CD8+ thymocyte stage. *Submitted.*

III Tuulasvaara A, Vanhanen R, Baldauf HM, Puntila J, Arstila TP. Interleukin-7 promotes human regulatory T cell development at the CD4+CD8+ double-positive thymocyte stage. *J Leukoc Biol*. 2016 Sep; 100:491-8.

IV Vanhanen R, Tuulasvaara A, Mattila J, Pätilä T, Arstila TP. Common gamma chain cytokines promote regulatory T cell development and survival at the CD4+ CD8+ stage in the human thymus. *Scand J Immunol*. 2018 Jun 15:e12681.

Publication III is also part of the Thesis of Anni Tuulasvaara (T Cell Development in the Human Thymus, University of Helsinki 2017).

INTRODUCTION

The human T cell repertoire is established in the thymus. It has to both defend the body against potential pathogens and maintain tolerance to self-antigens. The genetic recombination machinery has to generate a diverse peripheral T cell population that acts efficiently as an essential part of the adaptive immune response but does not lead to harmful autoimmune reactions. The education of thymocytes includes positive and negative selection in the thymus. Positive selection ensures that developing thymocytes are functional and negative selection eliminates autoreactive T cells.

Huge T cell receptor diversity is generated by somatic VDJ recombination. The specificity of a TCR is primarily determined by a hypervariable area called the third complementarity-determining region (CDR3) in the α and β chains. In the VDJ recombination the non-contiguous Vβ, Dβ and Jβ gene segments are rearranged in the β chain locus and Vα and Jα gene segments in the α chain locus. Theoretically possible TCRβ diversity has been calculated to be $5x10^{11}$ but the actual diversity of an individual remains unknown since it cannot be directly measured. Previous estimates of peripheral TCR diversity have been based on sequencing T cell compartments in blood samples.

Regulatory T cells are a suppressive T cell population responsible for peripheral tolerance. They were first identified in 1995 as a CD25 expressing population with suppressive capabilities. In 2001 they were reported to be characterised by the transcription factor FOXP3 that regulates Treg development and function in the periphery. Tregs can differentiate from peripheral conventional T cells but the majority of the regulatory population are natural Tregs that derive from the thymus. Their developmental pathway has been extensively studied in murine models whereas in humans further research is needed. Tregs develop as a separate cell lineage in the thymus. TCR-mediated signalling instructs the selection to the Treg lineage and cytokines and other cofactors have been recognised as being essential for Treg maturation.

In this thesis I have studied TCR diversity, regulatory T cell repertoire and Treg development in the human thymus. The primary TCR repertoire

generated in the thymus has not been previously measured although estimates of the peripheral diversity have been reported. Intrathymic TCR diversity gives insight into the mechanisms that generate a diverse functional TCR repertoire that can recognise the universe of pathogens and respond to the threat of them. Regulatory T cells are an indispensable part of tolerance in the human immune system. I have studied the timing of their commitment and the cytokines instructing their development in human thymic samples.

REVIEW OF THE LITERATURE

1. T CELL RECEPTOR

1.1 T lymphocytes

T cells are an essential part of the adaptive immune system and their development takes place in the thymus. B lymphocytes derive from the bone marrow and they are important effectors as part of humoral immunity. T cells are distinguished by the expression of a T cell receptor that in most cells consists of α and β chains. A 5% minority of T cells are γδ T cells. TCR is an αβ heterodimer that recognises peptide antigens presented in the major histocompatibility complex molecules (MHC).

Peripheral naive T cells are activated by antigen-presenting cells (APCs) when TCR recognises the target peptide presented on the MHC. The co-receptor expression of the TCR divides T cells into CD4 and CD8 populations. CD4 helper T cells provide activation signals to other cells and cytotoxic CD8 T cells kill cells infected by viruses. CD4 helper T cells recognise antigen peptides bound to MHC II molecules and cytotoxic CD8 T cells peptides bound to MHC I molecules. Regulatory T cells prevent autoimmunity reactions by suppressing other lymphocytes.

1.1.1 CD4 lymphocytes

CD4 T helper cells (Th cells) are an essential part of the adaptive immune system and form the majority of peripheral T cells. They activate other immune cells by providing cytokines, regulating immune responses, promoting B cell maturation and antibody secretion, enhancing CD8 T cell function and supporting macrophages. They express CD40L that binds to CD40 expressed by antigen-presenting dendritic cells, B cells, monocytes and macrophages. CD4 helper T cells recognise antigen peptides bound to MHC II molecules on the antigen presenting cells (APCs) leading to T cell activation in the lymph nodes. The second signal is provided by the interaction of co-stimulatory molecules CD28 and CD80/CD86 between the helper T cell and APC. This is required to prevent harmful autoimmune reactions. Following activation Th cells proliferate and secrete cytokines, particularly IL-2. The cytokine environment instructs Th cells to

differentiate into multiple T cell subpopulations. IL-12 stimulation drives Th1 and IL-4 Th2 maturation. Together IL-4 and transforming growth factor β (TGF-β) drive the differentiation of Th9 and IL-6 while TGF-β drives the differentiation of Th17 cells (Raphael, Nalawade et al. 2015).

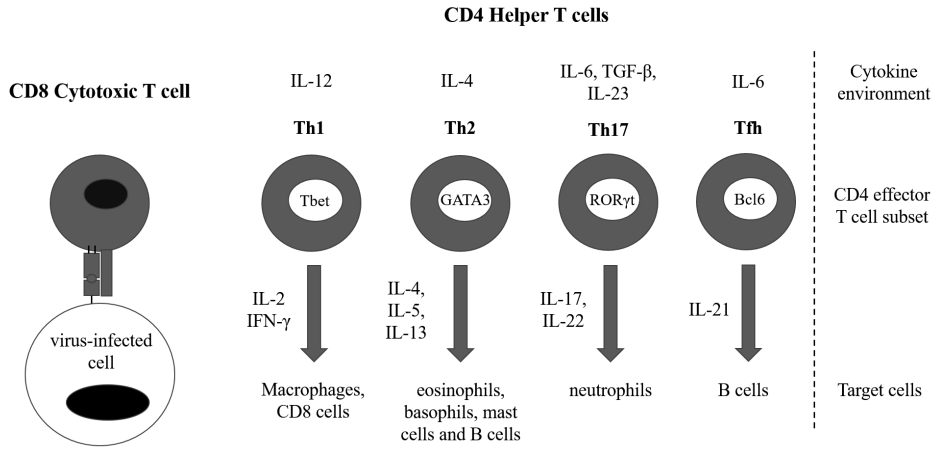
Th1 cells regulate immune responses against viruses and intracellular pathogens. IL-12 is the main triggering cytokine of Th1 cells that activate macrophages, CD8 T cells and B cells. Th1 cells drive macrophages to destroy intracellular bacteria by secreting IFN- γ and promoting the proliferation of CD8 cytotoxic T cells.

Th2 immune response is required against extracellular parasites. Th2 cells activate eosinophils, basophils, mast cells and B cells and produce IL-4, IL-5 and IL-13. Th2 response is triggered by IL-4 and plays a role in preventing the harmful allergy reactions. Th17 cells are a distinct cell lineage and are defined by their production of IL-17. They play an important role in mucosal barriers and they stimulate neutrophils to destroy extracellular pathogens.

Follicular helper T cells (Tfh cells) are a specialised subset of T helper cells that help B cells to produce antibodies (Crotty 2014). They are found in the lymph nodes, spleen and tonsils and they have an essential role in promoting germinal center formation and affinity maturation. Tfh cells are characterised by stable expression of transcription factor Bcl-6 (B cell lymphoma 6) and the cell surface markers CXCR5 (C-C chemokine receptor 5), PD-1 (programmed death 1 receptor) and ICOS (inducible T-cell costimulatory). ICOS-L and IL-6 promote commitment to the Tfh lineage.

Regulatory T cells are characterised by the expression of the forkhead/ winged-helix transcription factor FOXP3 and they maintain peripheral tolerance by suppressing other lymphocytes and harmful immune responses.

Figure 1. Cytotoxic CD8 T cell and the subsets of CD4 effector T cells. CD4 helper T cells differentiate into distinct subsets in response to the cytokine environment. The characteristic transcription factors are shown inside the cells. Activated helper T cells produce cytokines and enhance the functions of other cells.



1.1.2 CD8 lymphocytes

CD8 T cells recognise antigens produced by cancer cells and intracellular viruses. Recognition of an antigen on the MHC I causes CD8 lymphocyte to kill the infected or malignant cell. They use death receptor Fas (CD95) - Fas ligand (CD95L) interaction, perforin and granzymes to induce apoptosis. Perforin creates pores on the infected target cells and allows the granzymes A and B to enter the cell. Similarly to CD4 helper T cells, naive CD8 cells require activation by specialised antigen-presenting cells before their differentiation into cytotoxic CD8 cells. Successful CD8 T cell activation also requires a preceding interaction between the APC and the activated CD4 helper T cell. CD8 T cell proliferation is enhanced by IL-2, produced by CD4 helper T cells.

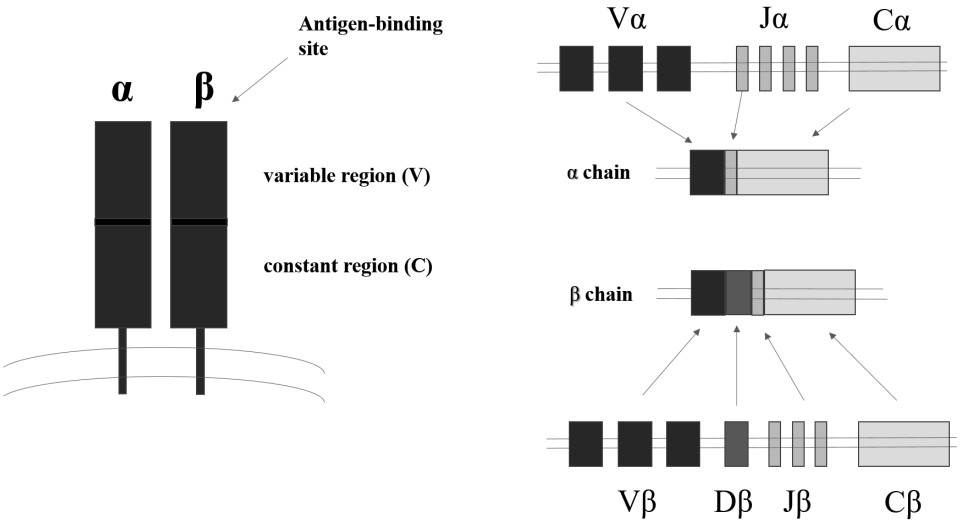
Regulatory FOXP3+ CD8 T cells have also been detected in the peripheral blood but their specific involvement remains unclear. They also express other Treg-associated markers CD25, GITR (glucocorticoid-induced tumor necrosis factor receptor) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and are identified by the expression of CD103 (Cosmi, Liotta et al. 2003) (Uss, Rowshani et al. 2006). They can suppress activated T cells via cell-to-cell contact and produce IL-10 (Smith and Kumar 2008).

1.2 T cell receptor structure

T cell receptors expressed by αβ T cells are heterodimeric proteins that consist of two polypeptide chains, α and β, that both contain one variable (V) and one constant (C) domain. The chains are linked by a disulphide bond. α and β chains each have three hypervariable complementarity-determining regions (CDR1, CDR2 and CDR3) of which the third region is the most polymorphic and takes the main role in recognising the peptide. Hypervariable CDR3 is generated by somatic recombination machinery that rearranges the non-contiguous Vβ, Dβ and Jβ gene segments in the β chain locus and Vα and Jα gene segments in the α chain locus. P- (palindromic) and N- (nontemplated) nucleotides are added and deleted at the Vβ-Dβ, Dβ-Jβ and Vα-Jα junction sites.

CDR1 and CDR2 derive from germ-line sequences and they bind mainly to the major histocompatibility complex (MHC) molecule whereas hypervariable CDR3 recognises the displayed peptide. However, all three CDRs can contact both peptide and MHC. The V domain in the α chain contacts the amino-terminal half of the displayed peptide while the Vβ domain contacts the carboxy-terminal half of the peptide (Hennecke and Wiley 2001). X-ray crystallography has shown the three-dimensional structure of TCR. It has a relatively flat ligand-binding surface for pMHC interaction (Wang and Reinherz 2012).

Figure 2. T cell receptor. T cell receptors consist of two polypeptide chains, α and β, that both contain one variable (V) and one constant (C) domain. α and β chain genes are composed of non-contiguous segments that are rearranged by somatic recombination machinery during T cell development.



1.3 Co-receptors

The formation of the immunological synapse creates the interface between a TCR and the presenting MHC molecule on the surface of the target cell and the formation requires coreceptors CD3, CD4 or CD8 and other costimulatory molecules. TCRs are associated on the cell surface with four CD3-subunits (gamma, delta, epsilon, zeta) that form the CD3 protein which has an essential role in signal transduction. Each of them consists of an extracellular Ig-like domain, a short stalk region connecting peptide, a transmembrane helix and a cytoplasmic tail. TCR-peptide-MHC interaction induces a cascade of downstream signalling events via the ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tail of the CD3 (Wang and Reinherz 2012).

The co-receptors CD4 and CD8 are essential in TCR-pMHC-interaction since they contact the MHC, stabilise this interaction and increase its duration (Das, Sheridan et al. 2001). CD4 is composed of four immunoglobulin-like

domains and it binds MHC II molecule with the first domain. TCR-pMHC interaction initiates downstream signalling events when CD4 delivers the tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase) into close proximity with the complex and allows the phosphorylation of ITAMs and the recruitment of Zap-70 (Wang and Reinherz 2012).

CD8A and CD8B genes encode the chains of the CD8 co-receptor. Each chain contains a single immunoglobulin-like domain that is linked to a glycosylated polypeptide chain. The isoform CD8ab is the most common but CD8a chains can also form a homodimer. CD8 binds the α3 domain of the MHC I molecule and also recruits Lck with the cytoplasmic tail to bring it into close proximity with the TCR-complex leading to downstream signalling events (Wang and Reinherz 2012).

1.4 Antigen recognition and MHC interaction

T cells recognise foreign antigens through presentation of antigen peptides by MHC molecules on the cell surface. There are more than 200 MHC genes located in chromosome 6 in humans. MHC class I and II proteins in humans are called human leukocyte antigens (HLA) and many autoimmune diseases are associated with a certain HLA allele. The HLA genes are HLA-A, -B and -C in MHC I and HLA-DP, -DQ and -DR in MHC II. One α-β superdomain and two immunoglobulin-like domains form a heterodimer in the MHC molecule. The superdomain binds the peptide and is composed of one heavy chain in the MHC I and two heavy chains in MHC II (Rudolph, Stanfield et al. 2006).

MHC I displays peptides primarily derived from intracellular degradation of proteins in the cytosol and is expressed by all the nucleated cells. The pathogen antigen presented in the MHC I molecule indicates intracellular infection and instructs cytotoxic CD8 T cell to kill the cell. Dendritic cells, macrophages, thymic epithelial cells (TECs) and B cells express MHC II. Specialised antigen-presenting cells are dendritic cells that display antigens to naive T cells that activate the immune response. Dendritic cells (DCs) process antigens collected from tissues and convey them to the peripheral lymphoid organs. Presentation of antigens to naive T cells leads to T cell activation and immune response. Three signals are essential for T cell activation: TCR-pMHC-interaction, a co-stimulatory signal from the interaction between CD28 and CD80/CD86 and cytokines. T cell

activation is carefully regulated since unnecessary activation can lead to autoimmune reaction. Antigen presentation, the expression of costimulatory molecules and the availability of cytokines set limits for the activation. Cross-presentation allows dendritic cells to capture, process and present exogenous antigens on the MHC I molecule. This is essential for activating viral- and tumor-specific CD8 T cell responses (Fehres, Unger et al. 2014).

2. REGULATORY T CELLS

2.1 Natural Regulatory T cells

Regulatory T cells have an essential role in regulating immune responses, maintaining immunological tolerance and preventing autoimmunity. They are divided into two groups: natural regulatory T cells (nTregs) that derive from the thymus and induced regulatory T cells (iTregs) that differentiate from conventional CD4 T cells in the periphery. Conventional CD4 T cells can transiently acquire the low expression of FOXP3 upon antigen stimulation and activation but the phenotype is not stable (Chen, Jin et al. 2003) (Walker, Kasprowicz et al. 2003, Gavin, Torgerson et al. 2006, Allan, Crome et al. 2007, Nagar, Vernitsky et al. 2008).

Regulatory T cells are characterised by the expression of the forkhead/ winged-helix transcription factor FOXP3 that regulates Treg development and function. Mutations in FOXP3 lead to early and severe immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) in humans (Bennett, Christie et al. 2001). Defects in suppressive Tregs can cause numerous other autoimmune diseases in humans and regulatory T cells are also involved in infection and cancer (Belkaid and Rouse 2005, Curiel 2007, Costantino, Baecher-Allan et al. 2008). In the future Treg-based immune therapies have huge potential for the treatment of immune-mediated diseases.

The majority of regulatory T cells present in the periphery are natural Tregs originating from the thymus (Hsieh, Zheng et al. 2006, Wong, Mathis et al. 2007). In mice neonatal thymectomy after birth prevented the emergence of natural Tregs (Asano, Toda et al. 1996). In 1995 Sakaguchi et al. reported the existence of a CD4 T cell population that expressed IL-2 receptor α

chain (CD25) and had a suppressor function (Sakaguchi, Sakaguchi et al. 1995). Human CD4+ $CD25_{high}$ Tregs were first identified and reported in 2001 (Baecher-Allan, Brown et al. 2001). The expression of Foxp3 as a regulatory T cell marker was reported in 2003 (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003).

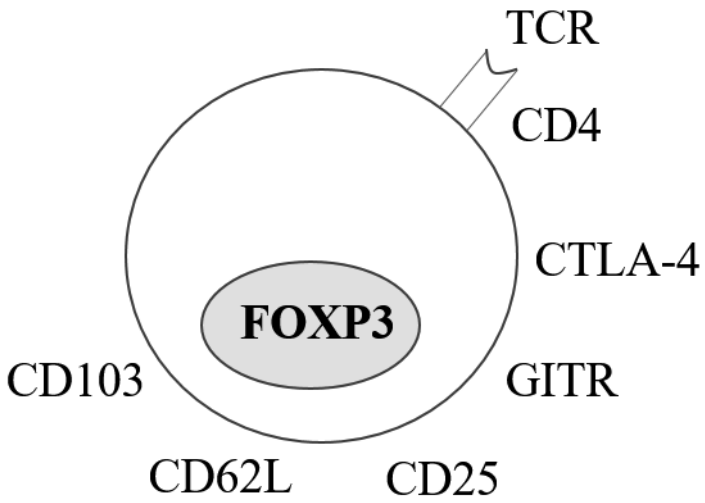
The constitutive expression of FOXP3 is the main feature of natural Tregs but the high expression of IL-2 receptor α chain (CD25) is also an important marker and useful in the purification of Tregs. Tregs require IL-2 from other cellular sources since they do not produce it themselves. However, CD25 is also expressed by antigen-activated non-regulatory CD4 T cells in peripheral blood and only 1-2% of highest expressing cells are Tregs (Baecher-Allan, Brown et al. 2001, Sakaguchi, Miyara et al. 2010). Deficiency of CD25 leads to autoimmunity and immunodeficiency in humans (Caudy, Reddy et al. 2007). Patients with CD25 deficiency have normal count of FOXP3+ cells but the Treg suppressive function is impaired.

Low expression of the α chain of the IL-7 receptor (CD127) is characteristic of Tregs, although conventional T cells can downregulate the expression of CD127 upon activation (Liu, Putnam et al. 2006, Seddiki, Santner-Nanan et al. 2006). A homing marker CD62L, CD103, GITR and CTLA-4 are also associated in regulatory T cells (Seddiki, Santner-Nanan et al. 2006, Sakaguchi, Miyara et al. 2010). An inhibitory receptor CTLA-4 is essential for Treg suppressor function but it can also be expressed by activated CD4 and CD8 T cells (Jago, Yates et al. 2004). The deficiency of CTLA-4 causes severe immune dysregulation and IPEX-like phenotype (Kuehn, Ouyang et al. 2014, Schubert, Bode et al. 2014).

Tregs have been studied extensively in murine models but there are significant differences between human and murine Tregs and their developmental pathways. Human Tregs can express alternatively spliced FOXP3 isoforms with distinct functions (Du, Huang et al. 2008) and they can be divided into functionally distinct subsets (Ito, Hanabuchi et al. 2008, Miyara, Yoshioka et al. 2009). $CD25_{high}$ $CD127_{low}$ CD45RO+ FOXP3+ Tregs have the strongest suppressive capacity and antigen stimulation leads to their apoptosis. CD25+ $CD127_{low}$ CD45RA+ $FOXP3_{low}$ CD31- Tregs have lower expression of CD25 and FOXP3, weaker suppressive capacity and stronger resistance to apoptosis upon TCR stimulation. They are also highly proliferative and express CD31, which is a marker of recent thymic

emigrants. It has been suggested that these cells are the predominant subset of Tregs that derive from the thymus, emigrate, proliferate and convert into effector Tregs (Cvetanovich and Hafler 2010). The human Treg population exhibits more heterogeneity than in mice. Human terminally differentiated $CD25_{high}$ $CD127_{low}$ CD45RO+ $FOXP3_{high}$ Treg population is identified by the expression of HLA-DR. They have significantly higher expression of FOXP3 than HLA DR- effector Tregs, use contact-dependent suppression mechanisms and do not produce IL-10 (Baecher-Allan, Wolf et al. 2006, Costantino, Baecher-Allan et al. 2008).

Figure 3. Treg-associated markers.



2.2 FOXP3

FOXP3 (Foxp3 in mice) is a key distinguishing marker of regulatory T cells in humans and is essential for Treg suppressor function, proliferation and differentiation (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Khattri, Cox et al. 2003, Yagi, Nomura et al. 2004, Fontenot, Dooley et al. 2005, Fontenot, Rasmussen et al. 2005, Gavin, Rasmussen et al. 2007). A loss-of-function mutation in the FOXP3 gene leads to absence of Tregs and IPEX syndrome in humans and deletion of the Foxp3 gene leads to scurfy phenotype in mice (Chatila, Blaeser et al. 2000, Bennett and Ochs 2001,

Wildin, Ramsdell et al. 2001, Gambineri, Torgerson et al. 2003). Nowadays there are over 20 known FOXP3 mutations causing IPEX syndrome (Myers, Perroni et al. 2006). In human peripheral blood, FOXP3 expression is strong in the CD4+ $CD25_{high}$ population, while low in activated CD4 T cells (Wang, Ioan-Facsinay et al. 2007). The CD8 T cell subset can also upregulate FOXP3 expression. FOXP3 is a regulator that prevents Tregs from deviating into other T cell lineages. Loss of it leads to acquisition of effector T cell properties including the cytokine production of IL-2, IL-4, IL-17 and IFNγ (Wan and Flavell 2007, Williams and Rudensky 2007). Forced expression of FOXP3 allows both regulatory and conventional T cells to acquire a suppressive function (Aarts-Riemens, Emmelot et al. 2008).

FOXP3 can regulate 700-1400 target genes, acting as a transcriptional activator and repressor in developing and established regulatory T cells. The target genes are essential for a functional phenotype including *Ctla4, Il2ra, Nrp1* and *Tnfrsf18* (GITR). In addition to direct binding to genomic site, FOXP3 controls transcription indirectly by interacting and collaborating with cofactors (NFAT, NF-kB). Mediating epigenetic modifications at the target gene loci has also been reported to be an important regulating mechanism of FOXP3. There are notable differences in the function of FOXP3 in the periphery and the thymus, where its role in FOXP3-dependent differentiation is emphasised (Zheng, Josefowicz et al. 2007). Lin et al. have also questioned the importance of Foxp3 in murine Treg development (Lin, Haribhai et al. 2007). They reported that in the absence of functional Foxp3, Treg maturation proceeded and mature T cells exhibited the same characteristics as Treg cells but lacked suppressor function.

The human FOXP3 protein encoding gene is found in the X chromosome and has 11 exons (Brunkow, Jeffery et al. 2001). The promoter area is activated by transcription factors NFAT (nuclear factor of activated T cells), AP-1 (activator protein 1) and FOXO proteins, but the most essential for initiation of transcription are the conserved non-coding sequences CNS1, CNS2 and CNS3. CNS1 is considered a TGF-β sensor element which binds the transcription factor SMAD3 and plays a role in the generation of induced Tregs. CNS2 is a completely demethylated region which ensures stable Treg phenotype (see below). CNS3 initiates the transcription of FOXP3 in both natural and induced Tregs and the deletion of it reduces thymic Treg numbers significantly. The role of TCR stimulation and cytokines is

important for the transcription and expression of FOXP3 (discussed further later).

There are multiple variant FOXP3 isoforms that are alternatively spliced in humans. Their production depends on TCR activation. FOXP3Δ2 lacks the region encoded by exon 2; FOXP3Δ7 lacks exon 7 and FOXP3Δ2Δ7 lacks both. The expression pattern of different isoforms remains unclear (Ramsdell and Ziegler 2014).

2.3 Treg function and peripheral tolerance

The mechanisms of central tolerance eliminate autoreactive T cells during thymic development but peripheral tolerance maintains immune homeostasis and prevents autoimmune reactions in the periphery. Treg suppressor function is crucial in mediating harmful immune reactions. Activated regulatory T cells suppress effector T cells, dendritic cells, B cells, NK cells and macrophages at the infection site. TCR activation of Tregs is required and the shared antigen specificity between Treg and the target T cell might increase the efficiency of suppression (Corthay 2009).

Regulatory T cells produce anti-inflammatory cytokines TGF-β, IL-10 and IL-35, use direct cell-cell contact and modulate the antigen-presenting cells to suppress too potent immune reactions (Costantino, Baecher-Allan et al. 2008, Shevach 2009). IL-10 decreases the production of IL-2, TNF-α and IL-5 and inhibits the function of DCs and macrophages (Couper, Blount et al. 2008). Suppression through granzymes induces apoptosis in B cells and effector T cells (Zhao, Thornton et al. 2006). Tregs can also eliminate growth factors by taking up IL-2 from the environment of the effector cells and disturb the effector cell metabolism by taking up scarce amino acids through expression of ectoenzymes CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (Kobie, Shah et al. 2006, Borsellino, Kleinewietfeld et al. 2007). In addition, Tregs are suggested to regulate immune responses to inflammation, infection and tumor growth (Josefowicz and Rudensky 2009).

Tregs express co-inhibitory CTLA-4 molecules that interact with CD80/CD86 ligands with higher affinity than CD28 leading to the downregulation of costimulatory molecules on antigen-presenting cells. This hinders the activation of effector T cells because of the lack of the second signal (Oderup,

Cederbom et al. 2006). Tregs can also use CTLA-4 to induce the expression of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase on APCs (Schmidt, Nino-Castro et al. 2012). LAG-3 (lymphocyte activation gene 3 protein) expressed by Tregs binds to MHC II molecules and suppresses DC maturation (Liang, Workman et al. 2008). In conventional T cells LAG-3 inhibits cellular proliferation and activation (Sega, Leveson-Gower et al. 2014). Through galectin-1 expression Tregs can arrest cell cycle, induce apoptosis and inhibit cytokine production in target cells (Garin, Chu et al. 2007).

Producing anti-inflammatory cytokines is a basic function of Tregs but recent studies have also detected a pro-inflammatory Foxp3+ regulatory population (Voo, Wang et al. 2009, Dominguez-Villar, Baecher-Allan et al. 2011). Under inflammatory conditions they produce pro-inflammatory cytokines IFN-γ and IL-17A. Their functions have been identified in various autoimmune diseases such as psoriasis and type 1 diabetes (Pandiyan and Zhu 2015).

2.4 Epigenetic regulation of Tregs

In regulatory T cells the expression of FOXP3 alone is not sufficient for generating a stable Treg phenotype. Some Treg-specific genes are expressed in Treg cells independently of FOXP3 expression and induced Tregs lack a significant part of the Treg signature genes (Sugimoto, Oida et al. 2006, Hill, Feuerer et al. 2007). Transient FOXP3 expression is also insufficient for naive T cells to acquire suppressor function. Epigenetic reprogramming seems to be significant for establishing Treg lineage. In general, epigenetic modifications can either silence target genes or increase their accessibility for transcription factors and initiate transcription. The mechanisms of epigenetics include DNA methylation, histone modifications and nucleosome positioning.

The human FOXP3 locus contains the promoter region and three conserved non-coding regions that regulate FOXP3 expression. CNS2, also known as Treg-specific-demethylated region (TSDR), is a CpG-rich intronic enhancer region that is completely demethylated in natural Tregs but methylated in conventional T cells and activated T cells that transiently upregulate FOXP3 (Baron, Floess et al. 2007). In addition, there are permissive histone marks present at the FOXP3 promoter in Tregs (Schmidl, Klug et al. 2009).

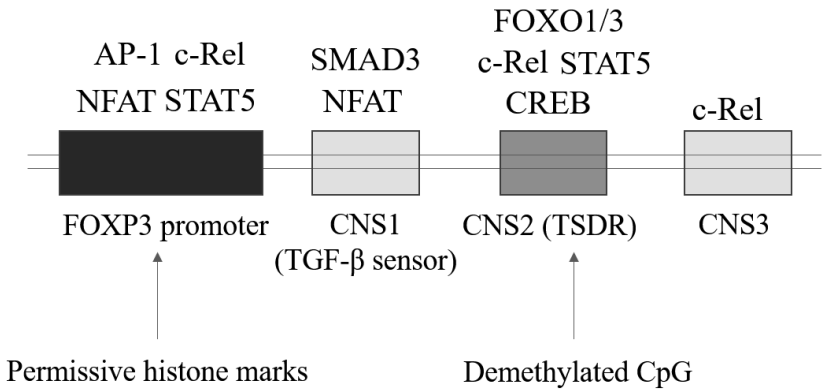
The maintenance of demethylated TSDR is a marker of stable FOXP3 phenotype (Floess, Freyer et al. 2007). In humans DNA methylation of TSDR mostly prevents stable transcription of FOXP3 in conventional T cells (Nagar, Vernitsky et al. 2008). Induced Tregs have more heterogeneous demethylation patterns in TSDR than natural Tregs and their phenotype is less stable. TSDR methylation analysis might become a useful clinical tool for quantitative evaluation of peripheral Tregs (Janson, Winerdal et al. 2008). Barzaghi et al. reported that IPEX-like patients (IPEX phenotype caused by gene defects other than FOXP3) exhibited reduced numbers of Tregs using TSDR demethylation assay (Barzaghi, Passerini et al. 2012).

The initiation of FOXP3 expression does not require the demethylation of TSDR but long-term stability is maintained by it (Huehn, Polansky et al. 2009, Zheng, Josefowicz et al. 2010). The forced demethylation of TSDR also leads to a stable phenotype (Polansky, Kretschmer et al. 2008). In murine models the demethylation of TSDR was initiated during the early stages of thymic development, leading to the generation of stable mature regulatory T cells. The mechanisms included 5-hydroxymethylcytosine (5hmC) and enzymes of the ten-eleven-translocation (Tet) family (Toker, Engelbert et al. 2013). Mice deficient of Tet2 and Tet3 have Tregs with unstable Foxp3 expression and they easily lose their suppressive function (Yue, Trifari et al. 2016). The pathways leading to demethylation of TSDR are only partially known. TCR signalling and downstream activation of the NF-kB family member transcription factor c-Rel are suggested to play a significant role in demethylation of CNS2 resulting in the induction of Foxp3 (Long, Park et al. 2009, Engel, Sidwell et al. 2013).

Studies in mice have shown that the demethylation of TSDR is essential during thymic Treg development (Ohkura, Hamaguchi et al. 2012, Kitagawa, Ohkura et al. 2015). Ohkura et al. recognised several regions within Treg-cell associated genes encoding proteins such as CD25, CTLA-4, Eos and GITR that were demethylated in regulatory lineage thymocytes in the thymus. Foxp3 expression alone was not sufficient for establishing stable Treg lineage. TSDR hypomethylation pattern is induced by TCR stimulation and can occur independently of Foxp3 expression. Toker et al. have shown that active demethylation of TSDR occurs in parallel with the induction of Treg-type gene expression and was completed as Tregs emigrated from the thymus (Toker, Engelbert et al. 2013). The loss of DNA methylation was initiated by TET2 and TET3 enzymes and led to

the oxidation of 5-methylcytocines. Treg precursors might have epigenetic modulation in their chromatin structure prior to Foxp3 induction since they are primed to induce DNA demethylation after stimulation with TCR and IL-2 during their development. A global chromatin organiser, Satb1, was recently recognised to bind to the CNS0 region in the Foxp3 locus in double positive thymocytes (Kitagawa, Ohkura et al. 2017). Epigenetic modifications seem to play a role in the early stages of Treg development.

Figure 4. Regulation of the FOXP3 gene. The FOXP3 locus contains the promoter region and three conserved non-coding regions that regulate FOXP3 expression. CNS2 is also known as Treg-specific-demethylated region (TSDR). TCR and IL-2 downstream signalling mediate the transcription of FOXP3. The transcription factors binding to the promoter and CNSs are shown above the figure.



2.5 Induced Tregs

Natural regulatory T cells are the main regulatory population in the periphery but naive CD4 T cells are also able to upregulate FOXP3 and differentiate into induced Tregs (iTregs). FOXP3 induction requires TCR signalling with appropriate cytokines, including transforming growth factor β (TGF-β) and interleukin-2 (Chen, Jin et al. 2003, Zheng, Wang et al. 2004, Kretschmer, Apostolou et al. 2005, Davidson, DiPaolo et al. 2007, Selvaraj and Geiger 2007, Horwitz, Zheng et al. 2008). Mice deficient of TGF-β1 exhibit normal Treg development in the thymus but in the periphery the number of Tregs are decreased (Marie, Letterio et al. 2005). There is evidence that

TCR specificity dictates FOXP3 induction (Lathrop, Santacruz et al. 2008) and functionally specialised dendritic cells are able to induce iTregs more efficiently (Coombes, Siddiqui et al. 2007, Maldonado and von Andrian 2010). Guo et al. have suggested a two-step iTreg differentiation in murine models. CD4+ CD25+ Foxp3+ iTregs precursors were generated by TCR activation and inhibition of IL-2 signalling. Exposure of these peripheral iTreg precursors to IL-2 led to Foxp3 expression via Stat5 phosphorylation (Guo, Khattar et al. 2013).

iTreg differentiation is mainly associated with bacterial or viral infections, tumors and oral tolerance in mucosal tissues. They are enriched in the intestine and maternal placenta and they play an essential role in maintaining immune homeostasis there (Hadis, Wahl et al. 2011, Samstein, Josefowicz et al. 2012). In the gut the availability of TGF-β and retinoid acid contributes to the induction of Tregs (Coombes, Siddiqui et al. 2007).

Both nTregs and iTregs feature Treg-associated markers CD25, GITR, CTLA-4 and CD103 but there are differences in the expression of PD-1, neuropilin-1 (Nrp1), Helios and CD73 (Yadav, Louvet et al. 2012). Natural Tregs have stronger suppressive capacity than iTregs and iTregs act more locally in mucosal barriers. The overlap in TCR repertoire between iTregs and conventional T cells is restricted (Yadav, Stephan et al. 2013). Significant difference between thymus-derived natural Tregs and iTregs is the stability of the phenotype. iTregs are unstable because of the methylated TSDR in the CNS2 region. In human studies the in vitro conversion of iTregs from conventional T cells is less efficient than in mice. Induced Tregs easily lost FOXP3 expression, were not suppressive and produced proinflammatory cytokines. In murine models TGF-β alone has not been sufficient to generate a stable demethylated TSDR and regulatory phenotype for iTregs but administration of IL-2 together with TCR stimulation enhanced demethylation (Chen, Kim et al. 2011).

3. T CELL DEVELOPMENT

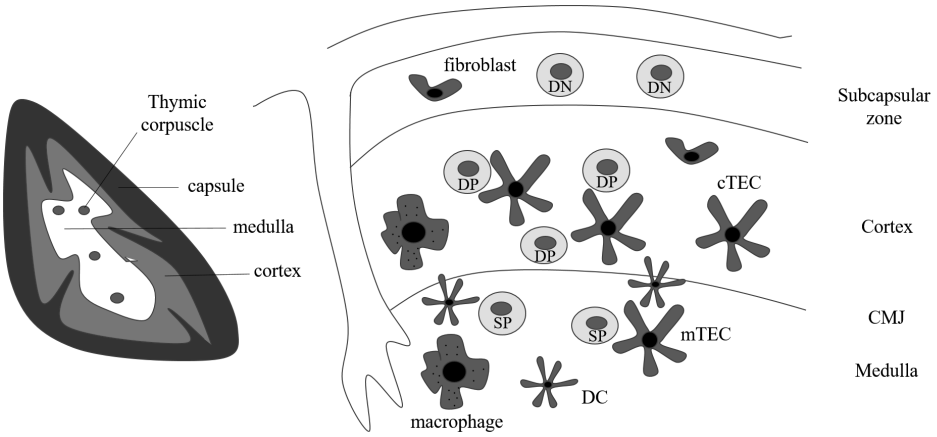
3.1 The structure of the thymus

T cell precursors derive from the bone marrow but migrate to the thymus during the early stage of development. The thymus is situated in the mediastinum in front of the heart and is divided structurally into the cortex and medulla. Thymic stroma provides the proper environment for thymocyte maturation. The most active phase of the thymus is during the neonatal period and childhood but the production of T cell continues throughout adult life. The thymus gradually atrophies and is replaced with adipose tissue with age.

The cortex includes immature thymocytes, branched cortical epithelial cells and macrophages that clear apoptotic thymocytes. Proliferating immature thymocytes are situated in the outer cortical layer before they migrate to the deeper cortical layer for thymic selection. Cortical thymic epithelial cells (cTECs) form a complex reticular network of stromal cells, supporting early and later stages of T cell development. The Notch ligand Delta-like 4 expressed by cTECs promotes the formation of double positive CD4+CD8+ thymocytes (Hozumi, Mailhos et al. 2008, Koch, Fiorini et al. 2008).

The medulla contains mature thymocytes, medullary epithelial cells, macrophages, dendritic cells and Hassall's corpuscles. Especially medullary thymic epithelial cells have a significant role in T cell development and can express tissue-specific-antigens that engage in negative selection. There are three different kinds of thymic dendritic cells, resident classical DCs, migratory classical DCs and migratory classical plasmacytoid DCs. Thymic nurse cells (TNCs) are comprised of multicellular complexes formed by cortical epithelial cells.

Figure 5. The structure and the cells of the thymus. The thymus is divided into a central medulla and an outer cortex. Precursor thymocytes enter the thymus via large blood vessels at the corticomedullary junction (CMJ). Double-negative (DN) thymocytes become double-positive (DP) thymocytes in the cortex and undergo positive selection mediated by cortical thymic epithelial cells (cTECs). Positively selected DP thymocytes differentiate into CD4+ or CD8+ single-positive (SP) thymocytes and migrate to the medulla.



3.2 T cell precursors

The precursor cells derive from hematopoietic stem cells (HSC) in the bone marrow and migrate via the bloodstream to the thymus. Thymus seeding progenitors (TSP) that express CD34+ CD45RA+ CD7+ enter through large venules at the border of the cortex and the medulla (corticomedullary junction CMJ). CD34+ $CD38_{low}$ early thymic progenitors (ETPs) occupy the outer cortex. The number of T cell precursor cells in the peripheral blood is extremely small (Krueger and von Boehmer 2007, Krueger, Zietara et al. 2017). Interaction with the thymic stromal cells triggers T cell differentiation. Notch1 is a transcription factor that has been identified as being essential in the commitment to different cell lineages. Sustained Notch signalling instructs thymocytes to commit to T cell lineage and lack of it leads to the arrest of T cell development in mice (Blom and Spits 2006).

Before commitment to T cell lineage ETPs lack the expression of RAG1 (recombination activating gene 1), CD1A, and cytoplasmic CD3, CD2 and CD7. The committed thymocytes express CD34, CD1a, CD7 and IL-7Ra. IL-7 has been shown to be essential for thymocyte development and survival at the early stage. During early development CD34+ thymocytes proliferate in response to IL-7. IL-7-deficient mice have reduced numbers of αβ T cells and lack γδ T cells and in humans IL-7R-deficient patients exhibit a severe combined immunodeficiency (SCID-X1) (von Freeden-Jeffry, Vieira et al. 1995, Bayer, Lee et al. 2008).

3.3 T cell development at the double negative stage

The development of thymocytes is divided into distinct phases that reflect the changes in the status of T cell receptor chains and in the expression of CD3 and co-receptors CD4 and CD8 on the cell surface. The commitment to the αβ or γδ T cell lineage takes place during early thymocyte development. TCR signal strength is essential for the lineage choice between αβ and γδ T cells. A strong TCR signal leads to γδ while a weak signal leads to αβ lineage commitment. In humans there are three distinct double negative stages: the most immature CD34+ CD38- CD1a-, CD34+ CD38+ CD1a-, and CD34+ CD38+ CD1a+.

Notch signalling promotes thymocyte proliferation and differentiation at the double negative stages. In mice, early thymic progenitors express a CD4- CD8- CD44+ CD25- CD117+ phenotype (Allman, Sambandam et al. 2003) and the double negative stage is subdivided into four stages based on the expression of CD44 and CD25. In the early DN1 phase thymocytes express the phenotype of CD44+ CD25- and the DN1 stage can be further subdivided on the basis of CD24 and CD117. The transcription factor Bcl11b is essential for T cell lineage commitment at the DN2 ($CD44_{low}$ CD25+) stage. The somatic recombination of the TCRβ genes V, D and J is also initiated at the DN2 phase by recombination activating genes RAG1 and RAG2. In humans TCRβ rearrangement starts at the CD34+ CD38+ CD1a- stage (Dik, Pike-Overzet et al. 2005).

3.3.1 Recombination of TCR genes

TCR diversity is generated by somatic rearrangements of the non-contiguous Vβ, Dβ and Jβ gene segments in the β chain locus and Vα and Jα gene

segments in the α chain locus. These multiple gene segments allow somatic recombination to encode a huge number of unique TCR sequences. To increase diversity, P- (palindromic) and N- (nontemplated) nucleotides are added and deleted at the Vβ-Dβ, Dβ-Jβ and Vα-Jα junction sites (Krangel 2009). Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase expressed in DN thymocytes that adds N-nucleotides at the junction sites. Theoretical diversity produced by the recombination machinery is huge, with over 10^{15} αβ receptors (Davis and Bjorkman 1988). The pairing of α and β chains that forms the TCR heterodimer, increases the diversity. TCRδ and TCRγ rearrange first before TCRβ and the commitment to TCRαβ lineage take place. Only a few γδ T cells have a rearranged TCRβ chain but TCRγ genes are rearranged in most TCRαβ cells (Sherwood, Desmarais et al. 2011).

β selection is the first major checkpoint in T cell development and in humans it takes place at the CD34+ CD38+ CD1a+ stage (Dik, Pike-Overzet et al. 2005). In mice it follows the somatic recombination of the TCR β locus at the DN3 (CD44-, CD25+) stage and terminates the β rearrangements. The selection ensures that thymocytes have a functional β chain on their surface and ends β-chain rearrangement, leading to allelic exclusion of the β locus (Khor and Sleckman 2002, Nishana and Raghavan 2012). A functional TCRβ gene must be assembled on only a single allele. Both alleles should not attempt VDJ recombination at the same time and a successful in-frame recombination on one allele gives a feedback signal that prevents further recombination events. Thymocytes with out-of-frame TCRβ chains do not pass the β selection and go to apoptosis. The successfully rearranged β chain allows the expression of pre-TCR, cell proliferation, and differentiation into DN4 stage. Signalling through IL-7 receptors ensures the survival of DN thymocytes.

3.4 The double positive stage

DN4 (CD44- CD25-) thymocytes become immature single positive (Petrie, Livak et al. 1993) CD4+ cells that have acquired the expression of CD4 but not yet CD8. The CD4ISP stage is followed by the expression of only CD8 α chain before the cells acquire both α and β chains. The expression of CD3-pre-TCR complex leads to cell proliferation, allelic exclusion and progression towards the double positive CD4+ CD8+ stage (Michie and Zuniga-Pflucker 2002). DP thymocytes comprise the majority of the

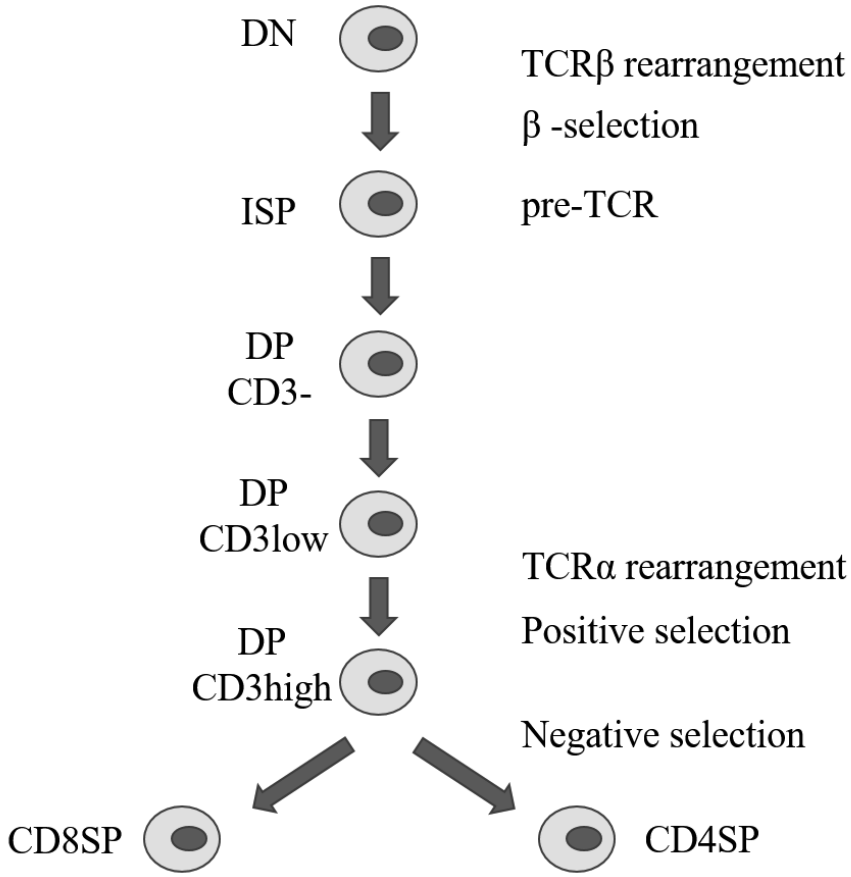
thymic population. The DP phase can be subdivided into different stages based on TCR-CD3 expression. At the early DP stage thymocytes lack CD3 expression but start to express it at the DP $CD3_{low}$ stage. After positive selection high expression of CD3 can be detected (Vandekerckhove, Barcena et al. 1994). CD69 is a C-type lectin-like signalling receptor that is a marker of positive selection (Sancho, Gomez et al. 2005). Most of the thymocyte proliferation during thymic development occurs during the late DN and early DP stages (Egerton, Scollay et al. 1990, Scollay and Godfrey 1995, Porritt, Gordon et al. 2003). In more mature DP populations the proliferation rate is low (Egerton, Scollay et al. 1990, Sinclair, Bains et al. 2013). In humans only 1-2 divisions were reported between DP $CD3_{low}$ stage and mature CD4SP and CD8SP thymocytes (Okamoto, Douek et al. 2002).

3.4.1 Rearrangement of the TCRα chain

The rearrangement of the α chain locus can pass through multiple attempts without allelic exclusion to achieve a functional TCR. The rearrangement initiates after cell proliferation, when thymocytes express both pre-TCRα and TCRβ at the CD34+ CD38+ CD1a+ stage (Dik, Pike-Overzet et al. 2005). In the α locus only V and J gene regions are rearranged and the process can be repeated until thymocytes acquire a functional α chain and can survive positive selection or are selected to die by neglect. On average five rounds of VJ recombination have been suggested on each allele (Warmflash and Dinner 2006).

Some thymocytes (approximately 30% of conventional thymocytes) can express two TCRs, consisting of two different α chains pairing with the same β chain. This is due to poor α chain allelic exclusion (Padovan, Casorati et al. 1993, Petrie, Livak et al. 1993). Dual TCR expression provides two distinct pMHC ligand specificities and a survival advantage in positive selection. During negative selection it can mask autoreactive TCRs from elimination. Mice deficient in secondary TCRs exhibited reduced numbers of thymocytes that survived positive selection, suggesting that simultaneous rearrangement of both TCRα loci is required for efficient T cell generation (Ni, Solomon et al. 2014). In the regulatory T cell population between 50% and 99% express two TCRs (Tuovinen, Salminen et al. 2006).

Figure 6. T cell development and thymic selections. The development of thymocytes is divided into distinct phases that reflect the changes in the status of T cell receptor chains and in the expression of CD3 and co-receptors CD4 and CD8 on the cell surface. DN thymocytes lack both CD4 and CD8 co-receptors. TCRβ recombination is initiated at the DN phase. DN thymocytes become immature single positive CD4+ cells. The expression of CD3-pre-TCR complex leads to cell proliferation, allelic exclusion and progression towards the double positive CD4+ CD8+ stage. The DP phase can be subdivided into different stages based on the TCR-CD3 expression. At the early DP stage thymocytes lack CD3 expression but start to express it at the DP $CD3_{low}$ stage. After positive selection high expression of CD3 can be detected. TCRα rearrangement occurs at the DP stage and can pass through multiple attempts.



3.5 Positive selection

Together, positive and negative selection eliminate over 90% of all thymocytes. The selections are dictated by TCR signalling strength and the peptide ligand pool composed primarily of self-peptides (Starr, Jameson et al. 2003). Thymic epithelial cells display these self-antigens complexed with MHC on their cell surface and mediate positive selection. The pathways that cTECs use to generate the diverse repertoire of selecting ligands include cathepsins, thymic-specific serine proteases, macroautophagy and thymoproteasome (Klein, Hinterberger et al. 2009). The peptides mediating positive selection are mainly low-affinity antagonists (ligands that block response) and weak agonists (ligands that have only partial efficacy).

According to the TCR affinity model the quality of the TCR-peptide-MHC interaction dictates whether a thymocyte survives positive selection or dies by neglect. The affinity threshold determining the outcome of the selection has been shown to be remarkably narrow. The affinity between TCR and self-peptide-MHC-complex must be sufficient to prevent the apoptosis of the cell in positive selection. This ensures that the developing thymocytes are capable of recognising peptide-MHC complexes with their TCR. A minimal signal is required for cell survival. Most T cells at this stage are eventually not able to bind to MHC and die by neglect. Co-receptors CD4 and CD8 ensure a stable binding in the TCR-pMHC interaction (Germain 2002). Themis (thymocyte-expressed molecule involved in selection) is one of the molecules modulating TCR signalling during selection (Fu, Vallee et al. 2009, Johnson, Aravind et al. 2009). It can reduce TCR signalling strength and selection threshold in response to low affinity MHC peptides. Themis is expressed predominantly at the early DP stage and the expression decreases after positive selection. Mice deficient of Themis exhibit a defect in positive selection and have reduced numbers of SP thymocytes and peripheral T cells (Gascoigne, Rybakin et al. 2016).

T cells respond to foreign antigens displayed by cells expressing a particular MHC which is called MHC restriction. Positive selection is a mechanism to ensure that the recognised antigen must be presented by the MHC molecule. Germline encoded CDR1 and CDR2 primarily bind the MHC molecule in the TCR-pMHC complex and it has been suggested that co-evolution of TCR and MHC favours TCRs capable of binding MHC molecules. The selection model is based on the role of co-receptors CD4

and CD8 in regulating MHC restriction (Rangarajan and Mariuzza 2014).

After positive selection CD69+ double positive thymocytes differentiate into CD4 or CD8 single positive cell lineage. TCR signalling regulates the lineage commitment. The partial downregulation of CD8 allows the thymocytes to choose whether its TCR is MHC I or MHC II restricted. Sustained TCR-MHC II signalling instructs thymocytes to commit to CD4 lineage but the recognition of MHC I leads to CD8 upregulation (Singer, Adoro et al. 2008). The migration to the medulla is regulated by cortical retaining and medullary attractant signals. Thymocytes upregulate CCR4 that leads them to CCL17 and CCL22-producing DCs (Hu, Lancaster et al. 2015). The chemokine receptors CCR7 and CCR9 also guide the migration.

3.6 Negative selection

Negative selection can occur simultaneously with positive selection in the cortex or after the cell migration to the medulla. It induces apoptosis in the thymocytes, recognising self-antigens with too high affinity. The expression of self-peptides derived from different tissues is required in the thymus to generate efficient negative selection. The elimination of autoreactive T cells maintains central tolerance. During negative selection between 3% and 30% of thymocytes are removed (Merkenschlager, Graf et al. 1997, van Meerwijk, Marguerat et al. 1997, Daley and Smith 2013, Stritesky, Xing et al. 2013). Medullary thymic epithelial cells (mTECs) are identified as the main intrathymic source of promiscuous gene expression and the autoimmune regulator (Aire) gene controls the expression of tissue-restricted antigens (TRAs) in the thymus (Anderson, Venanzi et al. 2002, Kisand, Boe Wolff et al. 2010). Aire can also mediate the maturation of mTECs. Conventional dendritic cells in the thymus can also capture the antigens released by mTECs and present them to T cells undergoing selections. Migratory DCs play a role in presenting peripheral self-antigens that are not included in the ligand repertoire of TRAs expressed by mTECs (Klein, Hinterberger et al. 2009).

The selection process creates a diverse TCR repertoire that is able to recognise a large array of peptides but is non-responsive to self-antigens. The mature single positive (SP) T cells emigrate from the thymus via post-capillary venules to the peripheral blood. They express sphingosine 1-phosphate receptor 1 (S1P1), mediating the export from the thymus

(Kurobe, Liu et al. 2006). CD69 can inhibit the emigration of mature single positive thymocytes to the periphery (Feng, Woodside et al. 2002). Recent thymic emigrants (RTEs) lose the expression of CD69 and CD1A but express high levels of CD3, CD27, CD45RA, CD62L and CD31. TCR excision circles (TRECs) formed during TCR rearrangement are molecular markers that identify RTEs.

3.7 Central tolerance

A diverse TCR repertoire is required to protect the body against potential pathogens but also has to maintain tolerance to prevent autoimmune reactions. Central tolerance eliminates autoreactive T cells via negative selection during thymic development. However, there are inevitably some autoreactive T cells that manage to avoid negative selection and emigrate from the thymus. Peripheral tolerance and the generation of regulatory T cells in the thymus compensate the faults of the central tolerance. Genetic mutations such as *AIRE* mutation in APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) that disturb the central tolerance in the thymus lead to autoimmune diseases.

3.8 TCR repertoire and diversity

The human T cell repertoire is established in the thymus. It has to protect the body against pathogens and maintain tolerance to self-antigens. The ability to recognise a huge number of potential foreign pathogens demands an enormous T cell receptor diversity. The majority of the TCR repertoire generated by somatic recombination is deleted during the selection process in the thymus (Bouneaud, Kourilsky et al. 2000). The shaping of the repertoire maintains central tolerance and prevents autoimmune reactions. The length of CDR3 shortens during thymic development when thymocytes pass through selections. Short near-germline CDR3 regions can be favoured by positive selection because of the more effective interaction with pMHC-complexes (Yassai, Ammon et al. 2002).

As previously described, the theoretical ability of the VDJ recombination machinery to create diversity is huge, with over 10^{15} different αβ receptors (Davis and Bjorkman 1988, Murugan, Mora et al. 2012, Sewell 2012, Zarnitsyna, Evavold et al. 2013). Robins et al. have calculated the theoretically possible TCRβ diversity to be $5x10^{11}$ (Robins, Srivastava et

al. 2010). The amount of distinct clonotypes depends on the number of TCR α chains that each β chain combines with. The theoretical upper limit of peripheral TCR repertoire has been estimated to range from 10^{12} to 10^{13} (Arstila, Casrouge et al. 1999, Nikolich-Zugich, Slifka et al. 2004).

However, the relationship between potential and actual TCR diversity requires further investigation because only a fraction of the potential repertoire can be found in each individual at a certain time point. 10^{15} T cells would weigh around 500 kg (Mason 1998). There are $4x10^{11}$ T cells circulating in the adult human body (Jenkins, Chu et al. 2010) and each T cell has many usually identical TCRs on its surface (Varma 2008). The group of T cells with a similar TCR defines a clonotype. In the periphery the number of different clonotypes depends on the balance between T cell loss and thymic input and the mechanisms controlling cell division and death (De Boer and Perelson 1997, Goldrath and Bevan 1999, Rudd, Venturi et al. 2011). Clonotypes compete for space and for stimuli from peptides presented by MHC molecules. Lythe et al. calculated the total number of clonotypes to be 10^{10} in the periphery (Lythe, Callard et al. 2016). They also suggest a mean clonal size only of order 10 even though some clonotypes may expand to thousands.

High-throughput sequencing has enabled the identification of large numbers of TCRs in one sample but the entire repertoire of one individual cannot be directly measured. Unseen species estimators are required to estimate the actual diversity from one blood sample (Laydon, Bangham et al. 2015). Rarefaction curves developed by Daley and Smith measure the number of observed unique TCRβ sequences as a function of the number of sequenced cells and the curves saturate when the complete diversity has been sampled (Daley and Smith 2013). Estimator DivE also uses rarefaction curves but fits a large number of functions to the curves and scores each with 4 criteria that measure the accuracy and consistency of fits to data and subsamples (Laydon, Melamed et al. 2014). The final estimate is the geometric mean of the five best models. In contrast to previous estimators, non-parametric Chao2 is based on the overlap of TCR sequence incidence data (Chao 1987).

3.8.1 TCR diversity in human peripheral blood

The earliest estimates of the TCR repertoire in human peripheral blood were

done using immunoscope techniques (Even, Lim et al. 1995, Pannetier, Even et al. 1995). Arstila et al. measured hundreds of sequences in blood and extrapolated the diversity to 1 million different TCRβ sequences and $0.5x10^5$ TCRα sequences (Arstila, Casrouge et al. 1999). In the highly diverse repertoire each β chain paired on average with at least 25 different α chains. In the memory subset, the diversity decreased to 200 000 different β chains each pairing with only a single α chain. Later Klarenbeek et al. observed that the memory subset is very diverse and comprises a large number of low-frequency clonotypes (Klarenbeek, Tak et al. 2010).

Next generation sequencing (NGS) has made possible greater sequencing depth and more accurate quantification of TCRs from single individuals. Using NGS Warren et al. calculated 1 million different TCRβ sequences from one adult blood donor (Warren, Freeman et al. 2011) and Robins et al. determined the number of unique TCRβ sequences to be 3 million in the peripheral blood of a healthy donor (Robins, Campregher et al. 2009). These reports place a lower limit on directly measured diversity. In a recent article Qian Qi et al. used high-throughput sequencing and Chao2 estimator to estimate 100 million unique TCRβ sequences in naïve CD4 and CD8 T cell repertoires of young adults (Qi, Liu et al. 2014). Practical and ethical considerations set limitations on obtaining large numbers of T cells from healthy donors; thus detecting rare clonotypes in low frequencies remains challenging.

Public T cells are T cell clonotypes that have identical TCRs and are shared among individuals. In theory, the likelihood of recombination machinery randomly generating identical TCRs should be extremely small but studies have shown that public T cells are quite common (Robins, Srivastava et al. 2010, Venturi, Quigley et al. 2011, Murugan, Mora et al. 2012). Warren et al. have reported that as much as 14% of an individual's repertoire is comprised of public sequences (Warren, Freeman et al. 2011). Public clonotypes have on average fewer junctional insertions, which indicates that the recombination machinery or selection process is biased toward near-germline sequences (Venturi, Kedzierska et al. 2006). Multiple nucleotide sequences can often encode the same public TCR amino acid sequence. Venturi et al. have proposed that public T cells are produced by convergent recombination: recombination machinery converge the production of the same nucleotide sequence and multiple nucleotide sequences converge to encode the same amino acid sequence (Venturi, Price et al. 2008).

4. REGULATORY T CELL DEVELOPMENT IN THE THYMUS

The majority of regulatory T cells arise in the thymus as a distinct T cell lineage, and therefore are referred to as natural Tregs. The significance of thymus in the production of Tregs was described in a study comparing Treg compartments in donors' thymus and blood samples. There was a direct correlation between the sizes of these two compartments (Machnes-Maayan, Lev et al. 2015). In the human fetus mature T cells are first detected in the thymus in the 12th gestational week and regulatory T cells can also be found at this stage (Cupedo, Nagasawa et al. 2005, Darrasse-Jeze, Marodon et al. 2005, Haddad, Guimiot et al. 2006, Michaelsson, Mold et al. 2006, Farley, Morris et al. 2013). Fetal human Tregs are capable of suppression and express FOXP3, CTLA-4 and GITR (Cupedo, Nagasawa et al. 2005, Darrasse-Jeze, Marodon et al. 2005).

Treg development in the thymus has been extensively studied in murine models but in humans the developmental pathway remains only partially known. There are notable differences between human and murine Treg development, including for example the timing of commitment. In the thymus Treg differentiation requires TCR signalling, co-stimulatory molecules and common γ chain cytokines. A two-step-model based on the murine models suggests that TCR signalling induces the expression of CD25 and thus allows the cytokines to promote Treg development (Burchill, Yang et al. 2008, Lio and Hsieh 2008).

4.1 Timing of commitment

During thymic development the first FOXP3+ cells can be detected in the CD4-CD8- double negative (DN) thymocyte subset before the expression of TCR (Tuovinen, Kekalainen et al. 2008, Liu, Li et al. 2014). The main population expressing Treg markers FOXP3 and CD25 comprises mature CD4 single positive thymocytes but this population can also be found at the DP and CD8SP stages. Most FOXP3+ cells localise in the medullary region (Annunziato, Cosmi et al. 2002, Cosmi, Liotta et al. 2003, Cupedo, Nagasawa et al. 2005, Darrasse-Jeze, Marodon et al. 2005, Liotta, Cosmi et al. 2005, Watanabe, Wang et al. 2005, Tuovinen, Pekkarinen et al. 2008, Martin-Gayo, Sierra-Filardi et al. 2010, Nunes-Cabaco, Caramalho et al. 2011). The majority of FOXP3+ CD4SP compartments develop Tregs in

the final stage but a proportion consists of recirculating activated peripheral Tregs (Thiault, Darrigues et al. 2015).

The generation of Foxp3-GFP (green fluorescent protein) reporter mice has established that in mice the induction of Foxp3 occurs at the CD4SP stage in the thymic medulla (Fontenot, Rasmussen et al. 2005). A small share of GFP+ cells are also detected in DP and CD8SP populations. Liston et al. have suggested that DP Foxp3+ subset could comprise as much as one fourth of the whole regulatory population, and they also seem to be positively selected and express CCR7 (Liston, Nutsch et al. 2008). Commitment to Treg lineage might not require the expression of Foxp3 so the lineage choice could precede the induction of Foxp3 in the cortex (Gavin, Rasmussen et al. 2007, Lin, Haribhai et al. 2007).

Although in murine models commitment to the Treg lineage takes place at the CD4+ single-positive (SP) stage, the evidence in humans suggests an earlier point of divergence at the CD4+ CD8+ double positive stage. A DP FOXP3+ population is clearly detected in the human thymus and they express a host of Treg-associated molecules, including CD25, CD39, GITR and CTLA-4, and exhibit suppressive capacity (Cupedo, Nagasawa et al. 2005, Darrasse-Jeze, Marodon et al. 2005, Liotta, Cosmi et al. 2005, Nunes-Cabaco, Caramalho et al. 2011). The majority of them are functionally mature and positively selected since they express high levels of CD3 and CD69 (Tuovinen, Pekkarinen et al. 2008, Lehtoviita, Rossi et al. 2009). Moreover, linear regression models reported by Nunes-Cabaco et al. indicate that the FOXP3+ DP population is the major contributor to the FOXP3+ CD4 SP subset (Nunes-Cabaco, Caramalho et al. 2011). Based on their computational analysis, Bains et al. suggest that an induction of Treg development and commitment occurs at the DP phase in the cortex (Bains, van Santen et al. 2013). The thymic cortex is capable of supporting Treg development in mice, too. The block of migration from the cortex to the medulla leads to an accumulation of CD4SP Foxp3+ thymocytes in the cortex (Liston, Nutsch et al. 2008, Cowan, Parnell et al. 2013). A similar effect was reported in the case of CCR7-deficient mice (Kurobe, Liu et al. 2006).

4.2 T cell receptor signalling

TCR signalling, including functional avidity and duration, dictates T cell

development, lineage choice to CD4 and CD8 subsets and differentiation of specialised T cell populations. The commitment to regulatory lineage is also dependent on TCR-mediated signalling, which is well established in murine models (Simons, Picca et al. 2010, Josefowicz, Lu et al. 2012, Kraj and Ignatowicz 2018). In negative selection developing conventional T cells that have high TCR affinity interaction with self-antigen/MHC complexes undergo apoptosis but thymocytes receiving low affinity TCR signals pass the selection. Thymic regulatory T cells express a diverse TCR repertoire that is also subjected to thymic selection. TCR transgenic mice studies have shown that self-reactive Foxp3-expressing thymocytes had a survival advantage in the negative selection compared to cells that received equal TCR signalling but no Foxp3 (Josefowicz, Lu et al. 2012). The selection to Treg lineage requires intermediate-to-high TCR affinity to self-antigens complexed with MHC molecules on the surfaces of the epithelial cells (Aschenbrenner, D'Cruz et al. 2007). The strength of the TCR signal can have a direct role in driving the extent of both elimination of autoreactive T cells and Treg differentiation (Caton, Kropf et al. 2014). Impaired negative selection process caused by reduced expression of MHC II molecules in medullary epithelial cells led to increased frequency of Tregs (Friedline, Brown et al. 2009).

In the human thymus the markers of positive selection, CD69 and CD27, are found in developing Tregs and the transcription of FOXP3 is activated by TCR stimulation and its downstream targets NFAT and AP-1 in the promoter region (Mantel, Ouaked et al. 2006). Tuovinen et al. have reported that natural Tregs often express two functional TCRs, which correlates to a higher level of FOXP3 expression (Tuovinen, Salminen et al. 2006). TCR signalling via two different TCRs could direct thymocytes to Treg lineage in the human thymus. The majority of FOXP3+ thymocytes are already positively selected since they are detected at DP $CD3_{high}$ and CD4SP stages. However, early FOXP3 expression can be detected at the DN stage without TCR. There is also a minor population of pre-DP cells which express both FOXP3 and TCR (Nunes-Cabaco, Ribot et al. 2010).

The same transgenic TCR can select both Treg and conventional T cells, and in non-transgenic settings there seems to be considerable TCR repertoire overlap between Treg and other T cells (Pacholczyk, Ignatowicz et al. 2006, Wong, Obst et al. 2007). Identical TCR with the same affinity for the selecting ligand can give rise to both Treg and conventional CD4+ cells

(Wojciech, Ignatowicz et al. 2014). TCR signalling alone is therefore not enough to instruct thymocytes to commit to Treg lineage.

4.3 Cytokines

Cytokines play an essential role in Treg development and particularly common γ chain cytokines induce Treg development and promote their survival. Mice deficient in IL-2, IL-7 and IL-15 exhibit absence of regulatory T cells in the thymus and periphery (Burchill, Yang et al. 2007). Similarly, lack of common γ receptors in mice prevents the generation of Tregs (Fontenot, Rasmussen et al. 2005, Burchill, Yang et al. 2008, Tai, Erman et al. 2013). IL-2Rα and IL-7Rα -deficient mice have the same phenotype (Bayer, Lee et al. 2008).

In murine models Foxp3 has been reported to induce thymocytes to express proapoptotic protein signature and decrease the expression of Bcl-2, which promotes cell survival. The lethal effect of Foxp3 can be prevented by common γ chain cytokines that upregulate Bcl-2 and protect thymocytes from apoptosis. Foxp3+ CD25- Treg precursor cells were most prone to apoptosis and required common γ chain cytokines to survive and complete their maturation (Tai, Erman et al. 2013).

STAT5 is a downstream transcription factor that is activated by common γ chain receptors. The binding sites of STAT5 are Foxp3 promoter and the CNS2 region. A constitutively active form of STAT5 in transgenic mice leads to an increase in the frequency of Foxp3 Tregs and also compensates the absence of IL-2Rβ in Treg development (Burchill, Goetz et al. 2003, Burchill, Yang et al. 2007). In mice the loss of the *Stat5* allele in DP thymocytes causes a dramatic reduction in Foxp3 CD4SP population (Yao, Cui et al. 2006, Burchill, Yang et al. 2007).

4.3.1 IL-2

IL-2 is a T cell growth factor that drives T cell proliferation and promotes the differentiation of CD4 Th1 and Th2 cells in the periphery (Cote-Sierra, Foucras et al. 2004, Liao, Lin et al. 2013). The main cellular sources of IL-2 are CD4 T cells and to a lesser extent CD8 T cells, NK T cells, activated DCs and mast cells. In the human thymus IL-2 was found both in the cortex and the medulla and the main producers are mature T cells

(Caramalho, Nunes-Silva et al. 2015). Three distinct subunits, the IL-2R α chain (CD25), IL-2R β chain (CD122) and common γ chain (CD132) form the IL-2 receptor. IL-2Rβ and common γ chain have a role in transducing intracellular signals. Natural Tregs express constitutively high levels of CD25, the α chain of IL-2 receptor, and IL-2 has been shown to be crucial for Treg homeostasis, expansion and function in the periphery, although they are unable to produce IL-2 themselves (Fontenot, Rasmussen et al. 2005, Malek, Yu et al. 2008, Liao, Lin et al. 2013). Resting T cells express low level CD25 but activation by TCR or IL-2 upregulates the expression. Following IL-2 engagement Janus family tyrosine kinases are activated, leading to the phosphorylation of IL-2Rβ and the recruitment of STAT5 proteins. In the periphery IL-2R-STAT5 signalling is essential for Treg suppressor function (Chinen, Kannan et al. 2016).

In mice IL-2 induces the activation of STAT5, promotes Foxp3 and CD25 expression, and drives Treg development in the thymus (Burchill, Yang et al. 2007, Cheng, Yu et al. 2013). IL-2- , IL-2Rα- and IL-2Rβ-deficient mice manifest severe autoimmunity and their number of Tregs is reduced, whereas transgenic IL-2Rβ expression in the thymus of IL-2Rβ-deficient mice restores Treg production and prevents autoimmune disease (Malek, Yu et al. 2002, Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Malek, Yu et al. 2008). The administration of anti-IL-2 antibodies also reduces the number of thymic Tregs (Bayer, Yu et al. 2005). Immature CD4+ Foxp3+ CD25- cells can be found in the thymus of IL2- and IL2R-deficient mice but these cells are not functional and have reduced numbers of key Treg-associated molecules that mediate suppressor function (Cheng, Yu et al. 2013).

Based on studies in murine models a two-step-model of Treg development in the thymus has been proposed (Burchill, Yang et al. 2008, Lio and Hsieh 2008). TCR signalling upregulates CD25 and increases the responsiveness of developing Tregs to IL-2. IL-2 induces the expression of Foxp3 and enhances the survival of Tregs. The expression of a Bcl2 transgene rescued the differentiation of STAT5-deficient Treg cells indicating that IL-2 and STAT5 signalling play important roles in facilitating the survival of Tregs. In human peripheral blood IL-2 selectively induced FOXP3 expression through STAT5 signalling pathway in CD4+ CD25+ cells but not in the counterparts (Zorn, Nelson et al. 2006), indicating that IL-2 directly targets FOXP3 gene in human Tregs. Demethylation of the TSDR

is required for stable Treg lineage and the role of cytokines in inducing demethylation remains unknown. Toker et al. reported that IL-2 did not drive demethylation of the TSDR in CD25+ Foxp3- precursors (Toker, Engelbert et al. 2013). Thiault et al. demonstrated that recirculating Tregs inhibit Treg development in the thymus by limiting the availability of IL-2 (Thiault, Darrigues et al. 2015).

4.3.2 IL-7

Human regulatory T cells express the high-affinity α chain of IL-7R (CD127) at very low levels. In general, responses to IL-7 are regulated by modulating the receptor level. Therefore, the role of IL-7 in Treg development remains unclear. A common γ chain and an α subunit form the IL-7 receptor. Following signals through the IL-7R STAT5 is phosphorylated and activated by JAK kinases. In the nucleus STAT5 induces the transcription by binding to the FOXP3 promoter area.

Epithelial cells are the source of IL-7 in the thymus (Hong, Luckey et al. 2012, Shitara, Hara et al. 2013). Early thymocyte development requires IL-7 signalling and lack of IL-7 causes a severe defect in T cell development. The absence of IL-7 leads to a massive reduction of thymocytes and peripheral T cells in murine models (von Freeden-Jeffry, Vieira et al. 1995, Bayer, Lee et al. 2008). The survival of DN cells is dictated through regulating the expression of Bcl-2 and the forced expression of Bcl-2 can restore thymic T cell development in IL-7R-deficient mice (Maraskovsky, O'Reilly et al. 1997). In murine models the later developmental stages are not dependent on IL-7 but IL-7 has been reported to promote the commitment to CD8 cytotoxic lineage (McCaughtry, Etzensperger et al. 2012).

At the CD4+CD8+ double positive (DP) stage murine pre-selection thymocytes downregulate CD127 and have been suggested to be unresponsive to IL-7 (Van De Wiele, Marino et al. 2004, Yu and Malek 2006). After positive selection DP thymocytes upregulate IL7Rα and downregulate SOCS1 (suppressor of cytokine signalling 1) (Chong, Cornish et al. 2003, Yu and Malek 2006). The more mature CD4+ single positive (SP) thymocytes again respond to IL-7 and lack of IL-7 disturbs Treg cell proliferation and leads to reduced numbers of Foxp3+ cells (Tani-ichi, Shimba et al. 2013). IL-7 can also induce Foxp3 expression in CD4 SP thymocytes (Wuest, Willette-Brown et al. 2008, Kim, Ligons et al. 2012).

In IL-7Rα- and IL-2Rβ-deficient mice the transgenic expression of IL-7R led to the development of CD4+ Foxp3+ CD25- cells but did not fully restore Treg development (Bayer, Lee et al. 2008).

In contrast to the murine models, in the human thymus double positive FOXP3+ thymocytes upregulate the expression of CD127. Exposure to IL-7 also led to the activation of intracellular STAT5 signalling (Nunes-Cabaco, Caramalho et al. 2011). However, IL-7 has been considered less important in regulatory T cell development than other common γ chain cytokines (Caramalho, Nunes-Silva et al. 2015).

4.3.3 IL-15

The receptors of IL-2 and IL-15 both contain IL-2Rβ and common γ chain and have a distinct α chain. They also activate common signalling pathways but in the periphery they seem to have different biological effects. IL-15 promotes the maintenance of long-lived memory phenotype cells (Waldmann 2006, Liao, Lin et al. 2013). IL-15-deficient mice do not exhibit lethal autoimmunity disease, but in the human thymus IL-15 was identified as an important cytokine in human Treg development. In murine T cell development IL-15 together with IL-7 are responsible for CD8 cytotoxic lineage specification (McCaughtry, Etzensperger et al. 2012). IL-15Rα is expressed by Treg precursors although to a lesser extent compared to the expression of IL-2R, and mice lacking both IL-2 and IL-15 have fewer Tregs than IL-2Rβ-deficient mice (Burchill, Yang et al. 2007).The lack of IL-15Rα did not alter the number of Tregs in mice (Soper, Kasprowicz et al. 2007).

IL-15 is capable of inducing STAT5 phosphorylation and the expression of FOXP3. Mice lacking IL-2 and IL-2β upregulated the expression of IL-15Rα, indicating that the absence of IL-2 can be partially compensated by IL-15 (Vang, Yang et al. 2008). In a recent human study IL-15 was shown to promote Treg commitment, proliferation and survival, though to a lesser degree than IL-2 (Caramalho, Nunes-Cabaco et al. 2015, Caramalho, Nunes-Silva et al. 2015). In the human thymus mTECs, macrophages and B lymphocytes produce cytokine, which is available both in the cortex and medulla.

4.4 Other signals

There are an array of different cofactors and signalling pathways that contribute to human Treg development. In the medullary region Hassall's corpuscles produce thymic stromal lymphopoietin (TSLP) that activates myeloid dendritic cells (mDCs) to promote Treg development from CD4SP $CD25_{neg}$ thymocytes (Watanabe, Wang et al. 2005, Hanabuchi, Ito et al. 2010). This induction requires the expression of CD80 and CD86 in the dendritic cells and IL-2. Thymic dendritic cells also produce TSLP and can be found in the cortex. In murine models TSLP has no direct effect on developing Tregs but it might act through other cell types as in humans (Vang, Yang et al. 2008). Plasmacytoid dendritic cells (pDCs) drive Treg differentiation from DP $CD69_{high}$ $CD3_{high}$ thymocytes with the stimulation of CD40L and IL-3 (Martin-Gayo, Sierra-Filardi et al. 2010). CD40L-CD40 interactions maintain normal numbers of thymic Tregs and a deficiency of them results in the reduction of Tregs (Williams, Tai et al. 2015). Medullary epithelial cells promote Treg generation by stimulating IL-2 production via ICOS ligand in the human thymus (Nazzal, Gradolatto et al. 2014).

CD28 is the main costimulatory molecule in T cells and plays a significant role in T cell activation in the periphery. Its ligands B7-1 (CD80) and B7-2 (CD86) are also expressed in the thymus and many studies in murine models have reported CD28 to be necessary for Treg development in the thymus (Williams, Tai et al. 2015). CD28-deficient mice have decreased frequencies of Tregs (Salomon, Lenschow et al. 2000) and TCR/CD28 downstream signalling including NFAT and AP-1 regulates Foxp3 transcription. In mice the expression level of CD28 is very low in double negative thymocytes that do not express TCRβ but it is upregulated at the DP phase (Williams, Hathcock et al. 2005). CD28 costimulation is required for IL-2 production but it also induces Foxp3 expression and upregulates CTLA-4 and GITR in TCR-signalled DP thymocytes in vitro (Tai, Cowan et al. 2005). Thus, CD28 is essential for Treg development by promoting IL-2 production (Jenkins, Taylor et al. 1991) but there is also an intrinsic requirement for CD28 costimulation.

The role of TGF-β in thymic Treg development has been debated (Goldstein 2013). In the periphery it converts conventional T cells to Tregs and upregulates the expression of Foxp3. In murine thymus TGF-β might confer survival advantage to Treg precursors but does not induce Foxp3

expression (Ouyang, Beckett et al. 2010). In contrast, Konkel et al. have suggested that thymocyte apoptosis leads to the production of TGF-β, which in turn promotes Foxp3 induction (Konkel, Jin et al. 2014). The loss of TGF-β receptor I reduced the number of CD4+CD25+Foxp3+ thymocytes but the effect was only temporary. Increased production of IL-2 rescued the number of Tregs (Liu, Zhang et al. 2008).

In addition to IL-2, IL-7 and IL-15, common γ chain cytokines also include IL-4, IL-9 and IL-21. The roles of IL-9 and IL-21 require further investigation. IL-9 signalling is essential for early thymocyte development but its role in Treg differentiation is unclear (De Smedt, Verhasselt et al. 2000).

AIMS OF THE STUDY

I To provide an estimate of TCR diversity in the human thymus

II To study the timing of commitment to the regulatory T cell lineage

III To study the effects of common γ chain cytokines on regulatory T cell development

SUMMARY OF MATERIALS AND METHODS

1 CELL ISOLATION AND CULTURE

1.1 Patient samples (I-IV)

Thymic tissue was obtained from a total of 99 children (47 males, 52 females) undergoing corrective cardiac surgery. Tissue is routinely removed for improved exposure during cardiac surgery. From one donor we also received a blood sample (I). The pediatric ethics committee of Helsinki University Hospital approved the study, and informed consent was obtained from the parents of the children. The study was performed in accordance with the Declaration of Helsinki.

Table I. Thymic samples.

	Number	**Median age (years)**	**Range (years)**	**Females**
I	4	0.5	0.1 - 0.7	1
II	13	0.3	0 - 9.3	10
III	42	0.5	0 - 10.8	20
IV	40	0.5	0 - 10.5	21

1.2 Cell isolation (I-IV)

Thymocytes were released within 6 hours of the thymectomy from the thymus tissue sample by mechanical homogenisation. The blood sample was prepared by lysing erythrocytes with brief incubation in sterile aqua.

1.3 Cell culture and apoptosis detection (III-IV)

Thymocytes were cultured in a 12-well plate with 1 millilitre human cell culture media (RPMI (Life Technologies, Paisley, UK), 10% heat-activated hAB serum (Finnish Red Cross Blood Service), 1% 100 x penicillin-

streptomycin, 1% HEPES, 0.1% L-glutamine, 0.1% MeEtOH) overnight. IL-2, IL-15 and IL-7 were purchased from Immunotools and were used at a final concentration of 25 ng/ml (IL-2) and 10 ng/ml (IL-15 and IL-7) in cell culture (Caramalho, Nunes-Silva et al. 2015).

Stimulation with anti-CD3 mAb was done in solution. The cells were incubated for 10 minutes on ice with 1 µg anti-CD3 x 10^6 cells, followed by cross-linking with 4 µg goat-antimouse IgG (Immunotools).

Apoptosis detection was done using the apoptosis staining kit with AnnexinV and PI (Beckton Dickinson) according to manufacturer's instructions. Since permeabilization cannot be used with apoptosis staining, Treg-associated marker CD25 was used instead of FOXP3. The populations of late apoptotic cells (AnnexinV+ PI+) and all apoptotic thymocytes (AnnexinV+PI+, AnnexinV+PI-, AnnexinV-PI+) were used in the analysis.

2 FLOW CYTOMETRY AND SORTING (I-IV)

2.1 Flow cytometry

The antibodies used in the experiments were direct fluorochrome conjugates: CD4-APC-Cy7, CD8-PE-Cy7, CD3-PE and CD3-APC, CD69-FITC (Immunotools, Friesoythe, Germany), CD4-PerCP, CD8-PE-Cy7, CD127-APC-Cy7, CD4-Biotin, Streptavidin-TexasRed, FOXP3-APC, CD5-PerCPCy5.5, ZAP-70-PE, CD4-AlexaFluor700, CD8-FITC, CD8-APC, FOXP3-PE, CTLA-4-PECy5, CTLA-4-APC, CD4-APC-Cy7, CD25-PECy5.5, CD25-FITC (Beckton Dickinson, San Jose, CA), CD25-PECy7, BCL-2-FITC, FOXP3-FITC (eBioscience, San Diego, CA) and Ki-67-PE (Santa Cruz Biotechnology, Dallas, Texas). Following the surface staining the cells were fixed, permeabilized and stained for FOXP3 and the other intracellular molecules Ki-67, CTLA-4 and BCL-2 using a FOXP3 staining kit from eBioscience (San Diego, CA), according to manufacturer's instructions.

Flow cytometry was performed using the Cyan ADP instrument (Beckman Coulter, USA). Analysis was done with Summit 4.3 and the FlowJo programs (FlowJo, LLC, Oregon, USA). Fluorescence compensation settings were optimised using BD Bioscience CompBeads (Beckton

Dickinson, San Jose, CA). Since doublets in the flow cytometric analysis of the DP population may lead to erroneous assignation of FOXP3+ cells (Lee and Hsieh 2009), we used minimal gating in the FSC vs SSC plot. In addition, we analysed the data using doublet exclusion by flow cytometric height and width parameters.

2.2 Sorting

The antibodies used in the flow cytometric sorting were direct fluorochrome conjugates: CD3-PE, CD8-APC, CD3-FITC, CD69-APC (Immunotools, Friesoythe, Germany), CD25-PE, CD4-APC-Cy7, CD8-PE-Cy7 (Beckton Dickinson, San Jose, CA) and CD4-Pacific Blue (eBioscience, San Diego, CA). Since with permeabilization and the intracellular staining of FOXP3 it is not possible to sort viable regulatory T cells, the surface marker CD25 was used instead of FOXP3.

Flow cytometry and sorting were performed using the FACSAria II instrument. Fluorescence compensation settings were optimised using BD Bioscience CompBeads (Beckton Dickinson, San Jose, CA). During sorting we excluded doublets by using flow cytometric height and width parameters.

3 PCR AND SEQUENCING

3.1 RNA extraction and quantitative PCR (IV)

Total RNA was isolated using RNeasy MiniKit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and first-strand cDNA was synthesised using oligo-dT-primer (Sigma) and AMV-reverse transcriptase (Finnzymes, Helsinki, Finland). The mRNA level of FOXP3 was quantified using Taqman Universal Master Mix (applied Biosystems) and Bio-Rad iCycler (Hercules, CA).The quantitative PCR data was normalised against β-actin expression levels. The primer-probe sets for FOXP3 and β-actin were bought from Applied Biosystems.

3.2 Methylation analysis (II-III)

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Bisulfite conversion was performed with Qiagen Epitect, according to the manufacturer's instructions. The demethylation

status of the TSDR region was quantified by real-time PCR as previously described with minor modifications (Wieczorek, Asemissen et al. 2009). PCR was performed with Bio-Rad iCycler (Hercules, CA).

3.3 Genomic DNA extraction and sequencing (I-II)

Frozen cell samples were either processed by Adaptive Biotechnologies (Seattle, USA) or genomic DNA extraction was performed in our laboratory according to the manufacturer's instructions (QIAsymphony, Qiagen, Germany). The amount of DNA and the quality of the samples were verified before sequencing.

The TCR α and β CDR3 region was amplified and sequenced from a standardised quantity of DNA. In the ImmunoSEQ assay of Adaptive Biotechnologies a multiplex PCR system was used to amplify the rearranged CDR3β and CDR3α sequences from sample DNA. This produced fragments of sufficient lenght to identify the VDJ region spanning each unique CDR3. Amplicons were sequenced using the Illumina platform. IMGT database (www.imgt.org) was used to define TCRβ V, D and J, and TCRα V and J genes. PCR and sequencing errors were minimised using PCR bias correction and a modified nearest-neighbour algorithm. Data was analysed using the ImmunoSEQ analyser toolset.

3.4 RNA-sequencing (II)

The method of RNA sequencing was based on the Drop-seq protocol (Macosko, Basu et al. 2015). 10 ng of RNA was mixed with Indexing Oligonucleotides (Integrated DNA Technologies) and cDNA was constructed. cDNA samples were amplified by PCR and the PCR products fragments were prepared using the Nextera XT (Illumina) tagmentation reaction. The concentration and quality of purified samples were analysed and the libraries were sequenced using Illumina NextSeq 500 at the Functional Genomics Unit of the University of Helsinki. The analysis pipeline suggested in Macosko et al. was used. Reads were filtered to remove polyA tails of length 6 or greater and aligned to the human (GRCh38) genome using STAR aligner (Dobin, Davis et al. 2013) with default settings. Uniquely mapped reads were grouped according to the 1-8 barcode, and gene transcripts were calculated by their Unique Molecular Identifiers (UMIs) to reduce the bias emerging from the PCR amplification.

Digital expression matrices (DGE) showed the number of transcripts per gene in a given sample.

4 STATISTICAL ANALYSIS

4.1 Extrapolation of total TCR diversity by rarefaction curves (I)

Rarefaction curves were constructed from the sequence data (number of unique TCRβ or α sequences as a function of observed cells) by generating a vector of TCR sequences with as many entries per sequence as observed in the data, randomly reordering the elements of the vector, and then reading the elements one by one while keeping track of the number of unique sequences. The resulting curves were extrapolated to $n = 1.3 \times 10^9$ cells with the software packages, preseq (Daley and Smith 2013) and DivE (Laydon, Melamed et al. 2014, Laydon, Bangham et al. 2015). The C++ package preseq computes a power-series formula that estimates how many times each sequence would be observed in a similar experiment of the same size. Using bootstrapping produces 95% confidence intervals. The R package DivE fits 58 different functions to the rarefaction curves and their subsamples. Then it scores each function and computes the geometric average of the extrapolations of the 5 best-scoring functions. Confidence intervals are not produced for individual functions but the spread of the 5 best-scoring functions can be considered as indicative of accuracy.

4.2 Extrapolation of total TCR diversity by incidence data (I)

The nonparametric estimator Chao 2 (Chao 1987) is mostly used to estimate species diversity in ecological unseen species studies. It considers each unique TCR sequence as a species and estimates the total species diversity based on incidence data. High overlap between species columns means that most of the diversity has been sampled. The R package *fossil* was used for implementing Chao 2 (Vavrek 2011).

4.3 Statistical analysis of the flow cytometric data (I-IV)

Statistical analysis was done with SPSS and GraphPad Prism software. The results are presented as arithmetic means and standard deviations. P-values for differences were calculated using Student´s two-tailed paired T-test. P-values <0.05 were considered significant.

RESULTS

1 TCR diversity in the human thymus (I)

The actual diversity of the total peripheral TCR repertoire in humans cannot be directly measured. Previously reported assessments of TCR repertoire are based on sequencing T cell compartments in blood samples and extrapolation to actual peripheral diversity, but human intrathymic diversity has not been previously estimated. We provide the first estimation of human TCR α and β diversity in the thymus and a new directly measured lower bound for T cell diversity.

1.1 Sequencing of TCR genes

This study included four thymus samples from otherwise healthy children undergoing corrective cardiac surgery. Flow cytometric assessment of thymic populations showed a normal distribution. Thymus sample 1 was isolated in its entirety and contained a total of 1.3×10^9 thymocytes. Genomic DNA was extracted from the total amount of cells and three samples, each the equivalent of 12×10^6 cells, were analysed. In the thymus samples 2, 3 and 4, 10×10^6 thymocytes were isolated and the gDNA equivalent of 4×10^6 cells analysed. From thymus 2, two separate samples of 10×10^6 thymocytes were extracted and analysed.

The complementarity-determining region 3 sequences of TCRβ were amplified and sequenced from all four thymus samples and CDR3 sequences of TCRα/δ from thymus samples 2-4. The number of observed clonotypes directly yields a lower bound of T cell diversity. Since some T cells express α chains utilising Vδ or Jδ gene segments (Chien et al. 1987, Satyanarayana et al. 1988) it was not possible to reliably separate TCRα and TCRδ loci. A small part of the sequences was therefore likely to be derived from γδ T cells.

From sample 1 we obtained an average of 47.4×10^6 TCRβ reads per replicate and 17.1×10^6 reads from thymuses 2-4 and approximately 80% of the sequences were productive (I, Table I). The number of unique productive TCRβ sequences found ranged from 1.5×10^6 in thymus 4 to 10.3×10^6 in thymus 1 when all three replicates were combined, the highest TCRβ diversity so far directly measured. From thymus samples 2-4 we obtained

an average of 34.9 x 10^6 TCRα reads. Only 32% of them were productive; this is most likely due to the lack of allelic exclusion in the TCRα locus (I, Table II). From the two combined replicates of thymus 2 we measured 3.7 x 10^6 unique productive sequences which is the highest directly measured TCRα diversity to date. In addition, we received a blood sample from the donor of thymus 1. Sequencing of 3.4 x 10^6 total TCRβ reads produced 86 000 unique productive sequences and 4000 of them were shared with the thymus repertoire.

1.2 Estimation of total TCRβ diversity

Since the sample size in sequencing is limited we used three computational estimators to extrapolate the total diversity in the entire thymus. The replicates of thymus 1 shared only a small fraction of the repertoire, indicating significantly larger total diversity. Estimators preseq and DivE were based on measuring the saturation of rarefaction curves and Chao2 on the size of the overlap between samples. Rarefaction curves measure the number of observed unique TCRβ sequences as a function of the number of sequenced cells and they saturate when the complete diversity has been sampled. Estimator preseq (Daley and Smith 2013) produced 73.1 x 10^6 unique TCRβ sequences in the thymus 1, with 95% confidence intervals of 43.3 - 129.9 x 10^6, and between 13.9 x 10^6 and 37.6 x 10^6 in thymus samples 2-4 (Table II).

DivE is also based on rarefaction curves but fits a large number of functions to the curves, scores each with 4 criteria and uses the geometric mean of the five best models as the final estimate (Laydon, Melamed et al. 2014). In the thymus 1 DivE extrapolated 42.5 x 10^6 unique TCRβ sequences and 9.2 - 10.5 x 10^6 unique sequences in samples 2-4. Chao2 is based on incidence data and it estimated a total diversity of 46.4 x 10^6 unique sequences in the thymus 1 and 30.0 x 10^6, 10.0 x 10^6 and 9.6 x 10^6 sequences in thymuses 2-4 (Table II).

Table II. TCR diversity estimates (x10^6)

Sample	Preseq	DivE	Chao2
TCRβ			
1	73.1	42.5	46.4
2	37.6	10.5	30.0
3	18.7	9.6	10.1
4	13.9	9.2	9.6
TCRα			
2	83.4	28.8	103.0
3		18.6	16.9
4	37.7	22.7	19.5

1.3 Estimation of total TCRα diversity

Estimates of the TCRα repertoire in thymuses 2-4 yielded consistently higher numbers of unique sequences than corresponding TCRβ diversity. Preseq produced 83.4 x 10^6 sequences in thymus 2 and 37.7 x 10^6 in thymus 4 but failed for thymus 3. DivE extrapolated to 28.8 x 10^6, 18.6 x 10^6 and 22.7 x 10^6 unique TCRα sequences in thymuses 2-4 and the estimates based on Chao2 were 103 x 10^6, 16.9 x 10^6 and 19.5 x 10^6 TCRα sequences in thymuses 2-4, respectively (Table II).

2 TCR repertoire in the regulatory T cells in the human thymus (II)

2.1 Sequencing of TCR genes in the sorted thymic populations

It has been previously shown that DP regulatory T cells contribute to the CD4SP Treg pool in the human thymus (Nunes-Cabaco, Caramalho et al. 2011). To analyse the Treg developmental pathway we sorted CD25+ DP and CD25+ CD4SP subsets as well as their CD25- counterparts from 5 pediatric thymus samples (samples A-E). The donors were otherwise healthy children undergoing corrective cardiac surgery. To avoid analysing doublets as DP cells a strict gating strategy was used. Since with permeabilization and the intracellular staining of FOXP3 it is not possible to sort viable regulatory T cells, the surface marker CD25 was used instead of FOXP3. The mean frequency of CD25+ cells was 0.7% (range 0.5-0.9%) in the DP population and 14.2% (range 11.9-15.7%) in the CD4SP population.

Genomic DNA was extracted from the sorted populations. The complementarity-determining region 3 (CDR3) sequences of TCRβ were then amplified and sequenced. The obtained reads depended on the size of the cell subset in the thymus. 70-80% of the sequences were productive. The number of unique productive TCRβ sequences found ranged from 600 to 50 000. We sorted thymus samples A and B to six different populations: CD4SP, CD8SP, DP $CD3_{high}$, DP $CD3_{low}$, DP CD25+ and CD4+ CD25+ cells and the samples C, D and E to four populations: DP CD25+, DP CD25-, CD4SP CD25+ and CD4SP CD25-. In samples A and B we combined the data in the populations DP $CD3_{high}$ and DP $CD3_{low}$ to form a DP CD25- subset. In these combined cell subsets the CD25+ cells were not excluded in the sorting.

Consistent with the previously reported shortening of CDR3 during thymic selection, the CDR3 length was shorter in the productive rearrangements than in the nonproductive rearrangements in all populations. At the DP stage the mean CDR3 was longest 45.6bp and the length shortened during development. The shortest lengths were detected in DP CD25+ (mean 43.4bp) and CD4 CD25+ (mean 43.7bp) cells.

2.2 DP CD25+ and CD4 CD25+ thymocytes share nucleotide and amino acid sequences

To track the developmental pathway of the regulatory T cells at the DP stage we compared the repertoire between DP CD25+ and other thymic populations by calculating the Jaccard index obtained by dividing the number of shared sequences by the combined size of the samples. We compared the overlap between the regulatory populations, between DP CD25+ and CD4SP populations and between DP CD25+ and the total DP population. Comparing the common unique and total number of nucleotide sequences showed that DP CD25+ and CD4 CD25+ populations were clearly closer to each other than any other population and the average Jaccard indexes 0.0022 (unique common sequences) and 0.0079 (total common sequences) were higher than between other populations. Thymic CD4SP population had unexpectedly low total reads which is one source of error in the analysis. In the nonproductive repertoire the regulatory populations were also closer to each other than any other populations.

In the repertoire of amino acid sequences the results were similar to nucleotide sequence analysis. In most of the samples the Jaccard index between DP CD25+ and CD4 CD25+ populations was higher than in the other two combinations and the average Jaccard indexes were 0.0049 (unique amino acid sequences) and 0.0130 (total amino acid sequences). This data supports the conclusion that especially at the nucleotide sequence level, DP CD25+ thymocytes share a similar TCR repertoire with the CD4 CD25+ population indicating that they are part of the common clonal and developmental pathway.

In samples A and B we compared the overlap between DP CD25+ thymocytes and DP $CD3_{high}$, DP $CD3_{low}$, CD4SP and CD8SP populations. In both nucleotide and amino acid sequence levels the highest Jaccard index was calculated with a DP $CD3_{high}$ subset. It has been previously shown that most Tregs express CD3 and are positively selected (Tuovinen, Pekkarinen et al. 2008, Lehtoviita, Rossi et al. 2009). The overlap with other thymic populations was quite small.

2.3 Methylation analysis of the DP and CD4SP CD25+ populations

We have previously shown that both DP and CD4SP FOXP3+ thymocytes give equal expression of FOXP3 on a single cell level measured with mean fluorescence intensity (Tuovinen, Pekkarinen et al. 2008). However, the epigenetic status of the Treg-specific demethylated region (TSDR) has also been shown to be essential in stabilising Treg phenotype (Toker, Engelbert et al. 2013). To test the demethylation status of the developing Tregs at the DP and CD4SP stage in the human thymus, we sorted four thymus samples (thymus samples F-I) to DP CD25+ and CD4SP CD25+ populations and isolated the genomic DNA. A real-time PCR method quantified the relative proportion of methylated and demethylated TSDR DNA using methylation-dependent amplification primers (Wieczorek, Asemissen et al. 2009). The mean delta Ct value ($\Delta Ct = Ct$ (demethylated DNA) – Ct (methylated DNA)) was 6.2 ± 0.2 in the DP CD25- and 6.0 ± 0.1 in the CD4 CD25- population. In both DP and CD4 CD25+ cells the mean ΔCt value was significantly lower (0.8 ± 0.2 and 0.8 ± 0.6, $p<0.001$, $n=4$). There was no difference between the DP and CD4 CD25+ populations in their demethylation status. Thus, a stable Treg phenotype can be detected already at the DP stage.

2.4 Differential gene expression analysis of DP and CD4SP Tregs

To further characterise the human regulatory populations in the thymus we used RNA sequencing. We again sorted DP CD25+ and CD4SP CD25+ thymocytes from 4 pediatric thymus samples (thymus samples J-M) and analysed gene expression changes between these populations using the edgeR Bioconductor package. In the analysis we found no statistically significantly expressed genes between DP CD25+ and CD4SP CD25+ cells.

3 The role of cytokines in regulatory T cell development (III-IV)

Regulatory T cell development has been extensively studied in murine models and common γ chain cytokines have been identified as essential (Burchill, Yang et al. 2007, Burchill, Yang et al. 2008). A two-step-model suggests that TCR signalling induces the expression of CD25 and allows the cytokines to promote Treg development at the CD4 single positive stage (Burchill, Yang et al. 2008, Lio and Hsieh 2008). However, human studies

favour an earlier emergence of Tregs at the CD4+CD8+ double positive stage. We have studied the role of cytokines IL-2, IL-7 and IL-15 at the DP phase in the human thymus.

3.1 Interleukin-2 (IV)

3.1.1 IL-2 selectively promotes the development of the most mature DP cells

IL-2 has been suggested to be the main cytokine enhancing Treg development at the CD4SP stage (Caramalho, Nunes-Cabaco et al. 2015) but FOXP3 upregulation has also been reported at the DP stage (Nunes-Cabaco, Caramalho et al. 2011). Since commitment to the regulatory lineage in the human thymus already starts at the DP stage, we analysed in detail the role of IL-2 in the development of FOXP3+ DP cells and subdivided DP thymocytes into three maturation stages based on CD3 expression levels. At the earliest stage thymocytes lack the CD3 molecule on their surface but start to express it at the $CD3_{low}$ phase. The transition to the CD4SP stage takes place after the most mature DP $CD3_{high}$ stage. The expression of the high-affinity IL-2 receptor CD25 in the FOXP3+ thymocytes varied between the different developmental stages. It was very low at the $CD3_{neg}$ stage, but then increased to 26.0% ± 13.9% at the $CD3_{low}$ and 39.8% ± 11.2% at the $CD3_{high}$ stage (IV, Figure 3A).

Thymocytes were cultured overnight with or without stimulation with IL-2. In general, IL-2 produced a significant increase in the FOXP3 mRNA level in the thymocyte population. The relative amount of FOXP3 mRNA was measured with quantitative PCR, using β-actin to normalise the data. In the flow cytometric analysis IL-2 increased the frequency of FOXP3+ cells from 0.7 % ± 0.5% to 1.0% ± 0.6% ($p<0.01$, $n=6$) within the DP population and also the FOXP3 content on a single cell level, as measured by mean fluorescence intensity (MFI) (IV, Figure 1). To analyse this in more detail we subdivided DP thymocytes into three maturation stages. Based on the expression of CD25, IL-2 has the potential to influence the developing thymocytes from at least the DP $CD3_{low}$ stage onwards. The highest FOXP3+ frequency was detected in the positively selected DP $CD3_{high}$ population. IL-2 did not have an effect on FOXP3 expression at the early DP stages but increased both FOXP3+ frequency from 2.4% ± 1.4%

to 3.5% ± 2.0% ($p<0.05$) and FOXP3 MFI from 36.1 ± 21.1 to 53.1 ± 24.5 ($p<0.01$) at the most mature $CD3_{high}$ stage (IV, Figure 3B).

IL-2 also promoted the expression of Treg-associated markers CTLA-4 (cytotoxic T-lymphocyte-associated molecule-4) and CD25. Within the FOXP3+ DP population the expression of intracellular CTLA-4 increased from 9.3% ± 1.6% to 13.2% ± 2.6% ($p<0.01$, n=8) and the CTLA-4 MFI from 156.6 ± 16.3 to 182.0 ± 22.4 ($p<0.05$). The biggest increase was detected at the DP $CD3_{high}$ stage (IV, Figure 3C). A minor increase in the frequency of CTLA-4 expressing cells was seen already at the $CD3_{low}$ stage but not in CTLA-4 MFI. Likewise, the frequency of CD25+ cells and CD25 MFI increased in the DP population after IL-2 stimulation (IV, Figure 2C). Thus, exposure to IL-2 at the DP stage both upregulates the expression of FOXP3 and promotes Treg associated markers and Treg phenotype. Together, these results suggest that IL-2 both upregulates the expression of FOXP3 and promotes Treg associated markers and Treg phenotype at the DP stage but only at the most mature DP $CD3_{high}$ stage.

3.1.2 Interleukin-2 enhances DP FOXP3+ cell survival

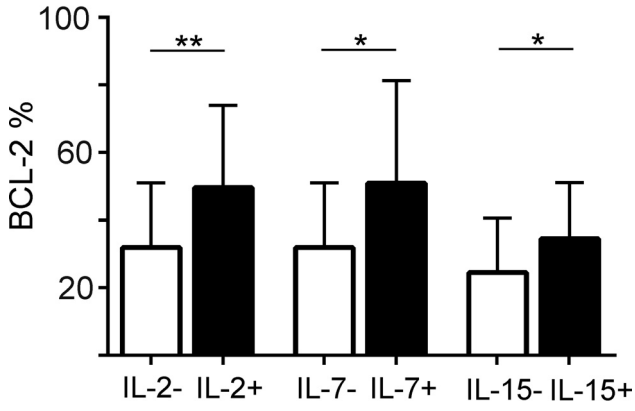
To study the effects of IL-2 on cell survival, we used Annexin V / propidium iodide staining for apoptosis detection and measured the expression of anti-apoptotic protein BCL-2 (B-Cell Lymphoma 2). The surface marker CD25 was used instead of FOXP3, since permeabilization of the cells is not compatible with the detection of apoptotic cells. The overnight culture with IL-2 decreased the frequency of Annexin V+PI+ late apoptotic cells within the CD25+ DP population from 50.5% ± 20.4% to 33.1% ± 18.5% (n=11, $p<0.001$) (IV, Figure 5A-B). In their counterparts (CD25- DP subset) very few apoptotic cells were observed, and IL-2 had no detectable effect.

BCL-2 expression was sharply downregulated during the transition from DN to DP stage, but in the DP FOXP3+ subset the frequency of BCL-2+ cells was significantly higher than in their FOXP3- counterparts, with an increasing trend in subsequent maturation stages. There was also an inverse correlation with cell proliferation, with most of the DP FOXP3+ cells expressing either BCL-2 or Ki-67 but not both (IV, Figure 5D). Overnight culture with added IL-2 induced a clear increase both in the expression of BCL-2 and BCL-2 MFI in the DP FOXP3+ population (IV, Figure 5E-F). Again, the effects were most prominent in the most mature FOXP3+ DP

$CD3_{high}$ stage, in which IL-2 increased BCL-2 frequency from 57.5% ± 25.0% to 64.0% ± 26.1% ($p<0.05$, n=10) and BCL-2 MFI from 115.2 ± 45.8 to 166.9 ± 61.7 ($p<0.01$) (IV, Figure 7A). At the $CD3_{low}$ stage IL-2 had a minor increasing effect on BCL-2 MFI. Taken together, this data shows that DP CD25+ thymocytes were highly susceptible to apoptosis but IL-2 promoted their survival and induced anti-apoptotic BCL-2 at the most mature DP $CD3_{high}$ stage.

It has been previously reported that IL-2 increases proliferation of FOXP3+ thymocytes at day 4 of culture (Caramalho, Nunes-Silva et al. 2015). We also measured the expression of the cell cycle marker Ki-67 after overnight culture. DP FOXP3+ cells were rapidly dividing, with 37.8% ± 27.2% expressing Ki-67, but IL-2 had no significant effect on their proliferation in any of the FOXP3+ DP subsets. DP cells are also subject to TCR-mediated selection, and IL-2 might modulate TCR-signalling and thus promote Treg maturation. To analyse the impact of IL-2 on TCR signalling we co-cultured the cells with anti-CD3 mAb and IL-2. This increased the frequency of FOXP3+ cells in the DP population from 0.3% ± 0.3% to 0.8% ± 0.8% (n=6, $p<0.05$) but this did not differ significantly from the effects of IL-2 stimulation alone (IV, Figure 4B).

Figure 7. The effects of common γ chain cytokines on the expression of BCL-2 in the DP FOXP3+ thymocytes. The mean (± SD) frequency of BCL-2 + cells in the DP FOXP3+ population with and without each cytokine in overnight culture. * $P < 0.05$, ** $P < 0.01$



3.2 Interleukin-7 (III-IV)

3.2.1 The expression of IL7R-α during thymic development

Although human peripheral regulatory T cells are defined by the low expression of CD127, it has been shown that CD127 is upregulated in FOXP3+ thymocytes at the DP stage (Nunes-Cabaco, Caramalho et al. 2011). We analysed CD127 expression in FOXP3+ and FOXP3- thymocyte populations at the different DP stages. In the FOXP3+ population, the highest expression of CD127, measured as mean fluorescence intensity (MFI), was detected in the DN population. At the DP stage CD127 MFI had a declining trend from $CD3_{neg}$ to $CD3_{high}$ stage with the sharpest drop occurring in the transition to SP stage (III, Figure 1). In contrast, in the FOXP3- population CD127 MFI peaked at the DP $CD3_{high}$ stage. The expression patterns of FOXP3+ and FOXP3- significantly differed from each other. The culture of DP FOXP3+ thymocytes with IL-7 led to CD127 downregulation by 40.0% ± 25.0% ($p<0.05$), indicating that DP Tregs are capable of responding to IL-7.

3.2.2 IL-7 promotes the development of regulatory T cells at the DP stage

To study the effects of IL-7 at the DP stage, we cultured human thymocytes overnight with and without IL-7. The frequency of DP FOXP3+ cells increased from 0.2% + 0.1% to 0.4 % ± 0.2% ($p<0.008$, $n=9$) with IL-7 exposure (III, Figure 2C). The single-cell level expression of FOXP3 measured with MFI in the DP FOXP3+ population did not significantly differ from the CD4SP stage. FOXP3 MFI increased from 27.3 ± 4.3 to 35.2 ± 8.5 ($p<0.02$) at the DP stage.

A similar increase was detected in the Treg-associated markers cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4) and CD25 (IL-2R). Both the frequency of CD25+ cells in the DP population and the expression of intracellular CTLA-4 increased in the DP FOXP3+ cells (III, Figures 3 and 5). IL-7 also induced an increase of CTLA-4 MFI within DP FOXP3 cells, indicating that it promotes Treg-associated phenotype.

3.2.3 IL-7 prevents apoptosis of regulatory T cells

To study the mechanisms that IL-7 uses to promote Treg development, we measured the expression of BCL-2 and the frequency of apoptotic cells with Annexin V and propidium iodide staining, defining apoptotic cells as including all but Annexin V/propidium iodide double-negative cells. DP CD25+ cells are highly susceptible to apoptosis, with 57.6% ± 16.9% apoptotic cells after overnight culture. IL-7 significantly decreased the frequency of apoptotic cells to 42.1% ± 13.5% ($p<0.03$, $n=13$) (III, Figure 6). In the DP CD25- subset IL-7 induced a minor decrease. The frequency of BCL-2 in the DP FOXP3+ population after overnight culture with IL-7 increased from 32.0% ± 19.0% to 50.9% ± 30.4% ($p<0.05$, $n=10$) and BCL-2 MFI from 172.1 ± 60.2 to 242.9 ± 17.4 ($p<0.01$) (IV, Figure 6F-G). Analysis of the different CD3 stages showed that the major effect of IL-7 was detected in the DP $CD3_{high}$ FOXP3+ subset but it also increased the BCL-2 frequency at the $CD3_{low}$ stage.

We then tested whether IL-7 affects TCR signalling, proliferation or methylation status of DP FOXP3 cells. We analysed whether preincubation with IL-7 changes the response of FOXP3+ DP cells to stimulation by anti-CD3 mAb. CD3 mAb stimulation alone induced an increase in CD69 MFI, a marker of positive selection, in DP thymocytes. Preincubation with IL-7 before stimulation via CD3 increased the frequency of FOXP3+ cells but these levels were not higher than those measured in cells incubated with IL-7 alone (III, Figure 4A). Similar to IL-2, IL-7 did not have an effect on TCR signalling. IL-7 is a growth factor and we measured the expression of the cell cycle marker Ki-67 in the DP FOXP3+ thymocytes. No increase was detected in the proliferation of DP FOXP3+ cells (III, Figure 4B).

Epigenetic changes in the Treg-specific demethylated region have been shown to be important in stabilising the Treg phenotype (Toker, Engelbert et al. 2013). To study whether IL-7 induces TSDR demethylation we used a real time quantitative PCR method previously described by Wierczorek et al. to measure the relative proportion of methylated and demethylated TSDR DNA (Wieczorek, Asemissen et al. 2009). IL-7 did not affect the methylation status but it is possible that subtle changes could have been missed since we used unsorted thymocytes.

3.2.4 IL-7 also increases FOXP3 expression in CD4 cells

The expression of CD127 in the CD4SP FOXP3+ thymocytes is low but we tested whether IL-7 has any effect on them. Surprisingly, stimulation with IL-7 resulted in a significant increase of FOXP3+ cells and FOXP3 MFI (III, Figure 7A-B). Similar effects were detected in the expression of intracellular CTLA-4 and CD25 (III, Figure 7D-F). CD4SP CD25+ cells were much less prone to apoptosis than DP cells. Only 10.2% ± 8.5% of them were apoptotic after overnight culture and adding IL-7 decreased the frequency to 3.2 % ± 1.4% ($p<0.02$). Thus the effects of IL-7 were very similar in the DP and SP populations, despite the low CD127 expression in CD4SP FOXP3 cells.

3.3 Interleukin-15 (IV)

Similar to IL-2 and IL-7, overnight culture with exogenous IL-15 increased the frequency of DP FOXP3+ cells from 0.4% ± 0.3% to 0.8% ± 0.6% ($p<0.05$, $n=8$) and FOXP3 MFI from 22.2 ± 4.1 to 27.4 ± 4.7 ($p<0.01$) (IV, Figure 6A-B). The most prominent increase was detected at the DP $CD3_{high}$ stage and no significant increase of FOXP3 was detected at the earlier DP stages. IL-15 also induced a decrease of late apoptotic Annexin V+ PI+ cells (IV, Figure 6C). The expression of BCL-2 increased, as well, but to a lesser degree than observed with IL-2 and IL-7, and there was no significant effect on BCL-2 MFI. In contrast to the other cytokines the increase in BCL-2 frequency was observed in the FOXP3+ DP $CD3_{low}$ cells and not in the $CD3_{high}$ cells (IV, Figure 7).

DISCUSSION

1 TCR diversity in the human thymus

A diverse TCR repertoire is essential for the immune system to recognise foreign antigens and defend the body against the universe of potential pathogens. This diversity is generated by somatic recombination of the TCR α and β gene segments in the thymus. Previous reports have estimated the total TCR diversity in the peripheral mature repertoire, identifying 1-3 x 10^6 unique TCRβ and 0.5 x 10^6 unique TCRα sequences. However, human intrathymic TCR repertoire diversity has not been previously studied.

High-throughput sequencing has enabled the identification of large numbers of TCR sequences from a single individual but total TCR diversity cannot be directly measured. In the peripheral blood only a small fraction of the lymphoid compartment can be sequenced in one sample. In pediatric thymus samples from children undergoing cardiac surgery the whole repertoire could be sampled and sequenced at least in theory. The limitations of the sequencing technology obliged to us to use mathematical estimators; we used three of these, preseq, DivE and Chao2, all with potential confounding factors. DivE fits 58 functions to the rarefaction curves, scores each function and selects the 5 best-scoring functions. The criteria for scoring are empirical and nonstandard. Low curvature in the rarefaction curves indicates too low sampled diversity and the results may be incorrect. Too low overlap between samples disturbs the estimation with Chao 2. However, the estimates from our thymus samples resulted in generally convergent outcomes.

The high-throughput sequencing of the thymus samples directly measured 10 million unique in-frame TCRβ sequences and 4 million TCRα sequences, the highest directly observed diversity so far for either chain. Computational estimators extrapolated the diversity to 40-70 x 10^6 unique TCRβ sequences and 60-100 x 10^6 TCRα sequences. Since each β chain can pair with at least 25 different α chains in the periphery (Arstila, Casrouge et al. 1999), actual TCRαβ diversity is significantly higher. In the thymus this pairing ratio is unlikely to be as high as in the periphery since the extensive thymocyte elimination complicates calculating the number of αβ heterodimers in the thymus.

Previous studies have reported peripheral TCRα diversity to be lower than in the β repertoire but in the thymus our data indicates the opposite. Our results showed thymic TCRα diversity to be 2-fold higher than TCRβ, which can be explained by the thymic maturation pathway and the mechanisms of the TCRα rearrangement and pairing with the β chain. The TCRβ chain rearrangement takes place at the double negative stage and the cells proliferate before the rearrangement of the V and J regions in the α chain locus. Therefore, thymocytes with the same β chain can pair with large numbers of different α chains. TCRα chain locus can go through multiple rearrangements whereas the recombination in the β locus occurs only once. Maximising the production of in-frame thymocytes is essential since positive and negative selection enormously limit the diversity. However, TCRα diversity probably contracts during positive and negative selection leading to lower peripheral α chain diversity.

Qian Qi et al. have previously used high-throughput sequencing and Chao2 estimator to estimate 100 million unique TCRβ sequences in naïve CD4 and CD8 T cell repertoires of young adults (Qi, Liu et al. 2014). Compared to this report, our results of 40-70 x 10^6 thymic β chains from the human thymus are smaller and the negative selection reduces the diversity even more. This is not surprising since selections and thymocyte egress constantly shape the transient repertoire. The extensive clonal deletion of thymocytes is apparent in the loss of TCRα diversity, when thymus and periphery are compared. Thymic diversity and clonal composition is continuously changing and our estimate describes the situation at one given time point. Dynamic thymic repertoire produces the clones for the peripheral T cell compartment. Comparing the repertoire in the thymus and blood sample from donor 1 showed that 4.7% of the peripheral β sequences were shared between them. This indicates some continuity between thymus and peripheral repertoire but recirculation may also contribute to the overlap.

We provide the highest directly measured diversity to date and the first estimate of the size of the human thymic TCR repertoire with 40-70 x 10^6 unique TCRβ sequences and 60-100 x 10^6 TCRα sequences. The thymic repertoire is thus extremely diverse, but based on the minor overlap between thymus and peripheral blood it is also likely to be transient and consists of variable clonal compositions with different clones produced at different times. The first steps have been made in measuring TCR diversity in different T cell compartments. In the future TCR sequencing provides

possibilities for characterising more deeply different T cell populations during thymic development.

Single-cell technologies have revealed the cellular heterogeneity of the immune cell populations (Islam, Zeisel et al. 2014, Proserpio and Lonnberg 2016) and identified unknown rare T cell subpopulations (Kakaradov, Arsenio et al. 2017). The analysis of rare cell types and TCR specificities of single thymocytes is essential for studying developmental pathways and selections in the thymus. In the future single cell studies are required to understand the complex T cell biology in autoimmunity and cancer.

2 Regulatory T cell development in the human thymus

Somatic recombination produces a diverse thymic TCR repertoire including also the thymocytes instructed to develop into regulatory T cells. Tregs and conventional T cells are suggested to arise as separate cell lineages and according to a two-step model TCR signalling increases the responsiveness of developing Tregs to IL-2 that induces the expression of Foxp3. The timing of the regulatory T cell commitment in the thymus is a matter of debate and the differences between human and mice are notable. The first FOXP3+ cells can be found already at the double negative stage (Tuovinen, Kekalainen et al. 2008, Liu, Li et al. 2014). In murine models Tregs are suggested to arise from Foxp3- precursors at the CD4 SP stage (Fontenot, Gavin et al. 2003, Fontenot, Dooley et al. 2005) with no direct link with DP Foxp3+ population in the thymic cortex. However, Treg cell commitment can occur in the cortex and the data from human studies favours this earlier commitment at the DP phase (Nunes-Cabaco, Caramalho et al. 2011). The main regulatory population comprises mature CD4 single positive thymocytes but the population is also clearly detected at the DP stage. Both the thymic medulla and cortex can support Treg development (Liston, Nutsch et al. 2008). DP FOXP3+ thymocytes express Treg-associated molecules, including CD25, CD39, GITR and CTLA-4 and exhibit suppressive capacity (Cupedo, Nagasawa et al. 2005, Darrasse-Jeze, Marodon et al. 2005, Liotta, Cosmi et al. 2005, Nunes-Cabaco, Caramalho et al. 2011). The majority of them are functionally mature and positively selected since they express high levels of CD3 and CD69 (Tuovinen, Pekkarinen et al. 2008, Lehtoviita, Rossi et al. 2009). Furthermore, linear regression models reported by Nunes-Cabaco et al. indicate that the FOXP3+ DP population

is the major contributor to the FOXP3+ CD4 SP subset (Nunes-Cabaco, Caramalho et al. 2011).

The TCR repertoire of Tregs has been reported to be specific to self-antigens (Hsieh, Zheng et al. 2006, Kim, Rasmussen et al. 2007, DiPaolo and Shevach 2009, Lee, Bautista et al. 2012) and the selection to Treg lineage requires intermediate-to-high TCR affinity to self-antigens (Caton, Kropf et al. 2014). Earlier studies have suggested partial overlapping between human peripheral Tregs and CD4 CD25- T cells (Fazilleau, Bachelez et al. 2007) but recent data indicates that regulatory and conventional T cells are completely distinct and non-overlapping (Golding, Darko et al. 2017). In murine thymus 6% of TCR sequences were shared between developing Tregs and conventional T cells (Wolf, Emerson et al. 2016). It was argued that since these cells share identical nucleotide code, they are derived from a common progenitor.

To track the developmental pathway of human regulatory T cells in the thymus we sorted DP CD25+ and CD4+ CD25+ thymocytes to compare the overlap between their TCR repertoires. CD25 is not a definitive marker for Tregs since it can be upregulated by conventional T cells. However, it is most commonly used for the purification of Tregs. Fazilleau et al. have detected a high level of FOXP3 in human peripheral CD4 $CD25_{high}$ cells (Fazilleau, Bachelez et al. 2007) and Tuovinen et al. have shown that high expression of CD25 is associated with FOXP3 in DP and CD4SP thymocytes (Tuovinen, Pekkarinen et al. 2008). To calculate the overlap between DP and CD4SP Tregs we used Jaccard index and the results showed that these populations were closest to each other on the nucleotide sequence level compared to other cell subsets. DP Tregs seem to be part of the common developmental pathway with CD4SP Tregs in the human thymus.

Demethylated TSDR is a key marker of natural regulatory T cells and it can be used to quantify Tregs in human peripheral blood instead of only FOXP3 expression. TSDR is a CpG-rich intronic enhancer region that regulates and maintains stable FOXP3 expression. Although conventional T cells can transiently induce FOXP3 expression, it is not sufficient to establish natural Treg phenotype since it does not induce all the Treg signature genes or lead to acquisition of suppressor function. Data from murine models has shown that the demethylation of TSDR is essential for thymic Treg development (Ohkura, Hamaguchi et al. 2012, Kitagawa, Ohkura et al. 2015), but the

demethylation pattern of TSDR during thymic development is unclear and the pathways leading to demethylation of TSDR are only partially known in humans. In murine thymus several Treg-associated gene regions such as CD25, CTLA-4, Eos and GITR have been recognised to be demethylated in regulatory lineage (Ohkura, Hamaguchi et al. 2012). The demethylation of TSDR was initiated during the early stages of thymic development and it could occur independently of FoxP3 expression (Ohkura, Hamaguchi et al. 2012). On the other hand, Toker et al. have shown that active demethylation of TSDR occurs in parallel with the induction of Treg-type gene expression and was completed as Tregs emigrated from the thymus (Toker, Engelbert et al. 2013). We compared the methylation status of the regulatory CD25+ populations between DP and CD4SP stages in the human thymus. Both Treg populations had a demethylated TSDR compared to their CD25- counterparts and no difference was seen between them. This data suggests that a stable Treg phenotype can be detected already at the DP stage supporting the previously reported data from the human studies. The commitment to Treg lineage can occur already at the double positive stage in the human thymus.

3 Cytokines dictate regulatory T cell development at the double positive stage

The factors inducing FOXP3 and enhancing commitment to the Treg lineage are only partially known in the human thymus. The development of the regulatory phenotype could also precede Foxp3 induction that stabilises the phenotype (Gavin, Rasmussen et al. 2007, Lin, Haribhai et al. 2007).The role of TCR-signals is essential and the selection to Treg lineage requires intermediate-to-high TCR affinity to self-antigens. Based on the data from murine models the two-step-model has been suggested. According to it TCR signalling upregulates IL-2R and increases the responsiveness of developing Tregs to IL-2 and other common γ chain cytokines that provide the second signal and induce the expression of Foxp3. The data from human studies supports earlier Treg commitment than in mice and thus I have concentrated in this thesis on the effects of cytokines IL-2, IL-7 and IL-15 at the double positive stage.

FOXP3+ thymocytes are first observed already at the CD4-CD8- double negative population stage (Liu, Li et al. 2014), where they lack TCR.

We have subdivided the DP phase into three stages based on the CD3 surface expression to study the effects of different cytokines. At the early DP stage thymocytes lack CD3 but start to express it at the DP $CD3_{low}$ stage. A functional TCR is first expressed at the DP $CD3_{low}$ stage and afterwards thymocytes are subjected to positive selection. At the most mature DP stage high expression of CD3 is detected. Positively selected FOXP3+ thymocytes are clearly found at the DP stage and they express Treg-associated markers. At the DP $CD3_{high}$ stage their FOXP3 expression measured with MFI is as high as in the CD4SP Tregs (Josefowicz, Lu et al. 2012). In this thesis I have studied at which stage the cytokines have an impact on Treg development.

Common γ chain cytokines are available for developing regulatory T cells in both thymic cortex and medulla. IL-7 has an essential role already in early T cell development and is secreted by thymic epithelial cells in the cortex (Mazzucchelli and Durum 2007, Tai, Erman et al. 2013). Caramalho et al. have also reported that IL-2 is expressed in both cortex and medulla and is thus available for developing T cells at the DP stage (Caramalho, Nunes-Silva et al. 2015). IL-15 is produced by macrophages and B cells in the thymus.

Regulatory T cells express high levels of CD25 and IL-2 is essential for the maintenance of the Treg population in the periphery. IL-2 signalling leads to phosphorylation of the transcription factors STAT3 and STAT5, which directly bind to a specific site in the first intron of the FOXP3 gene and initiate its expression (Zorn, Nelson et al. 2006). IL-2 has been previously identified as a key cytokine for Treg development. Nunes-Cabaco et al. have also shown that IL-7 signalling induces STAT5 activation in DP FOXP3+ thymocytes (Nunes-Cabaco, Caramalho et al. 2011). A recent study of human Treg development reported that IL-2 and IL-15 equally promoted Treg differentiation, focusing on the CD4SP stage (Caramalho, Nunes-Silva et al. 2015). In our study all three cytokines IL-2, IL-7 and IL-15 induced FOXP3 expression, promoted the regulatory phenotype and enhanced survival of FOXP3 thymocytes in the DP phase. The main impact was detected at the DP $CD3_{high}$ stage after positive selection.

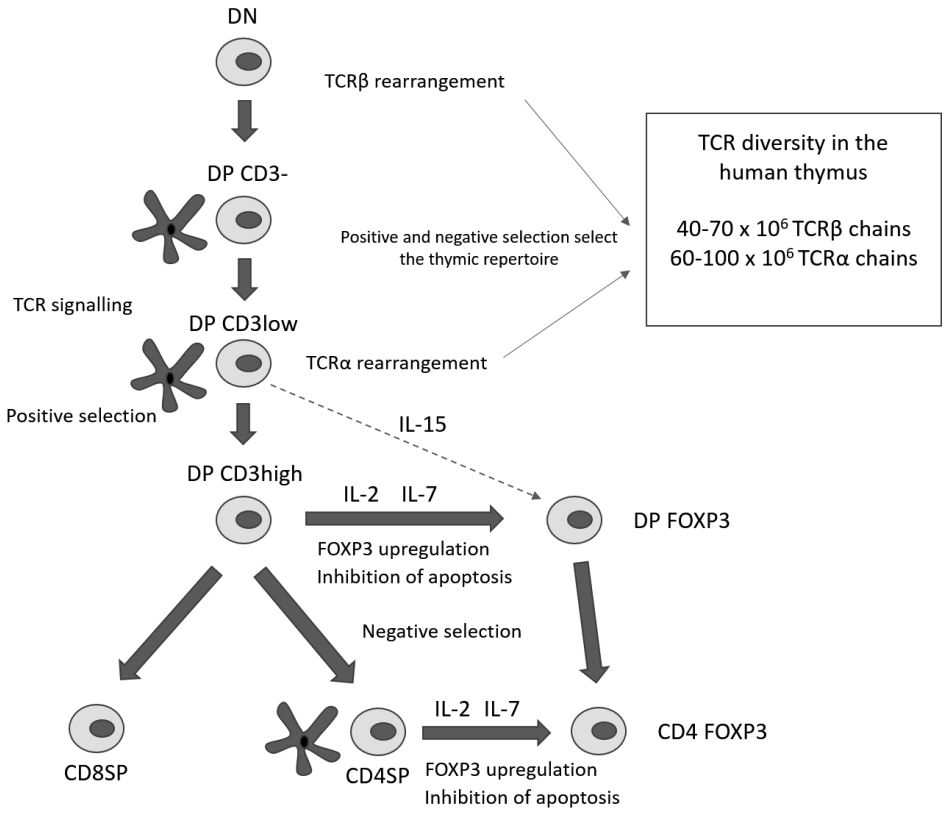
Peripheral mature human regulatory T cells are characterized as CD127 (IL-7Rα) negative and are not responsive to IL-7. However, in the human thymus developing DP FOXP3+ cells have relatively high expression of CD127

and stimulation with IL-7 upregulates STAT5 phosphorylation levels of DP FOXP3+ population. We also show that DP Tregs downregulate CD127 expression in response to IL-7. This indicates that DP FOXP3+ cells are responsive to IL-7 since it is able to directly modulate their receptor levels. SOCS1, a negative regulator in DP thymocytes, does not seem to have an effect on IL-7 signalling in DP FOXP3+ cells. A recent human in-vitro study considered IL-7 to play only a minor role in human Treg development (Caramalho, Nunes-Silva et al. 2015). In our study we show that human DP thymocytes are responsive to IL-7 and IL-7 increases the frequency of FOXP3+ cells and promotes Treg phenotype. Although CD127 was downregulated in the transition from DP to CD4SP stage, CD4SP FOXP3+ thymocytes remained responsive to IL-7. Exposure to IL-7 also increased their frequency and expression of Treg-associated markers.

To further investigate Treg development we studied the mechanisms of the different cytokines. At the CD4+CD8+ stage FOXP3+ thymocytes are highly susceptible to apoptosis but all three common γ chain cytokines selectively rescued them from cell death. They also upregulated the expression of anti-apoptotic BCL-2 in the DP population. In contrast to IL-2 and IL-7, IL-15 did not affect BCL-2 expression in the positively selected DP $CD3_{high}$ population but at the earlier $CD3_{low}$ stage. It has been previously reported that cytokines also increase proliferation of FOXP3+ cells (Caramalho, Nunes-Silva et al. 2015) but we did not detect an increase of Ki-67 expression in the Treg population. However, this might be due to shorter culture time. We used only overnight cultures and thus the effect of common γ chain cytokines on the proliferation rate cannot be ruled out.

Our results support a two-step model that has been well established in murine models. However, in the human thymus developing Tregs are clearly detected already at the DP stage. In the positive selection TCR signalling dictates Treg maturation and IL-15 enhances their survival. At the DP $CD3_{high}$ stage IL-2 and IL-7 increase the expression of BCL-2, suppress apoptosis and promote Treg phenotype before FOXP3+ cells migrate to the medulla.

Figure 8. TCR diversity and the development of FOXP3 regulatory T cells in the human thymus. Thymic TCR diversity is generated by somatic recombination of the TCR β chain locus at the DN stage and of the α chain locus at the DP stage. The diverse repertoire includes also the thymocytes instructed to develop into regulatory T cell lineage. Commitment to Treg lineage can occur at the DP or CD4SP stage during thymic development. The role of TCR-signals is essential for Treg development during positive selection. At the DP $CD3_{high}$ stage IL-2 and IL-7 upregulate the expression of FOXP3 and suppress apoptosis of DP FOXP3 Tregs.



CONCLUDING REMARKS

In this thesis I have studied T cell receptor diversity, the repertoire of developing regulatory T cells and the factors dictating regulatory T cell development in the human thymus. Analysis of TCR repertoire in the entire thymus provides knowledge of recombination machinery and thymic selections. Human intrathymic TCR repertoire diversity has not been previously studied and here we have estimated for the first time the total thymic diversity. Our estimation data showed an extremely diverse but transient TCR repertoire consisting of 40-70 x 10^6 unique TCRβ sequences and 60-100 x 10^6 TCRα sequences. Our directly measured diversity of 10.3 x 10^6 unique TCRβ sequences and 3.7 x 10^6 TCRα sequences sets a new lower limit for TCR repertoire diversity. Previous estimates have been based on sequencing peripheral repertoire in human blood samples. In contrast to the data from peripheral repertoire, we report TCRα diversity to be 2-fold higher than TCRβ diversity in the thymus. In the future TCR sequencing and single-cell technologies will provide methods for the deeper characterisation of different developmental stages and selections in the thymus. Studying TCR repertoires in autoimmune diseases and cancer can provide new insights into disease mechanisms and thus even new therapy options.

The diverse thymic repertoire also includes thymocytes that are instructed to differentiate into regulatory T cell lineage. The developmental pathway of natural regulatory T cells has been extensively studied in murine models but there are notable differences between human and murine Treg development. In the human thymus the commitment to Treg lineage can occur already at the CD4+CD8+ double positive stage and thus this thesis focuses on regulatory T cell differentiation and dictating cytokines at the DP stage. Tregs are characterised by the demethylation of Treg-specific-demethylated region and stable expression of the forkhead/winged-helix transcription factor FOXP3. To track the timing of Treg commitment we have used high-throughput TCR sequencing and methylation analysis of the TSDR. Comparing the common unique and total number of TCR nucleotide sequences showed that regulatory DP CD25+ and CD4SP CD25+ populations were clearly closer to each other than any other population, indicating that they are part of the common clone and developmental pathway. Comparing the methylation status of DP and CD4SP regulatory

populations showed no difference between them; a stable Treg phenotype can already be detected at the DP stage. This supports the previously reported human data which shows that the commitment to the regulatory lineage occurs at the CD4+CD8+ DP stage.

The common γ chain cytokines IL-2, IL-7 and IL-15 play an essential role in human regulatory T cell development. The significance of IL-2 for Treg differentiation has been well reported in murine models but IL-7 has been considered less important since mature peripheral Tregs express low levels of CD127. However, we show that during thymic development DP FOXP3+ thymocytes express CD127 and are responsive to IL-7. TCR signalling modulates Treg maturation at the DP stage but after positive selection cytokines upregulate FOXP3 expression, promote Treg phenotype and enhance the survival of DP FOXP3+ thymocytes. They are highly susceptible to apoptosis but IL-2, IL-7 and IL-15 avert cell death and upregulate the expression of anti-apoptotic protein BCL-2. Suppressing apoptosis seems to be a key mechanism for promoting Treg development at both the DP and CD4SP stage. Studying involvement of common γ chain cytokines in Treg development gives new insight into the timing of Treg commitment and selections of TCR repertoires.

Regulatory T cells maintain peripheral immunological tolerance and prevent harmful autoimmunity reactions. Research of normal Treg development provides the basis for recognising the mechanisms that lead to loss of tolerance. In the future manipulating Treg responses in humans could provide new applications for therapeutic interventions.

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