Autoimmune Regulator (AIRE) in the Maintenance of Immunological Tolerance

Eliisa Kekäläinen

Department of Bacteriology and Immunology, Haartman Institute, Faculty of Medicine, University of Helsinki, Finland

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty, University of Helsinki, in the lecture hall 2 of the Haartman Institute, Haartmaninkatu 3, on October 21st, 2011, at 12 o’clock noon

Helsinki 2011
Supervised by

Docent Petteri Arstila, M.D. Ph.D.
Department of Bacteriology and Immunology
Haartman Institute, University of Helsinki, Helsinki, Finland

Reviewed by

Professor Jorma Ilonen, M.D. Ph.D.
Department of Clinical Microbiology
University of Eastern Finland, Kuopio, Finland
and
Immunogenetics Laboratory
University of Turku, Turku, Finland

and

Professor Ilkka Julkunen, M.D. Ph.D.
Department of Viral Diseases and Immunology
The National Institute for Health and Welfare, Helsinki, Finland

Official opponent

Professor Olle Kämpe, M.D. Ph.D.
Department of Medical Sciences
Uppsala University, Uppsala, Sweden

© 2011 Eliisa Kekäläinen
Cover art: Tuisku-Tuulia Koivula
ISBN 978-952-10-7190-4 (PDF)
http://ethesis.helsinki.fi
Unigrafia 2011
To Mikael
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>TIIVISTELMÄ</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>LIST OF ORIGINAL PUBLICATIONS</strong></td>
<td>11</td>
</tr>
<tr>
<td><strong>ABBREVIATIONS</strong></td>
<td>12</td>
</tr>
<tr>
<td><strong>REVIEW OF THE LITERATURE</strong></td>
<td>14</td>
</tr>
<tr>
<td><strong>1 T LYMPHOCYTES AND THEIR FUNCTIONS</strong></td>
<td>14</td>
</tr>
<tr>
<td>1.1 <strong>ANTIGEN PRESENTATION AND T CELL ACTIVATION</strong></td>
<td>14</td>
</tr>
<tr>
<td>1.1.1 T Cell Receptors</td>
<td>14</td>
</tr>
<tr>
<td>1.1.2 Major Histocompatibility Complexes and Professional Antigen Presenting Cells</td>
<td>15</td>
</tr>
<tr>
<td>1.1.3 T Cell Activation</td>
<td>16</td>
</tr>
<tr>
<td>1.2 <strong>T CELL EFFECTOR SUBSETS AND THEIR FUNCTIONS IN THE IMMUNE SYSTEM</strong></td>
<td>17</td>
</tr>
<tr>
<td>1.2.1 CD8&lt;sup&gt;+&lt;/sup&gt; T Cells: Cytotoxic T Cells</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2 CD4&lt;sup&gt;+&lt;/sup&gt; T cells: T Helper Cells</td>
<td>18</td>
</tr>
<tr>
<td>1.3 <strong>T CELL DEVELOPMENT</strong></td>
<td>19</td>
</tr>
<tr>
<td>1.3.1 T Cell Development and T Cell Receptor Recombination</td>
<td>19</td>
</tr>
<tr>
<td><strong>2 T CELL TOLERANCE AND AUTOIMMUNE DISEASES</strong></td>
<td>25</td>
</tr>
<tr>
<td>2.1 <strong>CENTRAL VERSUS PERIPHERAL TOLERANCE</strong></td>
<td>25</td>
</tr>
<tr>
<td>2.2 <strong>PERIPHERAL TOLERANCE</strong></td>
<td>26</td>
</tr>
<tr>
<td>2.2.1 Restriction of Access to Tissue Specific Antigens and Tissues</td>
<td>26</td>
</tr>
<tr>
<td>2.2.2 Regulatory T Cells</td>
<td>26</td>
</tr>
<tr>
<td>2.3 <strong>AUTOIMMUNE DISEASES IN HUMANS</strong></td>
<td>36</td>
</tr>
<tr>
<td>2.3.1 Classification of Autoimmune Diseases</td>
<td>36</td>
</tr>
<tr>
<td>2.3.2 Genetic Background of Autoimmune Diseases</td>
<td>37</td>
</tr>
<tr>
<td>2.3.3 Environmental Factors in the Breakdown of Tolerance</td>
<td>38</td>
</tr>
<tr>
<td>2.3.4 Regulatory T cells in Human Autoimmune Diseases</td>
<td>44</td>
</tr>
<tr>
<td><strong>3 AUTOIMMUNE REGULATOR – GENE</strong></td>
<td>47</td>
</tr>
<tr>
<td>3.1 <strong>AUTOIMMUNE POLYENDOCRINOPATHY, ECTODERMAL DYSTROPHY, AND CANDIDIASIS (APECED)</strong></td>
<td>47</td>
</tr>
<tr>
<td>3.1.1 Clinical Phenotype of APECED</td>
<td>48</td>
</tr>
<tr>
<td>3.1.2 Autoantibodies in APECED</td>
<td>49</td>
</tr>
<tr>
<td>3.1.3 Other Genetic Factors Affecting APECED Phenotype</td>
<td>52</td>
</tr>
<tr>
<td>3.1.4 Other Human Diseases that Have Association with Reduced AIRE Expression, AIRE Mutations, or Polymorphisms in AIRE</td>
<td>53</td>
</tr>
<tr>
<td>3.2 <strong>MOUSE MODELS OF APECED</strong></td>
<td>54</td>
</tr>
<tr>
<td>3.2.1 Aire-deficient Mouse Models</td>
<td>54</td>
</tr>
<tr>
<td>3.2.2 Aire deficiency in Autoimmunity-prone Background Strains</td>
<td>55</td>
</tr>
<tr>
<td>3.2.3 Experimental Induction of Autoimmunity in Aire-deficient Mice</td>
<td>58</td>
</tr>
<tr>
<td>3.3 <strong>AUTOIMMUNE REGULATOR (AIRE) GENE</strong></td>
<td>59</td>
</tr>
<tr>
<td>3.3.1 The structure of AIRE and Structural Implications for Its Function</td>
<td>59</td>
</tr>
<tr>
<td>3.3.2 AIRE expression</td>
<td>61</td>
</tr>
<tr>
<td>3.3.3 The Functions of AIRE</td>
<td>65</td>
</tr>
<tr>
<td><strong>AIMS OF THE STUDY</strong></td>
<td>74</td>
</tr>
</tbody>
</table>
SUMMARY OF THE MATERIALS AND METHODS .................................................................75
1 SAMPLE MATERIAL ........................................................................................................75
  1.1 APECED PATIENTS AND HEALTHY CONTROLS (I) .........................................................75
  1.2 MICE (II-IV) .................................................................................................................. 75
  1.3 SAMPLE COLLECTION AND CELL ISOLATION (I-IV) .................................................. 76
2 MAGNETIC BEAD CELL SEPARATION AND \textit{IN VITRO} SUPPRESSION ASSAY (I) ....77
3 ADOPTIVE CELL TRANSFERS (II-IV) AND COLITIS CLINICAL SCORE (III-IV) ......77
4 POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING ..................................78
  4.1 RNA AND DNA ISOLATION, CDNA SYNTHESIS, AND REAL-TIME PCR (I-IV) ............78
  4.2 TCR REPERTOIRE ANALYSIS (I-II) .............................................................................. 79
  4.3 SEQUENCING (II) ........................................................................................................... 79
5 FLOW CYTOMETRY AND CELL SORTING WITH FLOW CYTOMETRY (I-IV) ..........80
6 HISTOLOGICAL ANALYSIS (II-IV) ..................................................................................80
7 IMMUNOHISTOCHEMISTRY STAINING (I, III) ...............................................................81
8 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (I, III-IV) ..........................82
9 STATISTICAL ANALYSIS (I-IV) .....................................................................................83
10 ETHICAL CONSIDERATIONS (I-IV) ..............................................................................83

RESULTS AND DISCUSSION ...........................................................................................84
1 DEFFICIENT REGULATORY T CELLS IN APECED PATIENTS (I) ...............................84
  1.1 AIRE EXPRESSION IN THE HUMAN THYMUS IS CONCENTRATED AROUND HASSALL’S CORPUSCLES ................................................................................................................... 84
  1.2 PHENOTYPE AND TCR REPERTOIRE OF TREGS IN APECED PATIENTS ..................86
    1.2.1 Reduced Expression of FOXP3 in Tregs from APECED Patients ............................... 86
    1.2.2 Tregs of APECED Patients Have a Naïve-like TCR Repertoire .............................. 87
  1.3 DEFICIENT SUPPRESSIVE FUNCTIONS OF APECED PATIENT TREGS ..................... 88
  1.4 FOXP3 EXPRESSION IN AIRE-/- MICE ........................................................................ 90
2 LYMPHOOPENIA-INDUCED PROLIFERATION AS A MODEL FOR STUDYING POLYCLONAL T CELL RESPONSES IN AUTOREACTIVITY-PRONE EXPERIMENTAL CONDITIONS (II) .................................................................................................91
  2.1 LYMPHOOPENIA-INDUCED PROLIFERATION AS A MODEL TO ENRICH SELF- OR NORMAL FLORA-REACTIVE CLONES FROM POLYCLONAL POPULATION ................................................. 92
  2.2 DUAL-SPECIFIC TCR REPERTOIRE GROWS MORE OLIGOCONAL IN LYMPHOOPENIA-INDUCED PROLIFERATION THAN SINGLE-SPECIFIC TCR REPERTOIRE .............................................. 93
  2.3 LYMPHOOPENIA-INDUCED PROLIFERATION CAN BE USED TO ENRICH AUTOACTIVE CLONES FROM A POLYCLONAL REPERTOIRE ................................................................................ 95
3 LOSS OF AIRE EXPRESSION IN THE THYMUS IS NOT SUFFICIENT TO CAUSE AUTOIMMUNITY IN LYMPHOOPENIA-INDUCED PROLIFERATION (III) ........................................ 98
  3.1 LYMPHOOPENIA AND AIRE – POSSIBLE SYNERGISTIC FACTORS IN THE BREAKDOWN OF TOLERANCE? .................................................................................................................. 98
  3.2 LYMPHOCYTES ADOPTIVELY TRANSFERRED FROM AIRE-/- DONORS TO NORMAL LYMPHOPEMIC HOSTS SHOW SIGNS OF IMMUNE DYSREGULATION ............................................. 100
  3.3 AIRE-GROUP SHOW A TH1-BIASED T CELL RESPONSE .............................................. 104
  3.4 HYPERPROLIFERATION OF AIRE-/- DONOR ORIGINATING TREGS IN GUT-DRAINING LYMPHOID TISSUE ....................................................................................................................... 105
4 LOSS OF AIRE EXPRESSION IN THE PERIPHERAL IMMUNE SYSTEM CAUSES SYMPTOMATIC AUTOIMMUNITY (IV) ........................................................................................................ 107

4.1 GENERATION OF LYMPHOPENIC AIRE-DEFICIENT MICE (AIRE-RAG1) ........................................... 107
4.2 ADOPTIVE TRANSFER OF UNSELECTED LYMPHOCYTES FROM A NORMAL DONOR GENERATES COLITIS IN AIRE-RAG1 MICE ........................................................................................................ 108
4.3 ADOPTIVELY TRANSFERRED NORMAL TREGS PROLIFERATE SLOWER IN AIRE-DEFICIENT LYMPHOPENIC MICE .............................................................. 111

5 SUMMARY OF THE RESULTS (III-IV) ................................................................................................ 113

CONCLUDING REMARKS AND FUTURE DIRECTIONS .................................................................... 116

ACKNOWLEDGEMENTS .................................................................................................................. 119

REFERENCES .................................................................................................................................. 122
ABSTRACT

Autoimmune regulator (AIRE) is the gene mutated in the human polyglandular autoimmune disease called Autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy (APECED) that belongs to the Finnish disease heritage. Murine Aire has been shown to be important in the generation of the T cell central tolerance in the thymus by promoting the expression of ectopic tissue-specific antigens in the thymic medulla. Aire is also involved in the thymus tissue organization during organogenesis. In addition to the thymus, AIRE/Aire is expressed in the secondary lymphoid organs. Accordingly, a role for AIRE/Aire in the maintenance of peripheral tolerance has been suggested. Peripheral tolerance involves mechanisms that suppress immune responses in secondary lymphoid organs. Regulatory T cells (Tregs) are an important suppressive T cell population mediating the peripheral tolerance. Tregs are generated in the thymus but also in the peripheral immune system T cells can acquire the Treg-phenotype.

The aim of this study was to characterize Tregs in APECED patients and in the APECED mouse model (Aire-deficient mice). In the mouse model, it was possible to separate Aire expression in the thymus and in the secondary lymphoid organs. The relative importance of thymic and peripheral Aire expression in the maintenance of immunological tolerance was studied in an experimental model that was strongly biased towards autoimmunity, i.e. lymphopenia-induced proliferation (LIP) of lymphocytes. This experimental model was also utilised to study the behaviour of T cells with dual-specific T cell receptors (TCR) during the proliferation.

The Treg phenotype was studied by flow cytometry and relative gene expression with real-time polymerase chain reaction. TCR repertoires of the Tregs isolated from APECED patients and healthy controls were also compared. The dual-specific TCRs were studied with the TCR repertoire analysis that was followed with sequencing of the chosen TCR genes in order to estimate changes in the dual-specific TCR diversity. The Treg function was tested with an in vitro suppression assay.

The APECED patients had normal numbers of Tregs but the phenotype and suppressive functions of the Tregs were impaired. In order to separate Aire functions in the thymus from its yet unknown role in the secondary lymphoid organs, the phenomenon of LIP was utilised. In this setting, the lymphocytes that are adoptively transferred to a lymphopenic recipient proliferate to stimuli from self-originating antigens. This proliferation can result in autoimmunity if peripheral tolerance is not fully functional. When lymphocytes that had matured without Aire in the thymus were transferred to lymphopenic Aire-sufficient recipients, no clinical autoimmunity followed. The Aire-deficient donor-originating lymphocytes hyperproliferated, and other
signs of immune dysregulation were also found in the recipients. Overt autoimmunity, however, was prevented by the Aire-deficient donor-originating Tregs that hyperproliferated in the recipients.

Aire-deficient lymphopenic mice were used to study whether peripheral loss of Aire had an impact on the maintenance of peripheral tolerance. When normal lymphocytes were transferred to these Aire-deficient lymphopenic recipients, the majority of recipients developed a clinically symptomatic colitis. The colitis was confirmed also by histological analysis of the colon tissue sections. In the Aire-deficient lymphopenic recipients Tregs were proliferating significantly less than in the control group’s recipients that had normal Aire expression in their secondary lymphoid organs.

This study shows that Aire is not only important in the central tolerance but is also has a significant role in the maintenance of the peripheral tolerance both in mice and men. Aire expressed in the secondary lymphoid organs is involved in the functions of Tregs during an immune response. This peripheral expression appears to be relatively more important in some situations since only those lymphopenic recipients that had lost peripheral expression of Aire developed a symptomatic autoimmune disease. This AIRE-related Treg defect could be clinically important in understanding the pathogenesis of APECED.
TIIVISTELMÄ


LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their Roman numerals.


The original articles are reprinted with the permission of their copyright holders.
ABBREVIATIONS

AIRE  Autoimmune regulator – gene/protein (human)
Aire  Autoimmune regulator – gene/protein (murine)
APC   antigen presenting cell
APECED autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy syndrome
BAFF  B cell activating factor of the TNF family
Card11 caspase recruitment domain family, member 11
Cbl-b  Casitas B-lineage lymphoma ubiquitin ligase
CD    cluster of differentiation
CDR   complement determining region
cTEC  cortical thymic epithelial cell
CTL   cytotoxic lymphocyte
CTLA-4 cytotoxic T Lymphocyte Antigen 4
DC    DC
dNA   deoxyribonucleic acid
ELISA Enzyme-linked immunosorbent assay
FOXP3 forkhead box P3 (human)
FoxP3 forkhead box P3 (mouse)
HIV   human immunodeficiency virus
HLA   human leukocyte antigen
HPRT  Hypoxanthine phosphoribosyl-transferase
IF    immunofluorescence
IFN-γ interferon γ
Ig    immunoglobulin
iNKT cells invariant natural killer T-cells
IL    interleukin
IPEX  Immunodysregulation – polyendocrinopathy – enteropathy – X-linked
iTreg induced regulatory T cell
KO    knockout mouse
LIP   lymphopenia-induced proliferation
mAb   monoclonal antibody
MHC   major histocompability complex
MLN   mesenteric lymph node
MS    multiple sclerosis
mTEC  medullary thymic epithelial cell
NK cell natural killer cell
NOD   non-obese diabetic
nTreg natural or thymic originating regulatory T cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>SAND</td>
<td>human Sp100, Aire1, NucP41/P75, and Drosophila DEAF1 domain</td>
</tr>
<tr>
<td>SAP</td>
<td>serum amyloid P component</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription protein</td>
</tr>
<tr>
<td>Tbet</td>
<td>T-box 21</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCR C</td>
<td>T cell receptor constant gene</td>
</tr>
<tr>
<td>TCR D</td>
<td>T cell receptor diversity gene</td>
</tr>
<tr>
<td>TCR J</td>
<td>T cell receptor joining gene</td>
</tr>
<tr>
<td>TCR V</td>
<td>T cell receptor variable gene</td>
</tr>
<tr>
<td>Tfhn</td>
<td>follicular T helper cell</td>
</tr>
<tr>
<td>TG</td>
<td>transgenic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TREC</td>
<td>T cell receptor excision circle</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSA</td>
<td>tissue specific antigen</td>
</tr>
<tr>
<td>Tr1</td>
<td>Type 1 regulatory T cells</td>
</tr>
</tbody>
</table>
REVIEW OF THE LITERATURE

1 T lymphocytes and Their Functions

T lymphocytes, or T cells for short, are a central part of adaptive immune system. All T cells express an antigen recognition receptor, the T cell receptor (TCR). T cells are divided into two main categories according to their co-receptor, the cluster of differentiation 4 or 8 (CD4 or CD8).

In order to mediate their many functions in the immune system, T cells must be activated. This process is strictly regulated and limited because once a T cell gets activated, it can activate other cells of the immune system. Uncontrolled or wrongly targeted T cell activation can lead to severe tissue destruction. T cell tolerance means that T cells are capable of recognizing self from foreign structures, and leaving host tissues unharmed.

1.1 Antigen Presentation and T Cell Activation

1.1.1 T Cell Receptors

T cells recognize their specific antigen with the cell-surface TCR that consists of two polypeptide chains, namely α and β, or γ and δ. In the peripheral immune system, surface expression of TCR is the best marker for mature T cells. Most effector T cells have an αβ receptor. The functions of γδ T cells are not yet fully understood. In the following sections when discussing T cell receptors, the focus will be on αβ receptors, if not otherwise indicated. Both α and β chains have three complement determining regions (CDRs). CDRs code for the parts of the TCR protein that eventually bind to the peptide – major histocompability complex (pMHC). CDR 1 and 2 bind to the MHC molecules, and CDR3, the most variable part of the chain, accounts for the binding of the peptide antigen. In the complete receptor there are in total six peptide loops (3 CDRs from the α chain and 3 from the β chain) that bind to the pMHC.

TCR spans through the cell membrane but its short cytoplasmic tail is incapable of transferring the TCR signal to intracellular signaling pathways. Thus the TCR is bound to the cell membrane by the CD3 molecule complex. This complex is crucial in the initiation of the intracellular signaling pathway that transfers the receptor signal into the nucleus. Without CD3 the receptor is unstable and cannot be transported to the cell membrane. The CD3 complex consists of several molecules that have the specialized intracellular motifs needed in signal transduction.
1.1.2 Major Histocompability Complexes and Professional Antigen Presenting Cells

T cells can only recognize antigens that are bound to the MHC protein. In humans the MHC molecules are called the human leukocyte antigens (HLA). The two main categories of MHC molecules are called type I and type II. Both types of molecules are highly unstable without a peptide antigen bound to the protein so MHC molecules are always expressed in the body as pMHC complexes. The general three-dimensional structures of both types of MHC molecules are very similar. They are heterodimeric proteins with a cleft that functions as an antigen-binding site. The antigen-binding cleft determines the type of the peptide that can be bound to the molecule: the MHC class II antigen-binding cleft is open at both ends so that it can bind longer peptide antigens, while only short peptide chains fit into the closed MHC class I molecules. Genetically MHC molecules are very polymorphic with the considerable variation in the antigen-binding site.

The CD8+ T cells can recognize their antigen only when it is bound to the MHC type I molecule that is expressed in all types of nucleated cells. The antigens presented in the MHC class I molecule are typically produced in the cell itself whereas antigens presented in MHC class II molecules originate from proteolysis of extracellular antigens in the endosomal compartment. MHC type II molecules are only expressed in the professional antigen presenting cells (APCs) and epithelial cells of the thymus. The CD4+ helper T cells can recognize antigens when they are expressed via MHC type II molecules.

The only cells capable of activating naïve T cells are called professional APCs. The most important population of APCs are the dendritic cells (DCs) that are found all over the body and especially on the body-environment interfaces, such as the skin and epithelial tissues. Other APCs are B cells and macrophages. DCs are divided into two main categories, myeloid and plasmacytoid DCs. These two main populations both comprise several subpopulations with different functions and anatomical distributions. In the following sections the myeloid DCs will be discussed briefly.

DCs are star-like cells that have many protrusions, which are used to sample the environment surrounding the cell. DCs express various innate pattern recognition receptors, such as Toll-like receptors (TLRs), for recognizing foreign microbial structures. DCs can engulf foreign material by macropinocytosis, phagocytosis, and receptor-mediated phagocytosis. DC can process the internalized material in endocytosomal vesicles and load antigenic peptides to an MHC class I or II molecules. MHC-antigen complex is then transported to the cell surface. If the surroundings of the DC have a high concentration of pro-inflammatory molecules, i.e. danger signals, the DC
becomes activated. Activated DCs are fully maturated and they express the co-stimulatory molecules required for naïve T cell activation. Co-stimulatory molecules include CD80 or CD86, which are collectively called the B7 molecules. The morphology of the DC changes upon activation; it becomes rounder and more mobile. It starts to express homing receptors guiding it to the local lymph node

The activated mature DCs produce large amounts of different cytokines. The cytokine profile they produce is determined by the initial signaling occurring via innate receptors. Therefore, DCs are important in initiating the correct T cell response to a given microbe. DCs can also secrete cytokines that can convert naïve T cells to suppressing cells that inhibit immune responses. It is not known what are the precise mechanisms for DCs to drive the pro-inflammatory or suppressive T cell responses.

1.1.3 T Cell Activation

T cell activation is restricted in many ways. T cell can recognize its specific antigen only when the antigen is bound to an MHC molecule. For full T cell activation costimulatory factors produced by interacting APC are needed. Due to these restrictions, T cell activation can in practice take place only in secondary organized lymphoid organs, mainly in lymph nodes and the spleen.

T cells need four different signals in order to become activated: i) TCR binding its specific antigen bound to the MHC, ii) co-stimulation from the APC, iii) activated co-receptor CD4 or CD8 signaling, and iv) the presence of T cell stimulating cytokines at the site of activation. The first signal is from the TCR binding the pMHC complex it is specific for. The second signal comes from the T cell surface molecule CD28 binding to the co-stimulatory factors CD80 or CD86 on the surface of the activated professional APCs. CD28 is constitutively expressed on most T cells. Only professional antigen-presenting cells can give the co-stimulation via B7 molecules that the T cells need to get fully activated. If only the first signal is present, the T cell can go into an anergic state, where it is not apoptotic but not functional either.

There are certain differences in the T cell requirement of co-stimulation. For instance, in certain conditions CD8+ T cells need less CD28-mediated co-stimulation than CD4+ cells. Meanwhile, suppressive regulatory T cells (Treg) are very dependent on CD28-mediated co-stimulation. Recently, inhibitory molecules of T cell activation, such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death ligand 1, have received increasing attention since they are involved in controlling the immune responses. Co-stimulation is therefore not an on-off switch, but more like a network of activating and inhibiting signals.
The third signal for T cell activation comes from the co-receptors CD4 or CD8 that bind pMHC class II and I, respectively. The co-receptor binding stabilizes the receptor complex so that sufficient signaling can take place. This binding also enhances TCR-derived intracellular signaling. Thus, TCR is about hundredfold more sensitive to the MHC-antigen complex-derived signal as compared with the pMHC binding alone\textsuperscript{141}.

The fourth signal for T cell activation comes from the different cytokines which APC is producing. The cytokines will determine what kind of effector functions T cell will acquire. The local cytokine milieu is very important in the CD4$^+$ T helper (Th) cell differentiation\textsuperscript{510} but it is also important in priming CD8$^+$ cytotoxic lymphocytes' (CTL) functions\textsuperscript{469}. Activated T cells are very prone to apoptosis and need high amounts of pro-inflammatory cytokines in order to survive. They recognize antigens like naïve cells, but there is no more a strict need for co-stimulation. When the inflammatory response wanes, the cytokine levels also go down. Activated T cells die when they do not get enough pro-survival signaling through the TCR or cytokine receptors. This greatly limits lymphocyte-driven immune response\textsuperscript{134}.

1.2 T Cell Effector Subsets and Their Functions in the Immune System

Different functions of T cells are mediated by various effector subtypes of T cells. The major populations of T cells, the CD4- and CD8-positive T cells, are defined by the expression of either one of the co-receptors. Both CD4 and CD8 positive T cells differentiate upon activation to effector cells that have different subpopulations. These subpopulations are characterized by certain transcription factors that drive the expression of genes needed for their specific functions, especially the production of lineage-specific cytokines.

1.2.1 CD8$^+$ T Cells: Cytotoxic T Cells

CD8$^+$ cells differentiate upon encounter with their specific antigen to CTLs. The CTLs destroy their target cells by driving them to apoptosis. Hence CD8$^+$ T cells are the most important cell type in fighting against intracellular microbes, for example viruses. CD8$^+$ T cells can be activated by APCs alone, but usually the effective proliferation of CTLs requires additional help from cytokines produced by activated CD4$^+$ cells. After activation and proliferation, which usually takes place in a lymph node, CTLs migrate to the site of infection and kill infected cells that have their specific antigen bound to a MHC I molecule on the cell surface\textsuperscript{229, 395}.

CTLs can induce apoptosis in targeted cells with different mechanisms, but all of them require close cell-to-cell contact\textsuperscript{32}. CTLs can produce so called cytotoxic granules that contain various cytotoxic molecules. The cytotoxic
substances are the primary effector molecules of CTLs. CTLs can also induce apoptosis in the target cells via specific apoptosis-inducing cell-surface molecules such as Fas-ligand. CTLs produce various cytokines that e.g. activate other components of the immune system, enhance antigen expression, or prevent viral replication in infected cells.

CD8$^+$ cytotoxic cells are important in tissue-specific autoimmune diseases because the chronic lymphocyte infiltration in inflamed tissues is often dominated by CTLs. Some autoimmune diseases also have an association with the expression of certain HLA type I molecules that present antigens to CD8$^+$ cells. However, since CTLs often require help from CD4-positive helper T cells for optimal cytotoxicity, it is believed that CTLs alone are not sufficient to break self tolerance.

1.2.2 CD4$^+$ T cells: T Helper Cells

The majority of CD4$^+$ T cells differentiate upon activation to Th cells. The name describes their action quite accurately since Th cells do not act directly on the microbe or infected cell but instead enhance or “help” other cells of the immune system to manage the microbial invasion properly. Many different subsets of CD4$^+$ Th cells have been described and new subsets still emerge. It is, however, not certain that all newly-characterized subsets represent stable end-stage differentiation of Th cells or whether they are merely at different phases of their differentiation. There is also a certain level of plasticity in the Th lineages so that under certain circumstances the cells can change their effector phenotype.

Naive CD4$^+$ T cells have the potential to differentiate into the effector Th-subsets depending on the cytokine signals they receive from their surroundings upon activation. Differentiation to a certain lineage often requires more than one cytokine. Thus, it is actually the local cytokine milieu in the lymph node, where the cell gets activated, that eventually will determine what kind of immune response dominates a given infection. Different subsets of Th cells are functionally characterized by their production of effector cytokines that activate or suppress other cells of the immune system. Th cell populations can be identified according to their cytokine production and phenotype. Each Th-subtype utilises specific signal transducer and activator of transcription (STAT) proteins, whose activity is controlled by their phosphorylation status. Phosphorylated STAT proteins mediate the cytokine signals to the nucleus, and control the expression of Th population specific genes. STATs are especially important in Th differentiation as they control the up-regulation of the Th population specific transcription factors. These master transcription factors further drive the expression of Th subtype-specific genes. Table 1 summarises the main Th populations, the cytokines
and cells driving their differentiation, as well as their major phenotypic characteristics.

Originally Th differentiation or polarization emerged between Th type 1 and Th type 2 cells that are important in the initiation and maintenance of cellular and humoral immune responses, respectively\(^\text{177}\). An overactive Th1 type response is believed to be important in autoimmune diseases while Th2 overreactivity is responsible for the generation of allergy\(^\text{512}\). New Th-subtypes Th17 and T follicular helper (Tfh) cells are, if uncontrolled, also important in the breakdown of self tolerance\(^\text{86, 91}\). One subtype of CD4\(^+\) T cells are suppressive regulatory T cells (Treg) that will be discussed in detail later. They are important in the maintenance of tolerance.

Table 1: Summary of the main non-suppressive effector Th subpopulations. IFN = interferon, IL = interleukin, TGF = transforming growth factor

<table>
<thead>
<tr>
<th>Cytokines driving differentiation</th>
<th>Th1</th>
<th>Th2</th>
<th>Th17</th>
<th>Tfh</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma), IL-12</td>
<td></td>
<td>IL-4, IL-13, IL-21 ?</td>
<td>IL-6, TGF-(\beta)</td>
<td>?</td>
</tr>
<tr>
<td>Macrophages, DCs, NK-cells</td>
<td></td>
<td>Dendritic and epithelial cells, other innate cells</td>
<td>DCs</td>
<td>?</td>
</tr>
<tr>
<td>Master transcription factor</td>
<td>Tbet</td>
<td>GATA3</td>
<td>ROR(\gamma)T</td>
<td>Bcl6</td>
</tr>
<tr>
<td>STAT protein(s)</td>
<td>STAT1, STAT4</td>
<td>STAT6</td>
<td>STAT3</td>
<td>STAT3</td>
</tr>
<tr>
<td>Effector cytokines</td>
<td>IFN-(\gamma), TNF-(\alpha)</td>
<td>IL-4, IL-5, IL-9, IL-10, IL-13</td>
<td>IL-17A, IL-17F, IL-21</td>
<td>IL-10, IL-21</td>
</tr>
<tr>
<td>Microbial defense</td>
<td>Intracellular microbes</td>
<td>Helminths</td>
<td>Extracellular microbes, fungi</td>
<td>Viruses</td>
</tr>
<tr>
<td>Clinical outcome of dysregulation</td>
<td>Autoimmunity</td>
<td>Asthma, allergy</td>
<td>Autoimmunity</td>
<td>Autoimmunity, cancer</td>
</tr>
</tbody>
</table>

\(1.3\) T Cell Development

1.3.1 T Cell Development and T Cell Receptor Recombination

T cells get their name from the thymus, a two-lobed organ situated beneath the sternum, where they develop from bone marrow-derived lymphoid
progenitors. The thymus is large in childhood but it atrophies with age. Thymic involution reduces output of mature T cells, however, the production will not end completely. Histologically the thymus consists of multiple lobules that are separated by connective tissue septae. Each thymic lobule has two histologically distinct areas: the cortex and the medulla (see Figure 1). Different stages of T cell development take place in either the cortex or the medulla. Thus developing T cells, i.e. thymocytes, move between different areas in the thymus. Thymic stromal cells strictly control this thymocyte traffic with chemokines and sequential expression of homing receptors and ligands.

![Figure 1: Hematoxylin and eosin stained section and a diagram of the structure of a single human thymus lobule. Arrows indicate the connective tissue septae. C = cortex, M = medulla, * = Hassall’s corpuscle.](image)

The cortical and medullary thymic epithelial cells (cTECs and mTECs respectively) are, in addition to being thymocyte traffic controllers, the most important mediators of T cell development. The details of thymic stroma development and function are still mostly unknown. Prominent structures in the human thymus are keratinized nodules in the thymic medulla called the Hassall’s corpuscles. Similar structures are found in the mouse thymus, even though originally they were considered to be very rare. The significance of Hassall’s corpuscles is still unknown but they have been suggested to be important in disposal of apoptotic thymocytes, antigen expression, and production of several cytokines.

The T cell precursors called the thymus seeding progenitors originate from the bone marrow-derived common lymphoid precursoir cells. They enter thymus from the bloodstream through the cortico-medullary junction and become the early thymic progenitors. Commitment to the T cell lineage takes
place at this stage of development\textsuperscript{518}. These very immature thymocytes first proliferate vigorously in the thymic cortex. At first, thymocytes do not express either CD4 or CD8 surface molecules so they are called double-negative thymocytes. After the double-negative phase the earliest expression of the CD4 can be found. These immature single-positive cells soon start to express also the CD8 co-receptor and thus enter the double positive development stage\textsuperscript{61}.

In order to create the vast antigen recognition potential of mature T cells, the TCR recombination is a multi-phased process. During the process, each genetic combination can be made from a number of options and thus each step increases the variation in the resulting receptor. Both chains are recombined separately from multiple gene fragments. The TCR α locus contains variable (V), joining (J) and constant (C) genes, and in the β chain locus there are also additional diversity (D) genes. Gene segment joining sites make up the CDR3 region in both chains. The gene segments are not linked together in a precise manner. Instead nucleotides can be left out or added, which further increases the CDR3 sequency variety\textsuperscript{335}. This process is called junctional diversity, and it is an important factor in increasing T cell receptor diversity. Theoretically, it has been estimated that there are approximately $10^{15}$ different potential TCR recombinations. However, there are not enough T cells in the immune system to express all possible receptor variants. Studies from human peripheral TCR repertoire indicate that there are approximately $10^8$ TCRs in the naïve T cell repertoire\textsuperscript{23, 335}.

TCR gene recombination starts when the recombination activating genes (RAG1 and RAG2) are expressed in double-negative thymocytes. The β chain is recombined first. When the β chain recombination is successful, the other β locus is shut down to prevent additional recombinations in a process called allelic exclusion\textsuperscript{53}. It will guarantee that the mature T cells almost always express only one recombined TCR β chain.

The remaining TCR recombination is carried out in double-positive thymocytes\textsuperscript{61}. The α chain recombination is initiated by a second wave of RAG expression. When the α chain is recombined and successfully paired with the β chain, the cell and its newly recombined TCR are tested. Thymic selections will ensure that only functional and self-tolerant single-positive T cells exit the thymus and enter the peripheral immune system.

1.3.1.1 Positive Selection of Developing T Cells
The thymic positive selection takes place in thymic cortex where cTECs present self-peptide – self MHC-complexes to thymocytes. The developing thymocyte has to have a functional TCR that binds with low affinity to the self-antigen – MHC-complexes. If the receptor does not bind to the MHC
molecules on cTECs sufficiently, the cell will die by apoptosis, the programmed cell death. The exact self-peptides that drive positive selection are not known but the receptor-ligand affinity needed for positive selection is significantly lower than the affinity needed for T cell activation\textsuperscript{245}.

In the TCR recombination process, the functionality and pairing capacity of the α chain with the already recombined β chain is tested by positive selection. The cell can, however, continue to recombine the TCR α locus further or try to pair the recombination from the other allele with the β chain\textsuperscript{425}. The TCR α locus orientation allows multiple successive recombinations to occur so most of the developing double positive thymocytes succeed in recombining at least one functional α chain\textsuperscript{217}. TCR α chain is considered to be more important in MHC recognition than the β chain\textsuperscript{433}. The V genes, that code for the MHC binding CDR1 and CDR2 loops in the TCR, are inherently biased already in the germline towards producing peptide chains that bind MHC. This has been proved both in receptor crystallization, \textit{in vitro} fetal thymic organ cultures, and transgenic mouse models\textsuperscript{142, 230}.

Along with the positive selection thymocytes commit to the two main populations of mature T cells: CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. The same TCR contact and signaling that prevent apoptosis in positive selection instruct the cell to choose its co-receptor. After the lineage commitment, the thymocytes express only CD4 or CD8 and are called single-positive thymocytes\textsuperscript{329}.

\subsection*{1.3.1.2 Negative Selection of Developing T Cells}
Positively selected single-positive thymocytes migrate to the thymic medulla. At this stage, the T cell receptor repertoire has been selected to be reactive to at least some extent to the self-antigen – MHC complexes. Negative selection is a process where developing thymocytes that have a too high affinity to self-antigens will be selected out from the T cell repertoire before they enter the circulation\textsuperscript{477}. Negative selection is also called central T cell tolerance. It guarantees that the peripheral T cell repertoire is overtly tolerant towards self-antigens. Negative selection can take place at any phase of the thymocyte development once the TCR is fully recombined and expressed on the cell surface.

The concept of thymic negative selection was established using mouse models that have a transgenic (TG) TCR. The TG-TCR was reactive only with an external or endogenous antigen whose tissue expression pattern was controllable. When the model antigen was expressed in the peripheral immune system, it lead to proliferation and activation of the TG-TCR T cells. When the antigen was expressed in the thymus, it resulted in clonal deletion of developing thymocytes\textsuperscript{301}. However, this experimentsl model for negative selection has been criticized since it does not take into account the negative selection of the normal polyclonal TCR repertoire.
Negative selection in the thymus was further established when tissue specific antigen (TSA) expression was discovered in the thymic medulla. mTECs are a specialized stromal cell population that has the capacity to express ectopic TSAs. This process is also called promiscuous antigen expression. In the thymic medulla TSAs such as insulin, are expressed outside their normal functional location in order to present them to developing thymocytes. This situation can be described as an “immunological homunculus” of TSAs created by the mTECs in the thymic medulla. How these epithelial cells express such a wide range of antigens is mostly unknown. The only identified factor promoting this antigen expression is the transcription factor Autoimmune regulator (AIRE) and it will be discussed in depth later.

In addition to mTECs, macrophages and DCs accumulating from circulation to the thymic medulla take actively part in the TSA expression and negative selection. mTECs have a high turnover rate and especially medullary DCs phagocytose apoptotic mTECs. Thus, these DCs can acquire TSAs in their antigen processing machinery. A single TSA is expressed at a low level on mTECs, and thus the thymic DCs express TSAs for an extended period. Additionally DCs can express blood-borne antigens, or bring antigens with them from peripheral tissues. DCs can migrate to thymic medulla also from the gut and present antigens derived from the intestinal flora. This mechanism may be important in the generation of tolerance towards intestinal commensals.

The thymocytes that bind to self-pMHC complexes with a too high affinity will die by apoptosis. It is currently not known what triggers the apoptosis in the potentially autoreactive thymocytes. There is a view that high affinity binding of the TCR to a TSA-MHC complex itself would be sufficient to drive thymocytes to apoptosis. This would mean that there are some developmentally determined TCR affinity “windows” for different cell fates (see Figure 2). It is not known how the cell discriminates between different TCR-pMHC affinities. It is also possible that the TCR’s binding interactions with pMHC in positive or negative selection are qualitatively different. The different binding patterns could determine whether the cell matures further or dies by apoptosis. Other signals from co-stimulatory molecules or co-receptors have also an undetermined significance in the negative selection.
1.3.1.3 T Cells with Dual T Cell Receptors

Allelic exclusion in the β locus will guarantee that mature T cells have only one TCR β chain. However, this is not the case for the TCR α chain loci. The recombination in the two TCR α alleles will stop only when the cell is positive selected\textsuperscript{217}. Thus it is possible that a mature T cell has two fully recombined α chains if the cell is positively selected only after both loci have been recombined. Approximately 15% of mouse and human peripheral blood T cells express two α chains on the cell surface, but the significance or function of these dual-specific T cells have not yet been fully characterized\textsuperscript{199, 357}. Apparently it is quite common that both α loci are recombined in the cell genome, but the other one is not translated to protein\textsuperscript{9, 263}. This could result from preferential pairing of some β and α chains\textsuperscript{376}.

It has been speculated that T cells with two antigen receptors could be potentially harmful. The second receptor can enable non-functional and potentially autoreactive receptors to bypass thymic selection\textsuperscript{362, 504}. There are conflicting experimental data to support this theory. In some animal models of autoimmunity, the dual-specific T cells are important in generating self-destructive inflammation\textsuperscript{120} but in other models these cells have been shown to be protective or have no effect on autoimmunity\textsuperscript{85, 120}. Blichfeld \textit{et al.}\textsuperscript{43} showed that due to the split TCR expression the dual-specific T cells received less TCR-mediated signaling per one TCR. This could result in diminished TCR signaling and thus non-functional receptors. It has also been claimed
that dual-specific T cell receptors expand the global TCR repertoire thus increasing the recognition potential against foreign antigens.\(^{198}\)

Tuovinen et al.\(^{462}\) reported that human regulatory T cells (Tregs) are more often dual-specific than non-suppressive effector cells, indicating that two different receptors could in fact be necessary in Treg development or function. Studies done in TG-TCR mice also suggest that Treg development needs the second endogenous TCR\(^{212, 234, 345}\). These results, however, were not confirmed in another experimental setting\(^{443}\), so the true role of dual-specific TCRs in autoimmunity is still unknown.

## 2 T CELL TOLERANCE AND AUTOIMMUNE DISEASES

Immunological tolerance as a whole means the immune system’s ability to separate the organism’s own structures from external antigens, and leave them unharmed. T cell central tolerance, i.e. negative selection, takes place in the thymus during T cell development. In the next sections, the focus is on the peripheral tolerance and the mechanisms controlling mature lymphocytes in secondary lymphoid organs are described. The events leading to autoimmune diseases, i.e. when the tolerance fails, are also discussed.

### 2.1 Central Versus Peripheral Tolerance

Thymic negative selection is a very effective system for selecting out TCR clones with high affinity to self-antigen – MHC complexes. However, data from various transgenic and polyclonal experimental models demonstrate that autoreactive T cell clones with low or moderate affinity to self can be found in the peripheral immune system of also healthy individuals. Even clones with high affinity to self can be found. However, these cells comprise a very small proportion of the total T cell repertoire\(^{48, 139, 499}\). Therefore, it is clear that additional mechanisms are needed in order to maintain tolerance after T cells have left thymus. These mechanisms are generally called peripheral tolerance, since they are active in the secondary lymphoid organs.

The division between central and peripheral tolerance is becoming more and more difficult to determine. Natural regulatory T cells (nTregs) develop in the thymus and are subjected to thymic selection. However, they mediate suppressive peripheral tolerance. Therefore factors affecting the negative selection in the thymus can also affect the development of Tregs and the maintenance of peripheral tolerance\(^{323}\).

It is difficult to conclude which one, central or the peripheral tolerance, is more important in preventing autoimmune diseases. Even in genetically
modified animal models, in which all developing T cells are reactive against the self, the breakdown of tolerance is not automatic, but often requires additional strong inflammatory stimuli such as immunization or bacterial colonization\textsuperscript{70, 163}. Therefore mechanisms of tolerance work in different areas and are complementary to each other. Central tolerance provides the basic framework for tolerance, but thymic positive selection also guarantees that all T cells are at least weakly reactive to self-pMHC. That is why many different mechanisms of peripheral tolerance have to exist so that an organism can respond and suppress autoreactivity dynamically in changing situations such as infections. Only in rare situations can a single mutation or “hit” break the body’s multiple control mechanisms. Similarly to the development of cancer, the breakdown of immunological tolerance is better described by a multi-hit model where several cumulative events, genetic or environmental, result in an autoimmune disease\textsuperscript{254}.

2.2 Peripheral Tolerance

2.2.1 Restriction of Access to Tissue Specific Antigens and Tissues

T cell activation is anatomically restricted to lymph nodes, which is important in controlling unwanted cell activation. The low concentration of TSAs in lymph nodes is generally not sufficient to activate the potential rare self-reactive T cells\textsuperscript{323}. TSAs are abundant only in the specific tissues where naïve T cells do not normally circulate. The already activated T cells are able to reach the inflamed tissues only when pro-inflammatory cytokines and other molecules up-regulate homing receptors in endothelial cells and loose the cellular junctions\textsuperscript{323}.

Certain immune-privileged sites like the brain, eyes, and testicles are primarily beyond the reach of adaptive immune system. This is clearly evident in tissue transplantation; for example the eye accepts non-identical cornea crafts at a very high frequency when compared with skin which rejects non-identical crafts very efficiently. Immune privilege is an active process. It utilises peripheral tolerance-like mechanisms together with prevention of the access of adaptive immune system into the tissue\textsuperscript{434}.

2.2.2 Regulatory T Cells

Many identified populations of T cells have regulatory capabilities. For example cytokines produced by Th1 type cells inhibit Th2 differentiation. These cell populations, however, are also capable of activating immune responses. True regulatory cells are specialized in suppressing immune responses, and it is their main effector function. The most important CD4\textsuperscript{+}
regulatory T cell population is called Tregs. This population contains both nTregs, that have committed to this lineage already in the thymus, and induced Tregs (iTreg), that acquire the regulatory phenotype after certain peripheral differentiation signals. Tregs constitute 5-10% of the peripheral T cell pool\textsuperscript{400}. These two populations are discussed in detail in below.

The importance of regulatory T cells in various diseases has been established; Tregs have been reported to play a role in the pathogenesis of many autoimmune diseases\textsuperscript{55}, allergy and asthma\textsuperscript{208}, obesity\textsuperscript{294} and atherosclerosis\textsuperscript{190}. Tregs also control feto-maternal tolerance\textsuperscript{325} and mediate oral tolerance towards food\textsuperscript{365}. Their significance in controlling normal immune responses towards microbes has gained a lot of attention lately\textsuperscript{34}. However, Tregs can also be harmful as they can suppress immune responses against malignant cells and therefore aid the progression of cancer\textsuperscript{321}.

2.2.2.1 The Regulatory T Cell Phenotype

Currently, it is not possible to differentiate between CD4\textsuperscript{+} nTregs and iTregs according to phenotype. It is also not possible to reliably differentiate regulatory T cells from normal effector T cells since virtually all markers associated with regulatory T cells are also expressed on other T cell populations (as summarised in Figure 3). Furthermore, CD4\textsuperscript{+} Tregs comprise several subpopulations with distinct phenotypes and functional properties\textsuperscript{58}. However, some markers have been more accurately described and play important functional roles in suppressive cells. Most CD4\textsuperscript{+} T regulatory populations express high levels of interleukin (IL) 2 receptor \(\alpha\)-chain (CD25). Originally Tregs were characterized only by these two surface markers as CD4\textsuperscript{+}CD25\textsuperscript{high} cells\textsuperscript{402}. However, results from studies utilising only these markers for Tregs were biased because the CD25\textsuperscript{high} population always contains a subpopulation of activated effector Th cells. Currently the best marker for Tregs is an intracellular transcription factor called Forkhead box P3 (FOXP3), which will be further described below.

Tregs are highly dependent on IL-2 signaling and will die without it. Thus the CD25 surface molecule is important for the function of Tregs because it is the high-affinity IL-2 receptor \(\alpha\)-chain\textsuperscript{287}. IL-2 signaling is mediated into the nucleus of Tregs through the STAT5-signaling pathway, and for example the high expression of FoxP3 required for Treg functions is initiated by STAT5 signaling. IL-2 or IL-2 receptor knock-out (KO) mice have some FoxP3\textsuperscript{+} cells but their FoxP3 expression level is low and they are incapable of suppressing immune responses\textsuperscript{72}. The effects of IL-2 in Treg development and function are, however, not restricted to mere survival but high IL-2 consumption of Tregs has even been suggested to be a method of immune suppression.
Other Treg-associated markers have functional significance. Tregs express constitutively high levels of both surface and intracellular CTLA-4 that is important in the Treg-mediated immune suppression. It is a negative regulator for the co-stimulatory molecule CD28. Its role in immune suppression is highlighted in the CTLA-4-KO mice that die of a massive lymphoproliferative and tissue destructive inflammatory disease within 3-4 weeks after birth.\textsuperscript{455} CTLA-4-mediated immune suppression is important especially in Tregs since selective block of CTLA-4 in Tregs caused a similar lymphoproliferative, fatal autoimmune disease as global CTLA-4 deficiency\textsuperscript{490}. However, CTLA-4 is not the only molecule used by Tregs for suppression\textsuperscript{50}.

Expression of different homing receptors is essential for Treg-mediated suppression since Tregs need intimate cell-to-cell contact in order to suppress immune responses.\textsuperscript{56, 127} A number of different homing receptors are associated with Treg functions, but no uniform Treg-associated homing receptor expression pattern has yet been established. Tregs express various homing receptors dynamically in response to changing inflammatory signals. The expression of homing receptors is also different in different anatomical locations. This highlights the functional and phenotypical specialization of different Treg subpopulations\textsuperscript{58}.

![Figure 3: Transcription factors, cytokines, surface markers, and receptors associated with the functional Treg phenotype. Those highlighted in the text are underlined.](image)

### 2.2.2.2 Forkhead box P3 (FOXP3) Gene

The human syndrome called Immunodysregulation – polyendocrinopathy – enteropathy – X-linked (IPEX) is a severe multi-organ syndrome that is characterized by severe diarrhea, general lymphoproliferation, eczema, and various endocrinopathies\textsuperscript{309}. The patients, that are all males due to the X-linked inheritance of the disease, die at a very young age unless a bone
marrow transplant is carried out. The gene defect behind the disease was localized to a gene called \textit{FOXP3}, and nowadays it is evident that the product of this gene is actually an important transcription factor for Tregs. The many different autoimmune and immune dysregulation phenomena seen in IPEX are a result of complete lack of functional Tregs, even though some cells with a phenotype resembling Tregs or other suppressive T cells are present also in IPEX patients\textsuperscript{304, 361}. The mouse model for IPEX, that is called Scurfy, has a spontaneous loss-of-function mutation the mouse \textit{FOXP3} homologue \textit{FoxP3}\textsuperscript{415}.

\textit{FOXP3} controls the main suppressive functions of Tregs\textsuperscript{211, 508} and it is also believed to be essential for the development of nTregs in the thymus\textsuperscript{38}. In mice, the expression of FoxP3 is mainly restricted to suppressive Tregs. In humans, however, it has been shown that human effector T cells can up-regulate \textit{FOXP3} transiently after activation but these cells do not acquire the suppressive capabilities\textsuperscript{516}. This transient \textit{FOXP3} expression makes it difficult to differentiate between activated effector T cells and suppressive Tregs. The methylation status of the \textit{FOXP3} gene is currently considered the best way to identify terminally differentiated Tregs\textsuperscript{399}. The \textit{FOXP3} promoter and a certain area in the gene itself are almost completely demethylated in suppressive cells whereas in non-suppressive T cells these sites are methylated\textsuperscript{265}. There is increasing evidence that the high constant expression level of \textit{FOXP3} is important for Treg functions. Thus the transient low \textit{FOXP3} expression in activated T cells is not enough to give them the full suppressive capacity of Tregs\textsuperscript{516}.

Ectopic FoxP3 expression via a viral vector in naive murine CD4+ cells is sufficient to transform them into suppressive Tregs and therefore FoxP3 was long believed to be the major transcription factor for Tregs\textsuperscript{211}. However, when studies established the full genetic Treg transcription signature, it became evident that a significant proportion of the typical Treg gene expression is actually independent of FoxP3\textsuperscript{205}. There are also reports of suppressive Treg-like cells that do not express FoxP3\textsuperscript{127}. It has been postulated that FOXP3 is actually not the transcription factor that initiates the suppressive actions in human Treg cells but rather stabilizes them\textsuperscript{399}. The human \textit{FOXP3} gene produces two different isoforms\textsuperscript{517}. Therefore it is possible that the discrepancies noted between mouse FoxP3 and human FOXP3 expression profiles reflect the different functions of these protein variants\textsuperscript{399}.

FoxP3 can both activate and repress various genes directly or indirectly, e.g. by affecting other transcription factors\textsuperscript{507}. However, the exact functions of FoxP3 are still partially unknown. FoxP3 affects intracellular TCR signaling in many ways. The up-regulation of FoxP3 during thymic development of nTregs is believed to result from TCR signaling\textsuperscript{38}. FoxP3
constantly promotes the expression of CD25 and CTLA-4, and down-regulates CD127 expression. Therefore the main molecules of the currently described Treg phenotype are FoxP3 dependent.

FoxP3 selectively blocks the production of pro-inflammatory cytokines like IL-2 and interferon γ (IFN-γ)\(^{346}\). Originally it was thought that high and stable FoxP3 expression blocks Th effector functions in Tregs, and therefore FoxP3\(^+\) cells represent the terminal differentiation stage of Tregs. A recent report contrasting this finding showed that in human tonsils there is a FOXP3 positive population that produced pro-inflammatory cytokine IL-17\(^{478}\). In the gut a population that expresses Tbet and FoxP3 at the same time has been described\(^{78}\). Tregs from Tbet-KO mice failed to suppress efficiently Th1 type immune responses\(^{249}\). Tregs has also been reported to express GATA3\(^{481}\). Tregs can also lose their FoxP3 expression and become effector cells with a potential to cause pathological inflammation. Taken together, it is apparent that there is certain amount of plasticity between Treg and other Th populations at least in transcription factor expression\(^{322}\).

### 2.2.2.3 Regulatory T Cell Development in the Thymus

Tregs were originally identified by Sakaguchi\(^{402}\) in a mouse model of autoimmunity where a multi-organ autoimmune syndrome is generated by thymectomizing a neonatal mouse on the third day after the birth. Asano et al.\(^ {24}\) showed that this autoimmune attack was completely prevented by the transfer of CD4+CD25\(^{\text{high}}\) cells. This finding illustrates that the nTreg development in mice is a relatively late event since the nTregs are generated only in the latest part of gestation and especially right after the birth. In humans the earliest FOXP3 expression in thymocytes is noticeable already after 12 weeks of gestation\(^{93}\).

It has been well established in mouse studies, that a certain proportion of the single-positive CD4\(^{+}\) thymocytes constitutively express FoxP3 and that these cells are the progenitors of mature nTregs\(^{38}\). FoxP3 is the most important factor determining Treg development\(^ {239}\). FoxP3 positive cells can be found in a small percentage in all thymocyte populations. Thus, it is not clear what is the first stage of T cell development when FoxP3 is up-regulated\(^ {132}\).

TCR signaling is crucial for Treg development and possibly also for FoxP3 up-regulation\(^ {368}\). In models where TCR signaling is selectively blocked, no Tregs are produced\(^ {252}\). In a TCR transgenic system where endogenous TCR recombination is prevented, Tregs are only generated when the cognate antigen for the transgenic TCR is co-expressed\(^ {234}\). FoxP3 expression can be used to identify the earliest thymic progenitors of Tregs but the factors responsible for FoxP3 up-regulation are not known. Together with the TCR signaling, CD28-mediated co-stimulation has at least a partial role in the generation of thymic FoxP3\(^+\) Tregs\(^ {493}\). The cytokines IL-2 and TGF-β appear
to have a synergistic role in thymic Treg development. Mice deficient in IL-2 produce significantly less thymic Tregs, while mice deficient for both IL-2 and TGF-β are completely devoid of thymic Tregs. The sole TGF-β deficiency, however, did not affect the amount of thymic Tregs.

The data from our own group using the human thymus challenge this TCR-based differentiation model for nTregs. We have reported a small cortical double-negative population that expresses FOXP3 without functional TCR. These cells were devoid of natural killer (NK) cell or γδ T cell markers so they appeared to be the most immature form of nTregs. It is thus possible that the nTreg differentiation actually precedes TCR signaling. It is also possible that this population we have described is not connected with the normal nTreg population.

FoxP3+ cells have been found in all areas of the thymus. Stromal cells are important in Treg development and both cortical and medullary thymic epithelial cells have been reported to mediate Treg development. Most FoxP3+ cells are found in the thymic medulla. DCs residing in the thymus have an important role in the nTreg lineage differentiation, possibly by providing additional co-factors needed in the expression of FoxP3. Hassall’s corpuscles have been reported to be involved in the nTreg development. Hassall’s corpuscles are the only thymic structures producing thymic stromal lymphopoietin, which stimulates the DCs to induce Treg differentiation.

Taken together, Treg development in the thymus is a complex process in which TCR signaling, co-signaling, and various cytokines are involved. The exact mechanism of how thymic Tregs are generated is, however, still mostly unknown, especially in humans.

2.2.2.4 T Cell Receptor Repertoire and Antigen Specificity of Regulatory T Cells

The TCR repertoire of nTregs was long believed to be self-reactive. This hypothesis originated from observations of Treg development in the murine thymus. In some TG-TCR settings, thymic antigen expression model resulted in an increase in the proportion of Tregs instead of negative selection. This lead to the hypothesis that there is a specific TCR affinity window that guides the developing thymocyte to up-regulate FoxP3 and thereby differentiate towards the Treg lineage. This affinity window guiding the Treg lineage commitment is believed to be more autoreactive than the TCR affinity needed for mere positive selection. However, it should be noted that the data obtained from monoclonal transgenic experimental models have not been confirmed in a polyclonal TCR repertoire. T cells bearing Treg-originating TG-TCRs proliferated more efficiently in lymphopenic hosts than T cells with effector cell-originating TG-TCRs. This kind of
proliferation is believed to be dependent on self-originating antigens and it is discussed in detail below. On the other hand, it has been suggested that these findings in lymphopenic hosts could in fact be a reflection of preferential survival of Tregs instead of an increased TCR-mediated Treg activation\(^{469}\).

The view of increased autoreactivity of the Treg TCR repertoire was challenged when a larger number of Treg-derived TCRs were sequenced, and self-reactive clones were found to represent a minority in the total population\(^ {355}\). These studies also showed that there is a significant overlap of 10-20% in the TCR repertoires of Tregs and naïve T cells\(^ {215, 354, 356}\). It should be noted that contradicting results showing no significant overlapping repertoires of Tregs and effector T cells have also been published\(^ {255, 284}\).

Further, Tregs that have a microbial antigen-specific TCR have been reported, and they seem to control the immune response to various infectious agents\(^ {34}\). The current concept is that the Tregs have a wide polyclonal TCR repertoire that recognizes both self and non-self antigens\(^ {354}\).

It is not clear whether the Treg-mediated suppression of immune responses is antigen specific or non-specific. It is clear that Tregs must be activated through their antigen receptor in a similar fashion to that of normal effector cells before they can start their suppressive actions\(^ {399}\). Therefore, Treg-mediated suppression of immune responses is antigen specific. However, Tregs can also be indirectly activated by microbial products through pattern-expression molecules that they express\(^ {442}\). It is thus possible that during immune response Tregs are activated also in an antigen non-specific fashion.

It is not known whether the effector T cells, i.e. the target of the regulation, and the Treg need to have the same antigen specificity. This scenario is not supported by experimental evidence showing that suppressive and non-suppressive T cells have little overlap in their TCR repertoire\(^ {255, 284}\). Antigen-specific Treg suppression focusing on DCs initiating the immune response could explain how antigen-specific, i.e. TCR signaling-dependent, suppression appears to be more efficient even though their TCR repertoires are not shared\(^ {77, 498}\). Tregs can also suppress effector cells by producing immunosuppressive cytokines. This suppression is not restricted by shared antigen specificity and it is also called by-stander suppression. It is believed to be generally less efficient in the prevention of autoimmune pathology\(^ {354}\).

### 2.2.2.5 Induced FoxP3\(^ +\) Regulatory T Cells

After the finding of nTregs it soon became evident that there are also suppressive T cells that acquire their phenotype only after activation in the peripheral immune system\(^ {239}\). These suppressive cells can be called adaptive Tregs or iTregs. No population-specific markers for iTregs have yet been found. Recently a transcription factor called Helios has been associated
specifically with the nTreg population but its value as a tool to differentiate nTregs from iTregs remains to be confirmed. CD4+ iTregs can be defined by their peripherally acquired expression of FoxP3 while in nTregs FoxP3 is up-regulated already during thymic development. Also other iTreg phenotypic markers are almost completely shared with nTregs.

It has been shown especially in mouse models that naïve CD4+ T cells can acquire a Treg phenotype. TGF-β and IL-2 are the most important cytokines in directing T cell differentiation to iTreg lineage. Naïve CD4+ cells require also TCR-mediated signaling in order to up-regulate FoxP3, but compared to the TCR signaling required for full T cell effector activation, suboptimal TCR signaling appears to be essential for iTreg conversion. Thus minute doses of antigen are more efficient in driving the induction than large ones. Similarly to the non-suppressive effector T cell activation, co-stimulation is required for iTreg conversion. iTreg conversion is strongly dependent of CTLA-4 signaling while the nTregs require CD28 signaling during their development in the thymus.

DCs are important in directing the iTreg conversion. Several cell surface markers on the DCs, for example programmed death ligand 1, have been linked to this induction together with TGF-β production. A population of DCs called the tolerogenic DCs resides in epithelial tissues, such as the lungs and the gut. These cells have been shown to be potent in iTreg induction. FoxP3 up-regulation can, however, take place also in secondary lymphoid organs since iTreg conversion occurs if the antigen is administered systemically. iTregs can also develop during immune response with specificity to an infectious agent. It has been speculated that the confusing data about self- or non-self specificity of the Treg TCR repertoire would, in fact, be partly explained by contaminating iTregs. The iTreg repertoire appears to be more reactive than nTregs to non-self antigens such as allergens, commensal microbiota, and alloantigens.

iTreg conversion has been shown to occur in the gut-associated lymphoid tissue. This has been explained by tolerogenic DCs residing in the lamina propria and gut-associated lymphoid tissue. The tolerogenic DCs have been characterized by surface expression of mucosal lymphocyte antigen 1 (CD103). CD103 is a homing receptor directing lymphocytes into the gut and other epithelial compartments of the body. Constitutive production of various anti-inflammatory cytokines (IL-10 and TGF-β) by the gut epithelial cells is important in the conditioning of these specialized DCs to generate tolerance. Diet-derived retinoic acid, i.e. vitamin A, is very potent in inducing FoxP3 expression and suppressing naïve CD4+ cells. It is important to note that the gut has specialized ways of promoting antigen specific and unspecific tolerance independently of the thymus due to the huge antigen load the gut commensal microbiota provides. iTregs originating from
the gut have indeed been reported to be important in mediating oral tolerance towards dietary antigens\textsuperscript{98, 165}.

2.2.2.6 Other T cell Populations Specialized in Suppressing Immune Responses: IL-10 Secreting Tr1 Cells, Regulatory CD8\textsuperscript{+} Cells, and Invariant NK T-cells

CD4\textsuperscript{+} regulatory T cells are currently the best characterized T cell population that can suppress immune responses. However, several other T cell populations with regulatory functions have been found. For example, invariant natural killer T - cells (iNKT cells) are a specialized T cell population with reported regulatory and suppressive functions\textsuperscript{37}. They express TCRs with limited receptor specificity together with cell surface markers of both memory T cells and natural killer (NK) cells. These cells appear to be dysfunctional especially in the mouse model of autoimmune diabetes\textsuperscript{60}. However, it is not known whether iNKT cells are functionally important in human autoimmune diseases\textsuperscript{37}.

Type 1 regulatory T (Tr1) cells are a specialized population of CD4\textsuperscript{+} T cells that has a capacity to produce large amounts of immunosuppressive cytokine IL-10\textsuperscript{389}. Tr1 cells have a role in the generation of mucosal tolerance, i.e. oral tolerance, and they are also induced if the antigen is administered through the nasal route\textsuperscript{484}. This may explain why Tr1 cell defects appear to be important in the generation of allergy\textsuperscript{496}. Tr1 cells do not generally express high levels of FoxP3. High IL-10 production is the hallmark of Tr1 cells but they can also produce other cytokines\textsuperscript{389}. IL-10 has e.g. immunosuppressive effects in the immune system, and many types of cells are capable of producing it\textsuperscript{398}.

CD8\textsuperscript{-} positive cells with regulatory capacities were described earlier that CD4\textsuperscript{+} regulatory T cells were characterized\textsuperscript{108, 452}. Various populations of CD8\textsuperscript{+} regulatory cells have been found both in humans and mice with partially overlapping functions. Some CD8\textsuperscript{+} regulatory cells also express FoxP3\textsuperscript{108}.

2.2.2.7 Regulatory T Cells: Mechanisms of Suppression

Increasing evidence emphasizes the importance of Tregs in the control of various immune responses. However, it is still unknown how Tregs suppress their target cells\textsuperscript{83}. The exact activating signals that initiate Treg mediated immune suppression are elusive. It is clear that for suppression Tregs must be activated through their TCR and receive signaling from surrounding cytokines similarly as effector T cells do\textsuperscript{400}. However, the antigen doses needed for Treg activation are significantly lower than the ones needed for activation of other effector T cells\textsuperscript{401}.

Upon activation, Tregs secrete immunosuppressive cytokines that inhibit surrounding cells in an unselective way. Suppression of antigen-
presenting cells will eventually affect the immune response on a larger scale\textsuperscript{417}. It has been suggested that since Tregs are so dependent on IL-2, even though they do not produce it themselves, they could regulate the immune response by consuming IL-2. Effector T cells need high amounts of IL-2 in order to survive. There is also evidence that Tregs can trigger apoptosis in effector T cells or DCs by direct cell-to-cell contact\textsuperscript{401}. Certain surface molecules expressed on Treg, for example Galectin-1, can arrest the cell cycle in the proliferating effector cells\textsuperscript{382}.

One important suppressive mechanism is the Treg-mediated modulation of DC functions. For example CTLA-4 appears to have an important role in mediating Treg suppression of DCs\textsuperscript{50}. Tregs expressing CTLA-4 can block the up-regulation of the co-stimulatory molecules on DCs and thus prevent the activation of antigen specific naïve T cells. CTLA-4 can also induce the expression of other immunosuppressive molecules on APCs\textsuperscript{417}. CTLA-4 binding further prevents DCs from producing proinflammatory molecules\textsuperscript{457}. Tregs can also prevent APC maturation\textsuperscript{400}. Anergic T cells and Tregs have been postulated to be immunosuppressive in a non-selective and passive way by blocking the access of naïve T cells to DCs. There is some experimental evidence supporting this, especially for Tregs, since they accumulate into small clusters around the DCs\textsuperscript{417}. Figure 4 summarises reported cell-contact-dependent and independent mechanisms that Tregs use for immunesuppression.

\textit{Figure 4: Summary of Treg cell suppression mechanisms.}

Cell-contact independent mechanisms: 1) secretion of immunosuppressive cytokines that can silence both effector and APC responses, 2) consumption of IL-2, and 3) physical blockage that prevents effector cells’ access to DCs.

Direct cell-contact dependent mechanisms: 4) induction of apoptosis in effector cells, 5) induction of anergy in effector cells, 6) prevention of DC activation and maturation, and 7) CTLA-4-mediated inhibition of co-stimulation on DCs.
It appears that the mechanisms of suppression nTregs and iTregs use are somewhat different. For example, iTregs have been suggested to produce more immunosuppressive cytokines that mediate by-stander suppression, while nTregs utilise more cell-to-cell contacts to suppress DCs\textsuperscript{213}. The requirements for immune regulation in steady-state, chronic infection, or acute inflammation are very different and thus the mechanisms of immune control are versatile\textsuperscript{88}. In conclusion, Tregs use various methods of suppression and it is currently unknown which of these are functional \textit{in vivo}\textsuperscript{83, 400}.

2.3 Autoimmune Diseases in Humans

Autoimmune diseases are a large group of diseases with variable clinical pictures and outcomes. Even though most autoimmune diseases are rare, the overall impact of these diseases on health is significant. It has been estimated that approximately 5% of the Western population is affected with some kind of an autoimmune manifestation\textsuperscript{95}.

2.3.1 Classification of Autoimmune Diseases

Autoimmune diseases are classically divided into organ-specific and systemic diseases\textsuperscript{95}. In organ-specific autoimmune diseases, such as in type 1 diabetes mellitus, a tissue-specific antigen triggers the autoimmune inflammation, and it can completely destroy the cells producing the antigen. In a few of these diseases the target autoantigen has been identified. One example is Goodpasture’s syndrome where the autoantigen has been recognized to be a collagen chain found only in highly specialized basement membranes such as in kidney glomeruli and pulmonary alveoli\textsuperscript{366}. The syndrome’s clinical findings correlate with the anatomical distribution of the autoantigen expression. Almost all patients have autoantibodies targeted to this specific collagen chain, and they often have an increased proportion of circulating T cells reactive for it\textsuperscript{366}.

In contrast to organ-specific autoimmunity, in the systemic autoimmune diseases the autoantigen(s) are expressed in many tissues. The model for these diseases is systemic lupus erythematosus (SLE) where autoantibodies targeted against e.g. DNA can often be found\textsuperscript{170}. Even though this classification of organ-specific and systemic autoimmune diseases is artificial and does not necessarily correspond to a difference in causation, it is clinically relevant. Coombs-Gell hypersensitivity classification can also be used to describe different autoimmune diseases. It separates the diseases to four classes according to the main mechanisms of tissue damage that are causing the symptoms and findings\textsuperscript{229}. For example SLE can be classified...
according to Coombs-Gell as an immunocomplex disease (type III hypersensitivity).

However, some autoimmune diseases are difficult to classify into organ-specific or systemic ones. In some autoimmune diseases, such as in Crohn’s disease, a clear genetic defect in the innate immune system has been found. The new classification suggested for autoimmune-like diseases is an immunological disease continuum that ranges from classical autoimmune diseases, both organ-specific and systemic, to the newly designated auto-inflammatory diseases. Most common autoimmune diseases, such as rheumatoid arthritis, share features of both auto-inflammatory and autoimmune diseases, and are thus located in the middle of the continuum.

2.3.2 Genetic Background of Autoimmune Diseases

Autoimmune diseases can also be classified according to their genetics. Monogenetic autoimmune diseases are caused by a mutation in a single gene. Monogenetic autoimmune disease can be inherited in a dominant fashion, or be autosomal recessive. Some monogenetic autoimmune diseases are X-linked, so that only males carrying the defective gene in their X chromosome are affected. Autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy (APECED) is a monogenetic autosomal recessive autoimmune disease and it will be described in detail below. Monogenetic autoimmune diseases are rare but they have been excellent models for autoimmune research.

More common autoimmune diseases such as SLE and rheumatoid arthritis are polygenetic. Their inheritance is not autosomal recessive or dominant. Instead, in polygenetic diseases gene polymorphisms or alleles increase the risk for the disease. In the following sections the most common genetic risk factors for autoimmune diseases are briefly described.

2.3.2.1 Human Leukocyte Antigen Association with Autoimmune Diseases

The most thoroughly described genetic predisposition to autoimmune diseases is HLA-association. It was found that in some autoimmune diseases certain HLA alleles are significantly enriched in the diseased population compared with the rest of the population. The associations are generally more pronounced in HLA class II alleles but there are diseases that are connected with the class I alleles as well. One of these diseases is ankylosing spondylitis that is strongly associated with the HLA class I allele B27. However, HLA-association only increases the risk of acquiring a certain autoimmune disease, but it does not predict accurately whether an individual will get the disease or not. For instance, type 1 diabetes has a strong HLA-association, but the
disease concordance in monozygotic twins, who are genetically identical, has been shown to be only 27% in Finnish population\textsuperscript{223}. It should be noted that there are also HLA-associations that are protective for some autoimmune diseases.

2.3.2.2 Other Genetic Risk Factors for Autoimmunity

In many autoimmune diseases, for example in Hashimoto’s thyroiditis and SLE, females are more often affected\textsuperscript{505}. The autoimmune diseases with male predominance, like ankylosing spondylitis, are rare. Some diseases, such as type I diabetes, affect both genders equally. The reasons for the gender-bias in some autoimmune diseases are mostly unknown\textsuperscript{393} but both hormonal and X-chromosomal factors are thought to be involved.

Research in immunogenetics has focused on revealing single nucleotide polymorphisms (SNPs) that are enriched in autoimmune diseases\textsuperscript{275, 488}. The most often affected genes can be roughly divided into four functional categories: 1) antigen clearance and presentation, 2) cell intrinsic signaling, 3) co-stimulatory molecules and activation, and 4) apoptosis\textsuperscript{225}. SNPs in these crucial genes cause certain qualitative or slight quantitative changes in the respective proteins rather than a complete loss of function\textsuperscript{413}.

2.3.3 Environmental Factors in the Breakdown of Tolerance

Together with various genetic factors, environmental factors are important in the development of autoimmune diseases. This is best illustrated by the unequal geographical distribution of the autoimmune disease incidence\textsuperscript{95, 229}. Incidence of autoimmune disease is also growing in developed Western countries, which could not be explained by genetic factors\textsuperscript{229}. Environmental factors can partly explain the lower-than-expected concordance of autoimmune diseases between identical twins\textsuperscript{95, 196}, which has not been shown only for type I diabetes, but also for other polygenetic autoimmune diseases as well. For example, if an identical twin is affected with SLE, the risk that the other twin gets the disease is approximately 24\%\textsuperscript{97}. Paradoxically, identical twins can differ from each other genetically. For example, the X-chromosome inactivation in females can lead to different patterns of mosaicism. Also TCRs and antibodies are somatically recombined in a stochastic manner\textsuperscript{404}. These intrinsic epigenetic factors are important but there are also environmental factors involved in the generation of autoimmunity. Factors such as hormonal variation\textsuperscript{393}, emotional stress\textsuperscript{436}, toxins, and dietary factors\textsuperscript{412} have been found to affect the manifestations and outcome of human autoimmune diseases.
2.3.3.1 Microbes and Autoimmune Diseases

Since the immune system has evolved to protect the body against microbes, it can be assumed that the microbe–immune system interactions can also influence the immunological tolerance towards self. An association between an infection and the onset of an autoimmune disease has been shown for many diseases. For example, there is a significant correlation of enterovirus infections with autoimmune diabetes in individuals that are genetically at risk of developing the disease. Infections can predispose to autoimmunity through many mechanisms. The target cells can be chronically infected with the microbe and polyclonally activate lymphocytes, which could eventually lead to tissue destruction. This mechanism has been proposed to play a role in the development of type 1 diabetes since enteroviruses can infect pancreatic insulin-producing \( \beta \)-cells. Inflammation following microbial evasion can also change auto-antigen structures to make them more immunogenic, or reveal otherwise hidden self-structures. Infections can also generate conditions that favor autoreactivity, such as lymphopenia, which will be described in detail below.

Processing of microbial antigens can also be defective and lead to a failure in immunetolerance. For example, in SLE defective clearance of apoptotic cells and cellular debris is important in the appearance of autoantibodies and following autoimmunity. Molecular mimicry is a theory for the development of autoimmunity that connects infections and autoimmune diseases through antigen presentation. In molecular mimicry the microbial antigen is believed to resemble a self-antigen so closely that it can break the tolerance against self. Sometimes this could be achieved only when the antigen is presented in certain HLA molecules. A model disease for molecular mimicry is rheumatic fever where streptococcal antigens, namely two immunodominant peptides of the bacterial M5 protein, display cross-reactivity with heart valvular proteins and cardiac myosin peptides. It has been shown that CD4\(^+\) T cells of the patients preferentially recognize these proteins, especially when the proteins are presented in HLA-DR7 that is the HLA allele associated with increased risk for rheumatic fever.

2.3.3.2 Lymphopenia and Autoimmunity

Transient lymphopenia often follows for example viral infections. Persistent lymphopenia usually results in immunodeficiency. The sizes of different lymphocyte subpopulations are tightly controlled. In a normal immune system, the lymphocytes quickly sense the “empty space” around them and start to divide in order to reach their normal distribution. As the individual ages, the thymic output of naïve T lymphocytes decreases and the constant numbers of T cells is maintained by homeostatic proliferation of existing naïve and memory T cells. T cell homeostasis is remarkably stable for all different
subpopulations and is tightly controlled by cellular interactions and cytokines

2.3.3.2.1 Lymphopenia-induced Proliferation of T Cells

Lymphopenia-induced proliferation (LIP) was discovered in mouse models where mature T lymphocytes were transferred to sublethally irradiated or genetically lymphopenic recipients. In a lymphopenic environment, the lymphocytes proliferated faster than in steady-state lymphoreplete situations and the proliferation resembled strongly a normal immune response to external antigens. It also resulted in a stable memory-like phenotype in the proliferating lymphocytes.

There are many factors driving this proliferation: the self-antigen–MHC complexes, cytokines, and antigens originating from intestinal flora. These different factors are all driving the LIP of T cells, but their relative importance in each experimental setting is dependent on the method how the lymphopenia was generated. When mature lymphocytes are adoptively transferred to genetically lymphopenic animals, that are incapable of producing functional lymphocytes themselves, the resulting LIP is very fast. This proliferation is greatly reduced in animals that are kept under germ-free conditions so it appears that the fast burst of cell division and activation is mainly driven by antigens originating from commensal flora. The proliferation is also strongly dependent of DCs and co-stimulation. When transferring polyclonal T cells to genetically lymphopenic recipients with normal enteric flora, a small fraction of the transferred cells start to proliferate vigorously and acquires an activated effector phenotype. This mouse model of LIP is widely used in autoimmune studies.

There is also another form of LIP following acute lymphopenia after irradiation or lymphocyte depletion by monoclonal antibodies (mAbs). This proliferation is slow and it usually takes several weeks before the adoptively transferred or endogenous cells have reached the size of a normal lymphocyte pool. The signals that drive the proliferation are the same ones that are needed for T cell survival in a lymphoreplete environment, namely contacts with MHC-self-antigen complexes, and certain cytokines, especially IL-7 and to a lesser degree IL-15. During the proliferation the dividing cells acquire a stable memory-cell like phenotype and gene expression profile but they differ from true memory cells in their functions. Thus the proliferation does not fully restore the normal proportions of mature and naïve T cell populations; the naïve T cell pool is only restored in severe lymphopenia by de novo thymopoiesis.

Both types of LIP can take place simultaneously in the same lymphopenic recipient but the kinetics of the proliferation are different. It is not known what explains the difference between LIP following irradiation and in...
genetically lymphopenic animals. Irradiation disturbs the barrier function of the enteric mucosa so the intestinal bacterial antigens should be even more easily available after irradiation. Surh et al. speculated that the lymphopenia generated by irradiation is not complete but some normal T cells could remain in the intestinal tissue, and somehow prevent antigen leakage from the gut thus preventing the fast antigen specific proliferation. LIP as a model to study autoreactivity and peripheral tolerance has also been criticized because of these discrepancies between different experimental conditions.

Since the resources and signals needed for LIP are limited, the T cells must compete for them. Such competition takes also place during an immune response but during lymphopenia it becomes more pronounced, and only the cells with the highest affinity towards self- or gut microbiota-originating antigens proliferate. A lymphopenic environment can significantly lower the activation threshold needed for T cells and thus predispose to immunopathology. The resulting T cell repertoire after LIP is biased towards self-antigen recognition. LIP can predispose lymphopenic animals to autoimmune manifestations, such as colitis that is described in detail below.

2.3.3.2.2 Colitis Triggered by Adoptive Cell Transfer of Naïve T Cells to Lymphopenic Recipients

Adoptively transferred naïve CD4+ T cells cause chronic colitis in genetically lymphopenic recipient animals. This model is called the T cell transfer colitis and it is widely used for studying human inflammatory bowel diseases. Following the transfer of naïve T cells the majority of recipients develop a moderate to severe colitis in approximately five to eight weeks. The animals show symptoms such as weight loss, hunching, piloerection, bloody or watery diarrhea, and rectum prolapse. Histopathological inspection of distal colon obtained from mice with active disease reveals transmural inflammation, epithelial cell hyperplasia, polymorphonuclear and mononuclear leukocyte infiltration, crypt abscesses, and epithelial cell erosions. When the transfer is done to RAG-KO recipients, the small bowel can also be affected. The genetic background of both the donor and recipient can affect the disease outcome. For example, recipients that received an adoptive cell transfer from an IL-10 deficient donor, developed colitis faster. This finding also highlights the importance of IL-10 in the maintenance of intestinal homeostasis.

CD4+ Th cells are the most important cell population in mouse colitis since they are the main population infiltrating colon tissue in all colitis models examined so far. CD4+ T cells are both important as effector cells mediating the inflammation as well as regulators suppressing the inflammation. For example, when CD4+ T cells were depleted in vivo, the ongoing colitis was significantly reduced. T cell dominance in the inflammation has been also
shown in human inflammatory bowel diseases, both in colitis ulcerosa and Crohn’s disease\textsuperscript{33, 47}. When analyzed more closely, Th1 type cells appeared to be important in the initiation of colitis, since Tbet-KO mice failed to develop T cell transfer colitis\textsuperscript{332}. However, cytotoxic CD8\textsuperscript{+} T cells alone could also induce colitis when transferred to lymphopenic hosts\textsuperscript{446}. Th17 cells have recently been described to be important effector cells both in the T cell transfer model of colitis and Crohn’s disease\textsuperscript{299}. The relative importance of different T effector cell populations in the disease initiation remains to be shown.

The colon microbiota is essential in the generation of colitis. Animals that are housed in a germ-free environment do not develop colitis and pre-transfer antibiotic treatment ameliorates the disease\textsuperscript{439}. The disease severity and the time to symptom development after cell transfer also depend on the animal facility in which the recipients are kept\textsuperscript{188, 435}. The T cell transfer colitis most likely results from an overactive or dysregulated immune activation against gut-derived microbial antigens\textsuperscript{424}. There is evidence that also the human inflammatory bowel diseases result from loss of tolerance to self microbiota, which could follow from defective innate receptor signaling\textsuperscript{26}.

### 2.3.3.2.3 Regulatory T cells in Lymphopenia-induced Proliferation

T cell transfer colitis is prevented with a co-transfer of CD4\textsuperscript{+} memory-phenotype cells\textsuperscript{375}. This finding prompted researchers to dissect the population further and soon it became evident that one population preventing colitis is Tregs\textsuperscript{226}. The colitis could be initiated also by the depletion of CD25\textsuperscript{high} cells from the cell transfer\textsuperscript{16}. However, this depletion changed the scope of autoimmune manifestations after the transfer. For example gastritis became a more dominant symptom and if colitis developed, it was mild compared with the classic T cell transfer colitis\textsuperscript{16}. From these initial observations it was clear that Tregs have an important role in the maintenance of immune homeostasis and tolerance during the reconstitution of lymphocyte populations.

Tregs can partially suppress the fast burst of LIP\textsuperscript{49, 492}. Treg-mediated suppression of the fast proliferation is dependent on the amount of Tregs, i.e. the Treg / T effector cell ratio\textsuperscript{15}. Treg-mediated suppression in LIP is not uniform but, instead, it has been reported that Tregs suppress the fastest proliferating clones more efficiently, and thus maintain the TCR repertoire diversity during the proliferation\textsuperscript{491, 492}. Timing the transfer of Tregs a week before the adoptive transfer of naïve T cells seems to be more potent than co-transfer in the prevention of autoimmune colitis. Also \textit{in vitro} activation of Tregs prior to the adoptive transfer provides a more efficient protection from colitis\textsuperscript{94}. Therefore, it appears that the Tregs must be pre-conditioned or pre-activated in order to effectively suppress the fast LIP and especially the
proliferating colitogenic clones. The cytokine IL-10 is important in the efficient Treg-mediated prevention of colitis both as an immunosuppressive cytokine produced by the Tregs but also in maintaining the stable Treg pool. Innate immune cells, for example gut epithelial macrophages, produce IL-10 and this production appears to be important in maintaining the suppressive Treg phenotype.

There are also controversial reports showing that adoptively transferred FoxP3+ nTregs lose their suppressive potential as well as FoxP3 expression in LIP. These cells start to express effector cell markers and also produce effector cytokines. They have been called “ex-Tregs” and have been reported to acquire a Th1 phenotype and efficiently promote germinal center reactions in gut-associated lymphoid tissue. The reasons for this phenomenon are still unclear, but Th17 polarizing cytokines could prevent FoxP3 expression and thus bias the response towards a pro-inflammatory Th17 response. For example IL-6 has been identified as an important pro-inflammatory cytokine also driving the LIP. Summarising these conflicting data, Tregs are important in controlling the LIP, but their full importance and functions in a complex situation like T cell homeostasis are still under investigation.

2.3.3.2.4 Lymphopenia as a Co-factor in Human Autoimmunity

Lymphopenia has been associated with many human autoimmune or autoinflammatory diseases. Reduced numbers of lymphocytes have been reported in autoimmune diabetes, Crohn’s disease, and celiac disease. The reason for the lymphopenia is obscure in these diseases. It can both result from defective production of lymphocytes, as well as increased apoptosis, or other forms of increased loss of lymphocytes in the periphery.

Primary immunodeficiencies are rare genetic syndromes that are often complicated with autoimmune manifestations together with varying degree of lymphopenia. For example Omenn’s syndrome is caused by hypomorphic mutations in the RAG gene that results in severe defects in lymphocyte development. The patients are lymphopenic but also have many autoimmune manifestations such as erythrodema, lymphadenopathy, colitis, and alopecia. Patients also suffer from recurrent infections, chronic diarrhea, and failure to thrive, which are classical symptoms of immunodeficiency. The TCR repertoire of these patients is strongly biased with oligoclonal expansions of autoreactive T cells that also infiltrate into target organs. A similar combination of immunodeficiency and autoimmunity has been reported in the combined variable immunodeficiency–syndrome, severe combined immunodeficiency (SCID), Bruton agammaglobulinemia, hyper IgM syndrome, leukocyte adhesion deficiency, Wiskott-Aldrich syndrome, and chronic granulomatous disease. As the defects behind primary immunodeficiency
syndromes can also fundamentally affect the development of immune tolerance, it is not known if lymphopenia per se causes the autoimmune manifestations in these diseases. However, e.g. the animal models of Omenn’s and Wiskott-Aldrich syndrome have suggested that dysregulated homeostatic proliferation would be important in the breakdown of tolerance164, 290.

Some patients suffering from cancer or severe autoimmune diseases have lymphopenia following different treatments such as irradiation, chemotherapy, or monoclonal antibody therapy. For some solid organ transplants, e.g. the heart, patients must be depleted of lymphocytes in order to avoid rejection426. The immunosuppressive therapy following the transplantation can also cause lymphopenia. These patients can sometimes suffer from autoimmune manifestations during the immune reconstitution.

Patients infected with the human immunodeficiency virus (HIV) often acquire various disease manifestations that can be viewed as autoimmune or autoinflammatory386. For example diffuse infiltrative lymphocytosis syndrome is a HIV–specific disease that in many ways resembles Sjögren’s syndrome, a systemic autoimmune disease. This infiltrative lymphocytosis affects approximately 7% of HIV patients and it is associated with the HLA-DRBI alleles (most usually HLA-DRB1*1102, DRB1*1301, and DRB1*1302)386. Another HIV-specific auto-inflammatory manifestation is the immune reconstitution inflammatory syndrome that manifests after the initiation of the highly active anti-retroviral therapy. This therapy results in a dramatic and rapid increase in CD4+ T cell counts. The immune reconstitution inflammatory syndrome is constituted of acute worsening of inflammatory symptoms of pre-existing autoimmune disease or infections174. Dead or dying microbes, self-structures, or commensal flora initiate the exaggerated inflammation. Immune reconstitution syndrome is not a failure to control infection. The clinical picture is very variable and it depends on the individual’s previous history of opportunistic infections105, 254.

Altogether, it appears that in humans lymphopenia alone is not sufficient to break tolerance but it can be a co-factor in the progression towards autoimmunity.

2.3.4 Regulatory T cells in Human Autoimmune Diseases

Since the convincing discovery of human Tregs29, 30, several reports of dysfunctional or reduced Tregs in human autoimmune diseases have been published. Tregs can be dysfunctional in humans in several ways: 1) defects in thymic production of nTregs or peripheral conversion of iTregs results in inadequate numbers of Tregs, 2) impaired activation or suppressive function of Tregs, and 3) non-suppressive effector T cells can be resistant to Treg mediated suppression55.
2.3.4.1 Methodological Challenges in Studying Human Regulatory T Cells

Several research groups have published findings of reduced numbers of Tregs in different autoimmune diseases. However, older studies used non-specific Treg markers such as CD4+CD25<sup>high</sup> and thus the reported Treg population was significantly contaminated with activated effector T cells<sup>256, 399, 502</sup>. The lack of established clinical reference values for Treg numbers has made comparison between different studies difficult. The finding of the FOXP3 as a lineage specific marker solved the problem at least to some extent<sup>55</sup>. However, when it was found that the activated non-suppressive effector cells could transiently up-regulate FOXP3 without gaining the full suppressive phenotype, the role of FOXP3 as the only marker for human Tregs had to be reconsidered. The laborious analysis of the methylation status of the Treg-specific demethylated region is currently the best way to differentiate between transient FOXP3 up-regulation, and stable suppressive high expression of FOXP3 in thymus-originating nTregs<sup>265</sup>. However, the methylation status is impossible to measure in viable cells.

It is not known how well the amount of Tregs in circulating blood correlates with the number of Tregs in the inflamed tissue<sup>55</sup>. It is also challenging to select the correct controls for studying Tregs in inflammation. In non-inflamed tissues there are in general very low number of lymphocytes so the increase of Tregs in the inflamed tissue might be secondary to the general increase in lymphocyte numbers<sup>458</sup>. Therefore the control sample should be equally inflamed as the studied material, but the inflammation should not be autoreactive. For example osteoarthritis is used as a control for rheumatoid arthritis<sup>55</sup>. Studying Tregs in human autoimmune diseases is challenging because many diseases become symptomatic only after significant tissue destruction has taken place. This is often a late event considering the long path from the original brake in immune tolerance leading to symptoms<sup>458</sup>.

FOXP3 is expressed intracellularly, and cells need to be fixed and permeabilized before the analysis. Thus it is difficult to get viable FOXP3<sup>+</sup> cells for functional analysis from human patients<sup>399</sup>. When low CD127 expression was identified to be a Treg-specific marker<sup>283</sup> together with other surface markers, viable Treg isolation became more efficient<sup>90</sup>. However, since we do not know the exact mechanisms how Tregs mediate suppression <i>in vivo</i>, their function is difficult to measure, and all the suppression assays used <i>in vitro</i> are still unprecise<sup>399</sup>.

Most of these methodological problems faced in humans can be solved in mice by using a green fluorescent protein marker genetically linked to the FoxP3 expression<sup>131, 132</sup>. With this genetic construct it is possible to closely follow the fate of Foxp3 expressing cells <i>in vivo</i> and also to isolate viable suppressive cells. Since this is a powerful tool, a significant proportion of data...
on Treg development and functions has been gained from these mice. Also the importance of Tregs in the prevention of autoimmunity is mostly based on findings in mouse models of autoimmunity. However, it must be noted that mouse models of autoimmunity differ in many ways from the human diseases and represent simplified versions of the clinically diverse human conditions. There are several differences in the human Tregs compared to their mouse counterparts.

2.3.4.2 Regulatory T Cells in Human Autoimmune Diseases

Tregs have been extensively studied in multiple sclerosis (MS) that is a relapsing and remitting tissue specific autoimmune disease affecting the central nervous system. No reduction in the total number of circulating Treg has been reported, but when Treg subpopulations were analyzed, the naïve Treg population was decreased in MS patients as compared to healthy controls. Tregs in MS patients also failed to suppress auto-antigen driven antigen-specific proliferation as well as polyclonal proliferation. The percentage of Tregs among T cells in the cerebrospinal fluid of MS patients is comparable to the proportion seen in blood but still the sclerotic plaques in brain tissue contained very few FOXP3+ cells. Tregs isolated from MS patients also showed increased sensitivity towards apoptosis and dysregulated homeostasis. In this organ-specific autoimmune disease various defects in the functions and subpopulations of Tregs have been found, even though the overt Treg numbers originally appeared to be normal. In rheumatoid arthritis and type 1 diabetes the amount of Tregs in circulation is also generally reported to be normal, but their suppressive function is impaired.

Mouse studies have indicated that the accumulation of Tregs at the site of inflammation is required for effective suppression. The findings in MS support this cellular contact-dependent suppression mechanism in humans as well. Conflicting reports of increased amounts of Tregs in the target tissue during active inflammation have been published for synovial fluid in rheumatoid arthritis, skin biopsies of psoriatic lesions, and gut biopsies in inflammatory bowel diseases. It should be noted that these diseases, especially inflammatory bowel diseases, all share many features of the newly designated autoinflammatory diseases. For example, in Crohn’s disease Treg function has been found to be normal and the amount of Tregs in circulation to be increased. It is possible that the role of Tregs is different in these diseases compared with their role in autoimmune diseases.

Non-suppressive T cells isolated from type 1 diabetes patients show resistance to the Treg-mediated suppression. It is not yet known if the Tregs are functionally defective in SLE because the published data are very conflicting (reviewed by Gerli). However, in SLE the responder T cell
resistance to Treg-mediated suppression appears to be important\textsuperscript{470, 472}. The responder cell resistance as a mechanism of tolerance breakdown in humans is supported also by \textit{in vitro} findings with Tregs from healthy volunteers\textsuperscript{29, 30}.

The pathogenetic role of human Tregs in autoimmune and autoinflammatory diseases is still largely unknown. The Treg defects found are mostly qualitative rather than quantitative, and factors other than the Tregs themselves, such as responder T cell resistance, have to be taken into consideration.

3 AUTOIMMUNE REGULATOR – GENE

3.1 Autoimmune Polyendocrinopathy, Ectodermal Dystrophy, and Candidiasis (APECED)

APECED is an abbreviation for autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy. The other name for the syndrome is autoimmune polyendocrine syndrome 1 (APS-1)\textsuperscript{483}. The clinical phenotype of the disease was first described by M.F. Leonard in 1946\textsuperscript{273}. Finland has a high reported prevalence of the syndrome (1: 25 000) in the world\textsuperscript{228}, and only Iranian Jews (1: 9000)\textsuperscript{519} and Sardinians (1: 14 500)\textsuperscript{390} have a higher prevalence. Also the Norwegians have a high prevalence of the disease (1: 90 000)\textsuperscript{495} and numerous cases around the world have been reported (Ireland prevalence 1: 130 000\textsuperscript{112} and Poland 1: 129 000\textsuperscript{437}). The Finnish patients are the best described ones with 90 diagnosed cases with follow-up data for up to 60 years of age. APECED belongs to the Finnish Disease Heritage\textsuperscript{7}.

APECED is caused by loss-of-function mutations in the gene autoimmune regulator (\textit{AIRE})\textsuperscript{129, 328}. In the Finnish patients, 82 \% have the same mutation called the Finn major R257X indicating a founder effect in an isolated population. Over 60 other mutations of the \textit{AIRE} gene have been described\textsuperscript{65}. There are also patients that fulfill the clinical diagnostic criteria but do not carry the presently identified mutations\textsuperscript{203}. The Finn major mutation R257X is the most common identified mutation also outside Finland. The vast number of other mutations makes genetic diagnostics difficult\textsuperscript{65, 121, 410}.

APECED is inherited mainly in an autosomal recessive manner with homozygous carriers of the mutations affected with symptomatic disease\textsuperscript{7, 65}. There is one family with a dominant mutation in Italy. The syndrome is generally the same as in the autosomal form but this mutation increases the incidence of autoimmune thyroiditis\textsuperscript{66}. The dominant mutation inhibits selectively the function of the other \textit{AIRE} allele that could explain the different inheritance pattern\textsuperscript{224}. The heterozygous carriers of the recessive mutations appear to be generally healthy of the clinical manifestations of APECED, even
though the issue has not been extensively studied. There are conflicting results of the potentially increased risk for autoimmunity in the APECED patients’ siblings but these studies have been done with a small number of patients and thus need confirming57, 64, 411.

3.1.1 Clinical Phenotype of APECED

In monogenetic diseases the clinical phenotype is generally relatively constant. This, however, is not the case for APECED. The syndrome can be different even between siblings bearing the same mutation and living in the same environment. Symptoms manifest on average at the age of five, but the variation is from 0.2 to 18 years363. The first disease component is normally oral candidiasis and it is also the most common disease component among Finnish patients. The majority of patients have different mucosal or skin Candida infections at least periodically. The Finn major mutation is associated with a high incidence of candidiasis183 while in the Iranian patient cohort candidiasis is relatively rare519. Even though the patients have an immunodeficiency against Candida spp. they do not present general susceptibility towards other fungal, bacterial, or viral pathogens363. Candida infections are generally limited to the epithelial surfaces of the body, namely gastrointestinal tract, skin, nails, and mucosal surfaces of the genitalia. Deep invasive Candida infections, or other severe infections, are reported only rarely and they generally are associated with immunosuppressive treatment for autoimmune components of the syndrome378.

It is generally accepted that two of the most common disease components are required for the APECED diagnosis. Chronic mucocutaneous candidiasis is almost always present at the time of diagnosis, while hypoparathyroidism and Addison’s disease are the most common endocrinopathies. However, the accuracy of clinical diagnosis has been questioned, because very mild or atypical phenotypes in individuals with the disease causing genotype have been reported371. Currently, since the gene test is available, the diagnosis is confirmed with identification of the mutation. The mean diagnostic delay in Italian patients was 10.2 years from the first symptoms to genetically confirmed APECED diagnosis300. It has also been suggested that the presence of auto-antibodies against type I interferons could be used for diagnosis instead of laborious genotyping306.

The other common endocrine components in the syndrome include gonadal failure, hypothyroidism, and type 1 diabetes363. Patients often suffer from different gastrointestinal symptoms such as chronic diarrhea or constipation. Gastrointestinal manifestations are believed to be a result of autoimmunity towards the neuroendocrine cells of the gut. Another possible explanation is chronic exocrine pancreatitis and following malabsorption228. Other autoimmune manifestations found in APECED are hepatitis, gastritis,
pernicious anemia, and most recently autoimmune lung disease with varying symptoms\textsuperscript{12, 96}.

Ectodermal dystrophy is seen in patients as enamel hypoplasia, nail dystrophy, alopecia, and vitiligo\textsuperscript{241}. Keratopathy can result in blindness. Enamel dysplasia combined with the choric candidiasis in the mouth often results in poor oral health. Collins \textit{et al.}\textsuperscript{79} suggested that the classical ectodermal dystrophy components (nail and enamel dystrophy) found in APECED could in fact result from chronic infections. Alopecia and vitiligo can be classified also as autoimmune manifestations of the skin and hair follicles. Inflammation appears to have a role in the disease progression since immunosuppressive medication is often effective in the APECED-associated keratinopathy. Thus Collins \textit{et al.} have questioned whether APECED syndrome actually has an ectodermal dystrophy component at all\textsuperscript{79}.

The incidence of oral carcinoma is significantly higher in APECED patients\textsuperscript{385}. Apparently oral cancer is the result of chronic inflammation of the mucosa due to the \textit{Candida} infection and is not associated with the AIRE deficiency itself\textsuperscript{44, 466}. The incidence of other cancers appears to be normal amongst APECED patients except for anecdotal reports of hematological malignancies\textsuperscript{106, 204}.

Virtually all patients have more than two disease components, and cases of up to ten different endocrine or ectodermal components in one individual have been reported. On an average the patients have four disease components\textsuperscript{5}. Patients can acquire new disease components throughout their life, which is once again an indicator of the high variability of the syndrome. The disease components of AEPced are summarised in Table 2 on page 50.

3.1.2 \textbf{Autoantibodies in APECED}

APECED patients have multiple different autoantibodies\textsuperscript{428}. Their pathogenetic role in the development of the syndrome is largely unknown. Most tissue-specific autoantibodies are targeted against intracellular enzymes such as cytochrome P450 enzymes of the liver\textsuperscript{428}. A parathyroid antigen called NALP5 was identified to be the target of common autoantibodies found in APECED patients: 49\% of patients with APECED and hypoparathyroidism were positive for autoantibodies targeted to NALP5\textsuperscript{13}. Other APECED-associated autoantibodies and their known antigens are summarised in Table 3 on page 52.
Table 2: Clinical heterogeneity of APECED: classification of syndrome components. Prevalence of the most common disease components at the age of 30 is shown in parentheses.

<table>
<thead>
<tr>
<th>Immunodeficiencies</th>
<th>Ectodermal dystrophy</th>
<th>Endocrinopathy</th>
<th>Autoimmune manifestation</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucocutaneous candidiasis (98-100%)&lt;sup&gt;241, 363&lt;/sup&gt;</td>
<td>Enamel dysplasia (75%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Hypoparathyroidism (85%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Autoimmune gastritis (20%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Intestinal malabsorption&lt;sup&gt;371&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asplenia (20%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Nail dystrophy&lt;sup&gt;79&lt;/sup&gt; (50%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Addison's disease (81%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Autoimmune hepatitis (18%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Optic atrophy&lt;sup&gt;363&lt;/sup&gt;, retinitis pigmentosa&lt;sup&gt;349&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphopenia&lt;sup&gt;378&lt;/sup&gt;</td>
<td>Keratitis * (24%)&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Ovarian (36%) or testicular (25%) failure&lt;sup&gt;241&lt;/sup&gt;</td>
<td>Bronchiolitis&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Rash and arthralgia with fever (15%)&lt;sup&gt;363&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recurrent severe infections&lt;sup&gt;378&lt;/sup&gt;</td>
<td>Vitiligo * (79%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Type 1 diabetes (23%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Iridocyclitis, keratoconjunctivitis&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Metaphyseal dysplasia&lt;sup&gt;193&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Alopecia * (39%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Hypothyroidism (21%)&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Dry eyes, Sjögren's syndrome&lt;sup&gt;349&lt;/sup&gt;</td>
<td>Myopathy&lt;sup&gt;222&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Calcification of tympanic membrane&lt;sup&gt;222&lt;/sup&gt;</td>
<td>Pituitary failure&lt;sup&gt;222&lt;/sup&gt;</td>
<td>Rheumatoid arthritis&lt;sup&gt;363, 371&lt;/sup&gt;</td>
<td>Celiac disease&lt;sup&gt;222&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loss of intestinal neuroendocrine cells&lt;sup&gt;152&lt;/sup&gt;</td>
<td>Tubulointerstitial nephritis&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Chronic otitis&lt;sup&gt;371&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exocrine pancreatitis&lt;sup&gt;363&lt;/sup&gt;</td>
<td>Lymphangiectasia&lt;sup&gt;35&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vasculitis&lt;sup&gt;222&lt;/sup&gt;</td>
<td>Oral and oesophageal carcinoma&lt;sup&gt;44, 385&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Autoimmune red-cell aplasia&lt;sup&gt;204&lt;/sup&gt;</td>
<td>Hematological malignancy&lt;sup&gt;106, 204&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inflammatory demyelinating polyneuropathy&lt;sup&gt;468&lt;/sup&gt;</td>
<td>Epilepsy&lt;sup&gt;371&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Can also be classified as autoimmune component
The diagnostic value of autoantibodies in APECED varies a lot. Most autoantibodies are common in other autoimmune diseases as well and thus their diagnostic sensitivity for APECED is low. Autoantibodies can often be found before the onset of clinical symptoms and therefore they can be used to predict disease progression for gonadal failure or Addison’s disease. However, the predictive value of autoantibodies is quite low for diabetes.

Neutralizing autoantibodies targeted against type I interferons (IFN), especially IFN-ω seem to be an exception since these autoantibodies are found at an exceptionally high rate. 100% APECED patients are positive for autoantibodies targeted to IFN-ω, -α.8, or -α.2. Anti-IFN antibodies are even present before diagnosis in high titers. In addition to APECED, type I IFN auto-antibodies have been found only in patients that have thymomas, but with lower prevalence and titers. IFN antibodies may also have a role in the pathogenesis of APECED. Kisand et al. reported that patient-derived IFN-ω targeted autoantibodies caused a down-regulation of multiple IFN-regulated genes in immature DCs isolated from APECED patients. The effect was reversed when the cells were further cultured without the autoantibodies.

Recently, two independent groups showed that the majority of APECED patients have neutralizing autoantibodies against Th17-type cytokines IL-22 and IL-17. The patients also have less IL-22 producing cells. Th17 cells are important in the protection against Candida infections, so the autoantibodies could be associated with the Candida-specific immunodeficiency seen in APECED patients. Indeed, the autoantibodies were found in APECED patients that had chronic mucocutaneous candidiasis. Similar autoantibodies were also found in patients suffering from chronic mucocutaneous candidiasis without APECED. IL-22 and IL-17 cytokine antibodies were associated with the Finn major mutation. Puel et al. were able to identify autosomal recessive and dominant mutations in the IL-17 gene in patients with chronic mucocutaneous candidiasis disease that is not AIRE associated. This finding further consolidates the importance of IL-17 in the protection against Candida. Thus neutralizing auto-antibodies against IL-17 could explain at least partially the specific immunodeficiency towards Candida spp. found in APECED.

It is not known why candidiasis in APECED is only limited to skin and mucosal surfaces even though the autoantibodies are found systemically. Ahlgren et al. recently showed that peripheral blood monocytes isolated from APECED patients produce more IL-17A than healthy controls in response to Candida stimulation. However, Kisand et al. reported that peripheral blood mononuclear cells (PBMC) of APECED patients produce significantly less IL-17F, and one patient also showed reduced IL-17A production in a similar setting. IL-17A production varies also in healthy controls in response to
Candida-stimulation so the variance in results could reflect methodological differences. Both groups agreed that IL-22 production was significantly lower in patients with chronic mucocutaneous candidiasis with or without APECED as compared to healthy controls. IL-22 producing cells are preferentially found in the skin and mucosal surfaces. IL-22 therefore appears to be more important in the epithelial immune response towards Candida in APECED.

### Table 3: Autoantibodies in APECED

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Tissue expression</th>
<th>Clinical manifestation</th>
<th>Prevalence %</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-OH</td>
<td>adrenal cortex</td>
<td>Addison's disease</td>
<td>66</td>
<td>428</td>
</tr>
<tr>
<td>17α-OH</td>
<td>adrenal cortex, gonads</td>
<td>Addison's disease</td>
<td>44</td>
<td>428</td>
</tr>
<tr>
<td>SCC</td>
<td>adrenal cortex, gonads</td>
<td>Addison's disease, hypogonadism</td>
<td>52</td>
<td>428</td>
</tr>
<tr>
<td>AADC</td>
<td>liver</td>
<td>hepatitis</td>
<td>51</td>
<td>428</td>
</tr>
<tr>
<td>TPH</td>
<td>Intestinal serotonin producing cells</td>
<td>gastrointestinal dysfunction</td>
<td>48</td>
<td>118</td>
</tr>
<tr>
<td>HDC</td>
<td>gut</td>
<td>gastrointestinal dysfunction</td>
<td>37</td>
<td>241</td>
</tr>
<tr>
<td>TG</td>
<td>thyroid</td>
<td>hypothyroidism</td>
<td>36</td>
<td>241</td>
</tr>
<tr>
<td>TPO</td>
<td>thyroid</td>
<td>hypothyroidism</td>
<td>36</td>
<td>241</td>
</tr>
<tr>
<td>TDRD6</td>
<td>pituitary gland</td>
<td></td>
<td>49</td>
<td>241</td>
</tr>
<tr>
<td>TH</td>
<td>hair follicles</td>
<td>alopecia</td>
<td>44</td>
<td>201</td>
</tr>
<tr>
<td>GAD65</td>
<td>endocrine pancreas</td>
<td>diabetes</td>
<td>32</td>
<td>247</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>liver</td>
<td>hepatitis</td>
<td>8</td>
<td>428</td>
</tr>
<tr>
<td>TSGA10</td>
<td>testis</td>
<td></td>
<td>8</td>
<td>241</td>
</tr>
<tr>
<td>IA-2</td>
<td>endocrine pancreas</td>
<td>autoimmune diabetes</td>
<td>7</td>
<td>428</td>
</tr>
<tr>
<td>CaSR</td>
<td>parathyroid</td>
<td>hypoparathyroidism</td>
<td>50-86</td>
<td>144</td>
</tr>
<tr>
<td>SOX9</td>
<td>melanocytes</td>
<td>vitiligo</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td>SOX10</td>
<td>melanocytes</td>
<td>vitiligo</td>
<td>22</td>
<td>200</td>
</tr>
<tr>
<td>NALP5</td>
<td>parathyroid</td>
<td>hypoparathyroidism</td>
<td>49</td>
<td>13</td>
</tr>
<tr>
<td>KCNNR</td>
<td>bronchial epithelium</td>
<td>autoimmune lung disease</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>type I IFNs</td>
<td>soluble</td>
<td></td>
<td>100</td>
<td>305</td>
</tr>
<tr>
<td>IL-22</td>
<td>soluble</td>
<td>candidiasis</td>
<td>91</td>
<td>242</td>
</tr>
<tr>
<td>IL-17</td>
<td>soluble</td>
<td>candidiasis</td>
<td>75</td>
<td>242</td>
</tr>
</tbody>
</table>

### 3.1.3 Other Genetic Factors Affecting APECED Phenotype

HLA-association analyses with the disease components have shown that the incidence of type 1 diabetes, Addison’s disease, and alopecia are associated with the same HLA type II molecules as in non-APECED patients with these diseases. No HLA class II association could be found for hypoparathyroidism in Italian patients. The small number of patients makes large-scale HLA-association studies very hard. In the Finnish patient cohort
no clear HLA profile for other disease components or component combinations could be found\textsuperscript{183}

Associations between different APECED causing mutations and certain phenotypical patterns have been difficult to find. There is a high number of different APECED-causing mutations and a small number of patients with each mutation. However, the Finn major mutation R257X in the \textit{AIRE} gene has an association with a higher incidence of chronic mucocutaneous candidiasis\textsuperscript{183, 242}. On the other hand, in the unique Sicilian family with a dominant-negative p.G228W substitution in \textit{AIRE}, the patients have a low incidence of candidiasis but an exceptionally high incidence of autoimmune thyroiditis. This mutation is inherited in dominant fashion with a lower penetrance of the full APECED phenotype\textsuperscript{66, 306}. Another \textit{AIRE} mutation, that is common in the Italian APECED patients, was associated with a later onset of Addison’s disease\textsuperscript{65}.

Gender is an important risk factor in autoimmune diseases as generally females are more often affected. In APECED males have been reported to manifest a milder APECED phenotype characterized with later onset and a lower incidence of hypoparathyroidism\textsuperscript{178, 437}. Men also suffer less from hypogonadism\textsuperscript{428} than females but this finding could also result from the immuno-privileged status of testicles.

3.1.4 Other Human Diseases that Have Association with Reduced \textit{AIRE} Expression, \textit{AIRE} Mutations, or Polymorphisms in \textit{AIRE}

Since the discovery of \textit{AIRE} and the understanding of its vital role in maintaining immunological tolerance many autoimmune diseases have been analyzed for different \textit{AIRE}-haplotypes that might increase or decrease disease susceptibility. Associations of the increased risk for disease and SNPs in \textit{AIRE} have been found for systemic sclerosis associated with autoimmune thyroiditis\textsuperscript{125}. Recently, Terao \textit{et al.} reported also a strong association with two SNPs in \textit{AIRE} and rheumatoid arthritis in the Japanese patients\textsuperscript{451}. These polymorphisms in the Japanese population affected \textit{AIRE} expression level which could be the mechanism explaining the association\textsuperscript{451}. For melanoma, three protective SNPs in \textit{AIRE} were identified in healthy controls\textsuperscript{80}.

Contradictory reports on SNPs in \textit{AIRE} have been published for vitiligo\textsuperscript{233, 448}, alopecia\textsuperscript{367, 449, 485}, and childhood autoimmune hepatitis type 1\textsuperscript{110, 266}. The importance of \textit{AIRE} polymorphisms remains to be shown in these diseases. No association with \textit{AIRE} mutations or polymorphisms has been found in autoimmune polyendocrine syndrome type 2, isolated autoimmune Addison’s disease\textsuperscript{45, 467}, type 1 diabetes\textsuperscript{463}, sporadic idiopathic hypoparathyroidism\textsuperscript{160}, or polyglandular autoimmunity\textsuperscript{109}. In all, \textit{AIRE} polymorphisms are not associated
with common autoimmune single endocrinopathies even though all of these diseases are components of the APECED syndrome.

Hypomorphic mutations in the RAG genes in humans cause the Omenn's syndrome that is composed of both immunodeficiency and severe autoimmune manifestations290. Cavadini et al. reported that AIRE expression was significantly reduced in two patients with Omenn’s syndrome63. Loss of AIRE expression could explain some of the autoimmune manifestations in this syndrome even though the clinical pictures of these two diseases, Omenn’s syndrome and APECED, are not overlapping290.

Thymoma is a rare neoplastic tumor of the thymus. Thymomas are often complicated by various paraneoplastic autoimmune manifestations, of which myasthenia gravis is the most common one. Up to 95% of thymomas do not express AIRE, or the expression is greatly reduced407, 445. Thymoma patients are thus chimeric for AIRE expression because their remnant thymus tissue and secondary lymphoid organs maintain the normal AIRE expression. However, they do not generally develop APECED-like symptoms438, expect for one reported case73, even though the IFN auto-antibodies are commonly found in both diseases241. Myasthenia gravis per se is not associated with the loss of AIRE expression in thymoma293.

3.2 Mouse Models of APECED

3.2.1 Aire-deficient Mouse Models

When human AIRE was found, the mouse homologue Aire was quickly identified42, 480 and KO mouse models were created. The two first strains reported, Peltonen’s384 and Mathis’s20 mice, are both in the C57BL/6 background. Aire-deficient mice in B6 and B10 backgrounds have a relatively mild autoimmune phenotype, which is consistent with the reported resistance to autoimmune diseases in these inbred strains450. Aire−/− animals in BALB/c or SJL background have generally more pronounced autoimmune manifestations. Gastritis is typical for the BALB/c strain146. It is evident that the background strain has a significant impact on the scope and severity of autoimmunity in Aire-deficient mice232. The most common autoimmune manifestations are reduced fertility, autoimmunity against retina, gastritis, salivary gland inflammation, and exocrine pancreatitis. Aging increases the frequency of the autoimmune manifestations195.

Aire-deficient mice do not develop similar autoantibodies as the APECED patients220, 374. Ahlgren et al.4 reported that Aire-deficient mice are more susceptible to Candida albicans infection in the gastric mucosa. However, mice with the same genetic deletion in a different background are
not susceptible to Candida\textsuperscript{220}. Increased Candida susceptibility has not been found in any other Aire-deficient strain.

So far four different Aire-KO strains have been described and their phenotypes are summarised in Table 4 on pages 56-57.

### 3.2.2 Aire deficiency in Autoimmunity-prone Background Strains

Both Mathis’ mice and Matsumoto’s Aire-deficient mice have been crossed with the autoimmune diabetes prone NOD strain\textsuperscript{232, 334}. In both cases, Aire-deficient mice in NOD background developed a very severe autoimmune syndrome characterized by weight loss and increased mortality. Jiang et al.\textsuperscript{232} reported that generalized pneumonitis was the major reason for lethality. In Niki’s mice, the main autoimmune manifestation was a severe exocrine pancreatitis\textsuperscript{334} that was also evident in Jiang’s mice. Interestingly, even in the NOD background, both Aire\textsuperscript{-/-}NOD mice strains were completely protected from diabetes. Given the complex background of autoimmunity in NOD mice\textsuperscript{18}, it is hard to estimate why the loss of Aire has such contradicting effects on the NOD phenotype, namely protection from diabetes but acceleration of the other disease components.

Chen et al.\textsuperscript{71} crossed the Mathis’ Aire\textsuperscript{-/-} mice with Scurfy (FoxP3\textsuperscript{sf}) mice. Scurfy have a spontaneous mutation in FoxP3 gene and these mice do not have functional Tregs. Aire\textsuperscript{-/-}FoxP3\textsuperscript{sf} mice developed an autoimmune syndrome that was more severe than in either one of the parental strains alone. All Aire\textsuperscript{-/-}FoxP3\textsuperscript{sf} mice had to be euthanized before the age of 28 days. The lethal autoimmune manifestations in these double-deficient mice were lung inflammation and necrotizing hepatitis\textsuperscript{71}. The animals did not produce significant levels of autoantibodies. Also many organs affected in the Aire-deficient mice in the B6 background were almost completely devoid of infiltrates. This, however, could result from the young age of the studied animals\textsuperscript{71}. 


Table 4: Summary of the different Aire-/- mice phenotypes in different background strains. Bold: 100% of the animals affected.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Peltonen's mice</th>
<th>Mathis' mice</th>
<th>Matsumoto's mice</th>
<th>Scott's mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutation</strong></td>
<td>Termination codon in exon 6</td>
<td>Deletion of exon 2</td>
<td>Deletion from exon 5 to exon 12</td>
<td>Partial deletion of exon 8</td>
</tr>
<tr>
<td><strong>Background strain</strong></td>
<td>C57BL/6</td>
<td>B10.BR/SgSnJ</td>
<td>C57BL/6 mixed</td>
<td>C57BL/6</td>
</tr>
<tr>
<td><strong>The thymus morphology</strong></td>
<td>Increased incidence of thymic atrophy</td>
<td>Increased MECs</td>
<td>Normal</td>
<td>Increased MECs</td>
</tr>
<tr>
<td><strong>T cell irregularities</strong></td>
<td>Alterations in TCR repertoire, hyper-proliferation after immunization</td>
<td>Increased frequency of activated / memory T cells</td>
<td></td>
<td>Increased activated/ memory T cells, some alterations in TCR repertoire</td>
</tr>
<tr>
<td><strong>Auto-antibodies</strong></td>
<td>Liver, testis, pancreas, adrenal</td>
<td>Eyes, salivary gland, ovary, stomach</td>
<td>α-fodrin</td>
<td>Salivary gland, liver, salivary gland, eyes, pancreas</td>
</tr>
<tr>
<td><strong>Lymphocyte infiltrates in solid organs</strong></td>
<td>Liver, pancreas, lung, thyroid, ovary</td>
<td>Pancreas, stomach, salivary gland</td>
<td>Stomach, salivary gland, lung, liver, prostate, ovary, pancreas</td>
<td>Eyes, salivary gland, Gastric mucosa</td>
</tr>
</tbody>
</table>
Table 4 continued

<table>
<thead>
<tr>
<th>Strain</th>
<th>Peltonen’s mice</th>
<th>Mathis’ mice</th>
<th>Matsumoto's mice</th>
<th>Scott’s mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>C57BL/6</td>
<td>B10.BR/</td>
<td>C57BL/6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SgSnJ</td>
<td>BALB/c</td>
<td>mixed</td>
</tr>
<tr>
<td>Fertility</td>
<td>Reduced</td>
<td>Normal in</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>young mice,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduced in</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>aged mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs and</td>
<td>Reduced</td>
<td>Normal in</td>
<td>Normal in</td>
<td>Decreased</td>
</tr>
<tr>
<td>other</td>
<td></td>
<td>in vivo and</td>
<td>vitro Treg</td>
<td>proportion</td>
</tr>
<tr>
<td>regulatory</td>
<td></td>
<td>in vitro Treg</td>
<td>suppression</td>
<td>of Tregs in</td>
</tr>
<tr>
<td>populations</td>
<td></td>
<td>Treg suppression</td>
<td>activity</td>
<td>the thymus</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candidiasis</td>
<td>No</td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>susceptibility</td>
</tr>
<tr>
<td>Effect of</td>
<td>Reduced body</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>aging</td>
<td>weight,</td>
<td>incidence of</td>
<td>incidence of</td>
<td>incidence of</td>
</tr>
<tr>
<td></td>
<td>splenomegaly,</td>
<td>autoimmune</td>
<td>autoimmune</td>
<td>autoimmune</td>
</tr>
<tr>
<td></td>
<td>B cell lymphoma</td>
<td>findings</td>
<td>findings</td>
<td>findings</td>
</tr>
<tr>
<td>Other</td>
<td>B cell liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>infiltrates,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>increased BAFF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>References</td>
<td>195, 276, 277,</td>
<td>19, 20, 146,</td>
<td>103, 146,</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>384</td>
<td>168, 214</td>
<td>232</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td></td>
<td></td>
<td>259</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>259</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22, 220</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
FoxP3 deficiency combined with Aire deficiency thus resulted in severe autoimmunity. This finding could indicate that peripheral tolerance and especially Tregs are important in suppressing autoreactive T cell clones. Teh et al. studied the importance of peripheral tolerance with the loss of Aire by crossing Aire<sup>−/−</sup> mice in the B10 background with four different mouse strains with known defects in peripheral tolerance<sup>450</sup>. Defects in activation-induced cell death of mature T cells, or inhibition of inducible T cell co-stimulator – mediated co-stimulation and loss of Tf<sub>h</sub> cells did not affect the phenotype of Aire<sup>−/−</sup> mice<sup>449</sup>. The resulting double-deficient mice had comparable symptoms with their single-deficient counterparts. The authors also crossed Aire<sup>−/−</sup> mice with a strain that has a point-mutation in caspase recruitment domain family, member 11 (Card11) that is a critical scaffold protein needed for example in the TCR signaling. These Card11<sup>−/−</sup> mice have been reported to have defective TCR and CD28 signaling that results in a decreased amount of FoxP3<sup>+</sup> Tregs. Contrary to the results seen in Aire<sup>−/−</sup>FoxP3<sup>sf</sup> mice, these double-deficient animals stayed overtly healthy with no findings exceeding the phenotype of the parental strains<sup>450</sup>.

Casitas B-lineage lymphoma ubiquitin ligase (Cblb) is an important molecule in the generation of T cell anergy<sup>218</sup>. It is up-regulated in mature and anergic T cells where it inhibits co-stimulatory signaling and T cell proliferation, i.e. it is a negative controller of T cell activation like CTLA-4<sup>218</sup>. In mice that have a KO mutation in this molecule, T cells are released from the strict need for co-stimulation through CD28. These mice do not show any signs of overt autoimmunity or T cell hyperproliferation. However, when Teh et al.<sup>450</sup> crossed these mice with Aire-deficient mice, the resulting double-KOs developed a severe autoimmune exocrine pancreatitis that necessitated euthanaziation of animals on at the age of 25 days an average. The animals also had sialoadenitis and mild gastritis, together with autoantibodies targeted to exocrine parts of pancreas and salivary glands. All symptoms were transferable with total splenocytes from double-deficient animals to lymphopenic healthy recipients indicating that the disease was likely mediated by lymphocytes. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were required to transfer the disease<sup>450</sup>. Since Cblb has multiple functions in the adaptive immune system, it is difficult to estimate how the double-deficiency of Aire and Cblb resulted in synergistic development of autoimmunity.

### 3.2.3 Experimental Induction of Autoimmunity in Aire-deficient Mice

Considering the variable phenotypes and impact of aging in Aire-deficient mice, environmental factors are potentially important in the breakdown of tolerance. Gray et al.<sup>168</sup> tested if treatments with various innate immunity stimuli would have an effect on the autoimmunity in Aire<sup>−/−</sup> mice B6 and NOD
backgrounds (Mathis’ mice). Innate immune system signaling did not have any significant impact on the severity or onset of autoimmune manifestations. Also animals kept in a germ-free conditions developed similar disease as their counterparts kept in normal animal facility. Microbial colonization is therefore not needed for the development of autoimmunity in Aire⁻/⁻ mice.

Aire-deficient mice showed increased susceptibility to chemically induced autoimmune diabetes. Surprisingly, the mice heterozygous for Aire had the highest frequency of diabetes after the chemically induced pancreatic beta cell destruction.

Aire-deficient mice in the C57BL/6 background showed increased susceptibility to experimental autoimmune myasthenia gravis, where autoimmunity to the acetylcholine receptor is induced by immunization with the autoantigen. The disease susceptibility was not uniform, but younger Aire-deficient mice were partially protected from the disease compared with wild-type controls. On the contrary, aged Aire-deficient mice showed higher disease scores. In an experimental autoimmune encephalomyelitis model Aire-deficient mice became symptomatic significantly earlier than the wild-type mice, but the disease severity was not affected. In an experimental model for rheumatoid arthritis, the collagen-induced arthritis, the Aire-deficient mice became symptomatic faster and had a more severe disease after immunization. In all these immunization models of autoimmunity, the autoantigens used are expressed under the control of Aire in the thymus but Aire-deficient animals do not show signs of autoimmunity targeted to these autoantigens spontaneously. The tolerance is broken only after immunization with an adjuvant. Thus the breaking of tolerance in Aire-deficient animals is often dependent on environmental factors, such as strong inflammation that the adjuvant initiates.

3.3 Autoimmune Regulator (AIRE) Gene

3.3.1 The structure of AIRE and Structural Implications for Its Function

AIRE gene is located in the q22 region of chromosome 21. Its product AIRE is a nuclear protein 545 amino acids long. AIRE has several functional domains that indicate its function as a transcriptional regulator and chromatin-binding protein: caspase-recruitment domain, two plant homeodomain zinc fingers, four nuclear-receptor-binding motifs, and a proline-rich region that is common with transcription-associated proteins (see Figure 5). However, AIRE is not a typical transcription factor because it can promote the expression of thousands of genes and the transcriptional start sites can be different from those required for the genes’ expression outside the thymus.
AIRE influences a large number of genes when introduced into different cell lines, but the spectrum of genes affected is different from one cell type to another\textsuperscript{172, 248}. The genetic distribution of genes affected by AIRE is clustered, but the same cluster can contain AIRE-dependent and -independent genes\textsuperscript{101} further arguing against AIRE functioning as a classical sequence-specific transcription factor.

In the cell, AIRE is found mainly in the nucleus but it has also been found in fibrillar structures in the cytoplasm\textsuperscript{39}. In the nucleus, it is concentrated in nuclear bodies that are located in close proximity of nuclear speckles, but it is also found in the nucleoplasm\textsuperscript{40}. This kind of nuclear distribution is well in line with the functional domains indicating a role of AIRE in the transcriptional regulation\textsuperscript{184}.

The precise manner of how AIRE regulates gene expression is still elusive. The identified AIRE mutations in APECED patients have provided some insight into the functions of the AIRE protein\textsuperscript{182}. The regions in the gene associated with nuclear and cytoplasmic localization and zinc-finger domains are rather conserved between the murine and human homologues of AIRE\textsuperscript{42, 480} but especially the proline-rich region and the DNA-binding "human Sp100, Aire1, NucP41/P75, and Drosophila DEAF1 domain" (SAND) domain show little overlap between mammalian species\textsuperscript{403}. Most APECED mutations are found in evolutionally conserved regions of the AIRE gene\textsuperscript{403}.

The SAND domain seems to be needed for the nuclear localization of AIRE, and it possesses a regulatory function in the homomultimerization of AIRE\textsuperscript{182, 224, 384}. Two different SNPs located in SAND have been associated with decreased AIRE expression\textsuperscript{450}. Mutation in the SAND domain of AIRE is the only APECED mutation inherited in a dominant fashion. Autoimmune thyreoditis is especially common in the clinical phenotype following this mutation\textsuperscript{66, 224}. The mechanism behind this different inheritance pattern appears to be caused by an inhibition of the normal allele by the mutated protein\textsuperscript{224}. The same mutation also affected HLA class II expression in thymic epithelial cells\textsuperscript{456}.

In vitro experiments indicate that AIRE has a preferential DNA binding capacity both by the SAND domain and the zinc fingers\textsuperscript{257}. Considering the large pool of genes under AIRE control, it seems unlikely that AIRE functions only as a direct DNA binding transcription factor. As a proof of AIRE’s more general involvement in transcription regulation, many proteins that work in concert with AIRE have been identified\textsuperscript{2}. AIRE is found in large molecular weight complexes\textsuperscript{182}. Recently 45 molecular partners of AIRE were reported\textsuperscript{2}. They could be categorized to four different functional groups: nuclear transport, chromatin binding / structure, transcription, and messenger ribonucleic acid (mRNA) processing. AIRE was found to have the strongest impact on pre-mRNA splicing\textsuperscript{2} and this could explain how TSA expression is
more efficient in MECs expressing AIRE. The correctly spliced RNA is more stable and the translation can be carried out longer.

AIRE has a preferential binding capacity for unmethylated histones through the first plant homeodomain - type zinc finger\textsuperscript{348}. This indicates that AIRE has potentially a wider epigenetic control over the inactive parts of chromatin, and thus it promotes the expression of otherwise silent antigens\textsuperscript{251, 347}. However, binding to the unmethylated histones does not completely explain the transcriptional activity of Aire. In a mouse model with targeted mutation to the histone binding element in Aire, the mutation affected most but not all of the genes that the Aire-null mutation affected\textsuperscript{250}. AIRE has also been connected, together with other molecules, with the activation of stalled non-active RNA polymerase II molecules thus enabling transcription of otherwise dormant genes\textsuperscript{353}.

Figure 5: A schematic presentation of AIRE protein domain structure. Locations of the APECED causing mutations are indicated by arrows. CARD, caspase-recruitment domain; PHD, plant homeodomain zinc finger; LXXLL sequence, nuclear-receptor binding motif; PRR, proline rich region.

3.3.2 AIRE expression

3.3.2.1 Aire Expression in the Thymus

The highest level of AIRE expression can be found in the thymus in the mTECs\textsuperscript{40, 129, 328}. However, only a fraction of all mTECs express Aire\textsuperscript{100, 154}. Suzuki \textit{et al.} found AIRE expression in human double-positive thymocytes and thymic B lymphocytes\textsuperscript{444}. Also human thymic DCs express AIRE\textsuperscript{161}.

In the mouse, Aire expression in the thymus is restricted almost entirely to mTECs and these cells are enriched in the cortico-medullary junction\textsuperscript{521}. Murine thymic DCs show only very low expression of Aire\textsuperscript{521}. Aricha \textit{et al.}\textsuperscript{22} studied thymic Aire mRNA expression in different mouse strains and found that the C57BL/6 mice have spontaneously the lowest Aire expression as compared to SJL and BALB/c mice.
3.3.2.2 Aire Expression Outside the Thymus

The expression of AIRE outside of the thymus has been long debated. At mRNA level, Aire and AIRE transcripts were found from multiple solid tissues but the protein expression was more difficult to determine.\(^\text{119}\) This is most likely the result of different splice variants, and the varying affinity of the used poly- and monoclonal antibodies.\(^\text{119, 221}\) In human and mouse extra-thymic tissues AIRE and Aire expression has been confirmed both at mRNA and protein level in the spleen and lymph nodes.\(^\text{3, 184, 202, 203, 372}\) A few groups have been able to show AIRE expression in PBMCs.\(^\text{184, 373, 444}\) It can be concluded that AIRE and Aire are expressed in secondary lymphoid tissues outside the thymus but its expression in other tissues remains to be confirmed. Different methods used have given confusing reports especially in mouse solid tissues. The reported positive and negative AIRE protein expression in human and mouse tissues are summarised in Tables 5 (page 63) and 6 (page 64).

Lee et al.\(^\text{271}\) found that murine lymph node stroma contains Aire-expressing cells. Gardner et al. studied this population further in transgenic mice using a green fluorescent protein marker.\(^\text{143}\) The authors described Aire expression in mouse stroma-originating non-DCs in lymph nodes and the spleen that they called extrathymic Aire-expressing cells.\(^\text{143}\) These cells resembled DCs in their gene expression profiles and they also expressed MHC class II molecules, but no co-stimulatory factors (CD80 or CD86), or the DC marker protein CD11c. Interestingly, extrathymic Aire expressing cells also expressed a variety of tissue specific antigens like mTECs do but the expression profiles in these cells were different.\(^\text{143}\) These Aire\(^+\) cells were able to delete autoreactive T lymphocytes from peripheral T cell pool, even though the study did not confirm the role of Aire in the peripheral deletion.

3.3.2.3 Aire Expression in Embryos, Fetuses, and Neonates

There are conflicting reports on Aire expression during mouse embryogenesis. First, the expression was found to be associated with the initiation of the thymic organogenesis on the embryonic day 14.\(^\text{521}\) Another study did not detect Aire-expressing mTECs before embryonic day 16.\(^\text{487}\) However, recently it was shown that significant Aire expression can be found already on embryonic day 6.\(^\text{336}\) This Aire expression starts just before gastrulation that is the division of the three primary tissue layers, namely ectoderm, mesoderm, and endoderm. The authors speculated that if the expression pattern of human AIRE is similar to that in mouse, the lack of early AIRE expression might explain the ectodermal dystrophy in APECED.\(^\text{336}\)
Table 5: Reported positive AIRE and Aire protein expression in tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Human</th>
<th>Mouse</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>thymus</td>
<td>X</td>
<td>X</td>
<td>3, 184, 202, 203, 221, 372, 384, 408</td>
</tr>
<tr>
<td>spleen</td>
<td>X</td>
<td>X</td>
<td>3, 184, 203, 271</td>
</tr>
<tr>
<td>lymph node</td>
<td>X</td>
<td>X</td>
<td>3, 184, 203, 271, 372</td>
</tr>
<tr>
<td>tonsils</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>gonads</td>
<td>X</td>
<td></td>
<td>3, 408</td>
</tr>
<tr>
<td>kidney</td>
<td>X</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>lung</td>
<td>X</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>PBMC</td>
<td>X</td>
<td>X</td>
<td>184, 444</td>
</tr>
<tr>
<td>fetal liver</td>
<td>X</td>
<td>X</td>
<td>3, 203</td>
</tr>
<tr>
<td>liver</td>
<td>X</td>
<td></td>
<td>384</td>
</tr>
<tr>
<td>bone marrow</td>
<td>X</td>
<td></td>
<td>184</td>
</tr>
<tr>
<td>brain</td>
<td>X</td>
<td></td>
<td>3, 384</td>
</tr>
<tr>
<td>gut (goblet cells)</td>
<td>X</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>fallopian tubes</td>
<td>X</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>gut associated lymphoid tissue</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
</tbody>
</table>

One mouse model of conditional Aire expression has been developed in NOD background\textsuperscript{171}. Aire expression was conditionally turned off in the thymus epithelium at different time points, namely during organogenesis, neonatally, or in adult animals. In this model, the mice developed a full autoimmune syndrome only if Aire expression was turned off in the thymus during embryogenesis and the neonatal period. If thymic Aire expression was turned on after weaning of the mice, all mice were protected from the full autoimmune syndrome\textsuperscript{171}. In mice, Aire expression in the thymus before birth might therefore be more important than in later life. Adamson et al.\textsuperscript{3} found that there are dynamic changes in Aire expression in mice during the fetal period and Aire expression decreased towards delivery. In human fetal tissue, AIRE expression has been seen only in the thymus and liver\textsuperscript{203, 372}. The expression level of AIRE in relation to gestation time has not been studied.
Table 6: Reported negative AIRE and Aire protein expression in tissues. Reported contradicting results are indicated with an asterisk.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Human</th>
<th>Mouse</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>spleen</td>
<td>X</td>
<td>X*</td>
<td>202, 221, 372</td>
</tr>
<tr>
<td>lymph node</td>
<td></td>
<td>X*</td>
<td>202, 221</td>
</tr>
<tr>
<td>gonads</td>
<td>X</td>
<td>X*</td>
<td>202, 203, 372</td>
</tr>
<tr>
<td>kidney</td>
<td>X</td>
<td>X*</td>
<td>202, 203, 372</td>
</tr>
<tr>
<td>lung</td>
<td>X</td>
<td>X*</td>
<td>202, 203, 372</td>
</tr>
<tr>
<td>PBMC</td>
<td></td>
<td>X*</td>
<td>203, 372</td>
</tr>
<tr>
<td>liver</td>
<td>X</td>
<td>X*</td>
<td>3, 203</td>
</tr>
<tr>
<td>brain</td>
<td></td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>bone marrow</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>bowel</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>heart</td>
<td>X</td>
<td>X</td>
<td>3, 203, 372</td>
</tr>
<tr>
<td>pancreas</td>
<td>X</td>
<td></td>
<td>203, 372</td>
</tr>
<tr>
<td>skin</td>
<td>X</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>skeletal muscle</td>
<td>X</td>
<td>X</td>
<td>203, 372</td>
</tr>
<tr>
<td>adrenal gland</td>
<td>X</td>
<td>X</td>
<td>3, 184, 202, 203, 372</td>
</tr>
<tr>
<td>thyroid gland</td>
<td>X</td>
<td></td>
<td>203, 372</td>
</tr>
<tr>
<td>eye</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>mammary gland</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>salivary gland</td>
<td>X</td>
<td></td>
<td>372</td>
</tr>
</tbody>
</table>

* Contradictive results

3.3.2.4 Factors Controlling Aire Expression

Aire up-regulation in immature mTECs appears to be controlled by specific signals, such as Receptor Activator of Nuclear Factor κ B and CD40 signaling, but the exact molecular network controlling Aire expression is still unknown. The three-dimensional structure of the mTECs is important for Aire-expressing cells, since the expression of Aire is decreased when the thymic structure is disturbed. Mature thymocytes, however, are not needed for Aire expression, supporting the view that Aire-expressing mTECs are a specialized population that diverge from other thymic epithelial cells already during thymic organogenesis.

In several mouse models the expression of Aire has been found to be defective or lacking. From these models it has been possible to identify
signaling pathways and molecules that are involved in Aire expression: the lymphotoxin pathway,\textsuperscript{76} RelB transcription factor,\textsuperscript{274} adaptor molecule TRAF6,\textsuperscript{8} and NFκB.\textsuperscript{130,513} In these models the thymic architecture is disturbed and mTEC numbers are low. Therefore it is hard to differentiate whether the loss of Aire is secondary to the loss of mTECs in general. For example, the importance of the lymphotoxin pathway for the expression of Aire has been widely questioned.\textsuperscript{292,391,471}

3.3.3 The Functions of AIRE

Table 8 on page 73 briefly summarises the suggested functions of Aire in the thymus and peripheral immune system. In the following sections, these functions are described in more detail.

3.3.3.1 Aire in the Thymus Development and Tissue Organization

Originally the thymus architecture in Aire-deficient mice was considered normal\textsuperscript{20,384} and research focused mainly on the transcriptional aspects of Aire driving the expression of TSAs in mTECs. Gillard \textit{et al.}\textsuperscript{154} carried out a more detailed analysis of the thymic cellular departments and noticed several differences in the thymic morphology in Aire-deficient animals. For example, Aire-deficient animals lacked a mTEC population staining clearly positive for Ulex europaeus agglutinin 1, a marker generally used for the thymic epithelial cells\textsuperscript{154}. This could indicate that Aire-deficient mTECs are not fully maturated or functional since they do not express mature mTEC-associated markers\textsuperscript{311}. In Aire-deficient thymus, the morphology and distribution of mTECs is concentrated in the medulla, instead of cortico-medullary junction as in wild-type normal thymus\textsuperscript{500}. In the same study, the authors reported that Hassall’s corpuscle-like structures are almost completely absent in Aire-deficient thymuses. Therefore the loss of Aire significantly affects the thymus morphology, which indicates that Aire is necessary for normal thymus development.

Thymic epithelial cells, both cortical and medullary, originate from common bipotent progenitors. Aire-expressing cells have been generally considered to represent the terminally differentiated population of mTECs\textsuperscript{297}. Recently, however, it has been shown that Aire expression stops before the terminal differentiation of mTECs\textsuperscript{336}. Hamazaki \textit{et al.}\textsuperscript{185} found that tight junction components claudin-3 and claudin-4 are preferentially expressed in Aire-expressing mTECs but their expression is not dependent on Aire. During the thymic organogenesis claudin-3 and claudin-4 positive cells were identified as progenitors for Aire-expressing cells. This finding suggests a specific differentiation program for Aire expressing cells.\textsuperscript{185}
Supporting the view that Aire is essential for thymic organogenesis, Gillard et al.\textsuperscript{154} found that Aire controls the expression of Nanog, Oct4, and Sox2, which are transcription factors critical in maintaining the multipotentiality in progenitor cells\textsuperscript{154}. In addition to these genes, Aire also controls the expression of different transcription factors, for example a number of bone morphogenetic proteins that are involved in many important immunological processes such as cytokine production, lymphocyte homeostasis, and activation \textsuperscript{392}. Aire has been associated with the expression of various chemokines\textsuperscript{19} and cytokines\textsuperscript{406} in the thymus, and it takes part in the intrathymic migration of developing thymocytes\textsuperscript{262}. The precise role of Aire in thymic development and thymus function remains to be clarified.

3.3.3.2 Aire, Peripheral Tissue Antigen Expression in the Thymus, and Central Tolerance

Aire’s best-described function is in promoting the ectopic expression of peripheral TSAs in mTECs. TSAs are vital in the negative selection and clonal deletion of the autoreactive developing thymocytes as discussed previously\textsuperscript{477}. The data supporting this view come mainly from Aire-deficient mouse models. mTECs from Aire\textsuperscript{-/-} and wild-type mice were isolated and genome-wide micro-array analysis showed that the expression of large part of TSAs, such as insulin, was Aire-dependent\textsuperscript{20}. Many of these antigens have later been shown to be the targets for autoimmune attacks reported in Aire-deficient mice but there are also autoimmune manifestations targeted to antigens whose expression in the thymus was unaffected by the loss of Aire\textsuperscript{259, 334, 392}. Table 7 summarises the auto-antigens that are targets of autoimmunity in Aire-deficient mice and their expression in thymus.

Negative selection involves, in addition to ectopic TSA expression, the clonal deletion of thymocytes reactive to them. The association of Aire with clonal deletion was established in transgenic mouse systems. Liston et al.\textsuperscript{281} utilised a neo-self-antigen hen egg-lysozyme that was placed under Aire-dependent tissue specific transcription promoter (rat insulin promoter). The model-antigen transgenic mice had also their TCR loci replaced with a monogenic TCR specific for the neo-self antigen, so that all developing T lymphocytes were specific for it. With normal Aire expression, the model antigen was expressed in the thymus and all developing TCR-transgenic thymocytes were deleted, i.e. the animals were tolerized. When these mice were crossed with Aire-deficient mice, the model-antigen reactive TG-TCR expressing mature T cells escaped to periphery and autoimmune diabetes developed because the insulin promoter also drove the expression of the model antigen in pancreatic \(\beta\)-cells\textsuperscript{281}.
Table 7: The role of Aire in the expression of autoantigens in the murine thymus.

<table>
<thead>
<tr>
<th>Autoantigen</th>
<th>Disease association</th>
<th>Thymic expression in Aire-/-mice</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interphotoreceptor retinoid-binding protein</td>
<td>retinitis</td>
<td>reduced</td>
<td>102</td>
</tr>
<tr>
<td>Mucin 6</td>
<td>gastritis</td>
<td>reduced</td>
<td>146</td>
</tr>
<tr>
<td>Seminal vesicle secretory protein 2</td>
<td>prostatitis</td>
<td>reduced</td>
<td>214</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>collagen-induced arthritis</td>
<td>reduced</td>
<td>59</td>
</tr>
<tr>
<td>Myelin oligonucleotide glycoprotein</td>
<td>experimental autoimmune encephalomyelitis</td>
<td>reduced</td>
<td>248</td>
</tr>
<tr>
<td>Acetylcholine receptor</td>
<td>experimental myasthenia gravis</td>
<td>reduced</td>
<td>22</td>
</tr>
<tr>
<td>α-fodrin</td>
<td>Sjögren's syndrome like exocrine inflammation</td>
<td>unaltered</td>
<td>259</td>
</tr>
<tr>
<td>Odorant binding protein 1a</td>
<td>keratoconjunctivitis sicca</td>
<td>reduced</td>
<td>103</td>
</tr>
<tr>
<td>Pancreatic protein disulfide isomerase</td>
<td>exocrine pancreatitis</td>
<td>unaltered</td>
<td>334</td>
</tr>
<tr>
<td>Experimental autoimmune prostatitis antigen 2</td>
<td>experimental autoimmune prostatitis</td>
<td>unaltered</td>
<td>392</td>
</tr>
<tr>
<td>Vomeromodulin</td>
<td>interstitial lung disease</td>
<td>reduced</td>
<td>419</td>
</tr>
<tr>
<td>RIP-OVA (transgenic)</td>
<td>insulitis</td>
<td>unaltered</td>
<td>19</td>
</tr>
<tr>
<td>RIP-HEL (transgenic)</td>
<td>insulitis</td>
<td>reduced</td>
<td>281</td>
</tr>
</tbody>
</table>

Anderson et al.\textsuperscript{19} used a similar genetic construct as Liston et al., i.e. the rat insulin promoter, but used ovalbumin as the model antigen. Surprisingly, ovalbumin expression levels were unaffected by the loss of Aire expression. Even though the model antigen expression in the thymus was independent of Aire, the loss of Aire resulted in the escape of pancreatic ovalbumin-specific T cell clones to periphery where they caused a lethal autoimmune diabetes\textsuperscript{19}. Anderson et al. concluded that Aire has an important role in clonal T cell depletion, and it is not necessarily limited solely to the ectopic TSA expression\textsuperscript{19}. Indeed, they showed that the loss of Aire influenced an array of other genes as well, especially many genes associated with antigen processing and homing (for example chemokines). Aire did not only up-regulate these genes, but in some cases the gene was down-regulated in an Aire-dependent manner\textsuperscript{19}. 
In models where different antigens were abundantly expressed, for example the male antigen model, the loss of Aire had no effect on negative selection\(^\text{19}\). Also in transgenic systems using systemic promoters the neo-antigen-specific T cells were not deleted\(^\text{280}\). Using another TG-TCR and model antigen combination, Liston \textit{et al.} were able to demonstrate the role of Aire is negative T cell selection also in mice heterozygous for the Aire-KO mutation\(^\text{280}\). This dose-dependent effect of Aire on negative selection has not been reported in other mouse models. On the other hand, using a promoter that was specific for the thyroid tissue, autoreactive T cells escaped to periphery, but did not cause a disease\(^\text{280}\). Therefore, in transgenic models Aire is involved in the thymic expression of tissue specific antigens, but not that of systemic antigens. However, the impact of Aire on TSA expression in the thymus is not always predictable, as is highlighted by two different rat-insulin promoter constructs with contrasting results\(^\text{19, 281}\).

In transgenic models Aire is clearly involved in negative selection of T cells. Whether or not this is linked to Aire-driven TSA expression can be questioned. Current published data linking the loss of Aire expression to defective clonal deletion and following autoimmunity are all derived from transgenic models. Spontaneous autoimmunity against endogenous antigens, whose expression in the thymus is controlled by Aire in the thymus, has been reported\(^\text{102, 103, 146, 214, 419}\). These findings, however, are not sufficient to prove that the autoimmune inflammation results from defective negative selection \textit{per se}. Aire deficiency and loss of TSA expression in the thymus could also affect the maintenance of peripheral tolerance. However, autoimmune attacks in Aire-deficient animals are also targeted to antigens whose expression in the thymus is unaltered by the loss of Aire\(^\text{259, 334, 392}\). This discrepancy could be explained by the role of Aire in antigen expression on a more general level for example through decreased HLA expression\(^\text{19, 456}\).

A relatively small population of mTECs expresses Aire, and on a single cell level it has been shown that a given mTEC expresses only a limited array of TSAs\(^\text{100}\). The thymus must express a huge array of TSAs to maintain effective central tolerance. Thus, Aire cannot be the only transcription factor involved in TSA expression. It is not known how the constant wide pool of TSAs is maintained in the thymic medulla, and what is the role of Aire in this phenomenon. Aire\(^+\) mTECs are rapidly proliferating and they have a very short lifespan\(^\text{113, 124, 167}\). This has been suggested to facilitate TSA expression since the thymic DCs, that surround the mTECs, phagocytose apoptotic cells and can then cross-present the TSAs they acquire during this process\(^\text{140}\). Recently, Hubert \textit{et al.} showed that Aire drives the antigen transfer from mTECs to thymic DCs\(^\text{219}\). Aire has also been suggested to drive the accumulation of DC to the thymus medulla\(^\text{272}\). Thymic DCs have been shown to be important in the negative selection\(^\text{99}\). The mechanisms they utilise to
mediate deletional tolerance are still unknown. Approximately 50% of thymic DCs migrate from the circulation to the thymus and therefore can carry antigens from other parts of the body, including non-self antigens. Aire-dependent antigen transfer to thymic DCs expands the role of Aire in thymic negative selection even further and may explain some of the earlier conflicting results.

In the human system, there have been a few reports linking AIRE to TSA expression in the thymus. Giraud et al. showed that AIRE affects the thymic expression of CHRNA1, a gene that encodes for the α-subunit of muscle acetylcholine receptor, which is the main target of the pathogenetic autoantibodies in autoimmune myasthenia gravis. Aricha et al. showed that acetylcholine receptor expression is also reduced in the thymuses of Aire-deficient mice.

### 3.3.3.3 Aire and Cell Populations Developing in the Thymus

Li et al. found that there is a developmental arrest of thymocytes at late thymic developmental stages in the Aire-deficient mice. This finding was repeated by Hubert et al. The peripheral major T cell populations, however, have been reported to be normal in Aire-deficient mice. Aire deficiency apparently does not have any impact on the non-self antigen recognizing TCR repertoire even though some small alterations in the general peripheral TCR repertoire of the Aire-/- mice have been found. In the peripheral T cell pool, Aire-/- mice have more T cells with activated or memory phenotype but this is most likely the result of an inflammatory process seen in these mice. Therefore, it seems that Aire has a role in the thymic T cell development, which is a highly selective process and does not lead to overt imbalance in the peripheral T cell populations.

There are other cell populations than αβ T cells that originate and develop in the thymus. T cells with the γδ-TCR develop normally without Aire. Thymic metallophilic macrophages, which have been reported to act as APCs, also develop normally in Aire-deficient mice. Thymus-derived iNKT cells were first believed to develop normally in Aire-deficient mice, even though reduced numbers were noticed in the spleen. Recently Lindh et al. reported a detailed analysis of the iNKT cells in both Aire-deficient mice and APECED patients. The number of iNKT cells was reduced in both mice and human patients, and iNKT cells were dysfunctional in mice. They also found that thymus-originating NK cells developed normally without Aire, as well as conventional NK cells developing in the bone marrow. The lower frequency of iNKT cells in APECED patients was later confirmed in another study.

Motivated by the additive effect of FoxP3 deficiency in Aire-deficient mice, many groups have studied Aire’s influence on nTreg development.
Originally the Treg population was reported to be normal both in numbers and function in Aire-deficient mice\textsuperscript{19, 20}. A more detailed analysis showed that the TCR repertoires of Tregs were unaltered in Aire-deficient animals\textsuperscript{92}. Hubert \textit{et al.}\textsuperscript{220} summarised their own results and the data published by others and claimed that in Aire\textsuperscript{−/−} animals there is a trend of decreased Treg numbers in the periphery. Hubert's own data, however, did not reach statistical significance. Aricha \textit{et al.}\textsuperscript{22} found a clear reduction of FoxP3\textsuperscript{+} Tregs in Aire-deficient thymuses. The percentage of Tregs was not affected in the peripheral immune system in young mice, but it reduced when the mice aged. Also FoxP3 mRNA expression level was lower in aged Aire\textsuperscript{−/−} mice than in their wild-type controls. This finding could explain the earlier negative results since the previous studies have generally been done in younger animals.

Aschenbrenner \textit{et al.}\textsuperscript{25} were able to target model antigen expression to Aire\textsuperscript{+} mTECs by inserting their TG-antigen sequence under the control of an Aire promoter. When mice that had only these model antigen-expressing mTECs were crossed with the model antigen-specific TG-TCR mouse strain, the thymuses of these mice produced an increased proportion of FoxP3\textsuperscript{+} nTregs\textsuperscript{25}. Thus is is possible to speculate that Aire\textsuperscript{+} mTECs are involved in the generation of nTregs. It should be noted, however, that the setting Aschenbrenner used was highly modified and was far from a normal polyclonal nTreg development.

A recent study by Lei \textit{et al.}\textsuperscript{272} linked XCL-1 chemokine produced solely by Aire-expressing mTECs to the correct homing of thymic DCs that drive nTreg development. Without Aire, the chemokine was not produced in thymic medulla, no DCs accumulated in the medulla, and a reduced number of nTregs was produced. They also found that lymphocytes adoptively transferred to lymphopenic recipients from mice deficient in XCL-1 developed autoimmune dacryoadenitis similarly to Aire-deficient mice\textsuperscript{272}.

### 3.3.3.4 The Functions of Aire in Dendritic Cells and on Other Components of the Innate Immune System

Aire is expressed also in DCs both in mice and humans, and many functional disturbances in the Aire-deficient DCs have been found. APECED patients have a higher proportion of circulating monocytes, i.e. the precursors of tissue DCs, than healthy individuals\textsuperscript{209}. The populations of monocytes and both CD11b\textsuperscript{+} and CD11c\textsuperscript{+} DCs are increased in Aire\textsuperscript{−/−} mice in the body cavity and in the circulation\textsuperscript{383}. DCs from Aire-deficient mice also express high levels of the vascular adhesion molecule VCAM-1 indicating a potential defect in DC homing\textsuperscript{383}. AIRE over-expression in a transfection study using a monocyte cell line up-regulated several molecules involved in cell adhesion, chemokine and cytokine signaling indicating that AIRE-expression could be required for DC trafficking\textsuperscript{421}. 
In Aire-deficient mice less monocyte precursors were found in the bone marrow than in wild-type mice\textsuperscript{195}. These few precursors proliferated at a high rate\textsuperscript{195}. Therefore there appears to be a maturation defect in Aire\textsuperscript{-/-} mice, and also a premature exit of Aire\textsuperscript{-/-} monocytes from the bone marrow, which could explain the increase in the monocyte counts in the circulation\textsuperscript{195}. This finding should be considered with caution, as it is not generally accepted that Aire would be expressed in the bone marrow or in monocytes. Halonen et al.\textsuperscript{184} found murine Aire expression in the bone marrow but this finding has not been repeated. Poliani\textsuperscript{372} reported that human bone marrow was negative for AIRE expression.

DCs from patients or Aire-deficient mice activate T cell hybridomas in vitro more than normal DCs\textsuperscript{383}. Consistent with this, a study by Ryan et al. showed that monocyte-derived DCs from APECED patients produced more tumor necrosis factor $\alpha$ and expressed more co-stimulatory factor CD86 without any stimulation in comparison with healthy controls\textsuperscript{396}. Another study, however, described that DCs from APECED patients produced less cytokines as a response to microbial stimulus\textsuperscript{373} indicating that DCs do not reach the fully mature state without AIRE\textsuperscript{421}.

The immunodeficiency in APECED towards Candida spp. has been hypothesised to result from defects in AIRE-dependent signaling pathways in DCs\textsuperscript{54}. For example, the initiation of a proper Th17 response towards Candida is initiated in DCs through activation of the innate receptor Dectin-1\textsuperscript{331}. It is not known whether AIRE is involved in this process even though the pattern recognition receptors, that are important in initiating the innate immunity response towards Candida, are unaffected in APECED patients\textsuperscript{209}. There is some experimental evidence supporting defective DC functions in response to Candida challenge in APECED patients\textsuperscript{54, 209}. Monocytes from APECED patients showed a delayed receptor-mediated endocytosis of Candida-antigens that resulted in decreased and altered intracellular signaling pathway activation in comparison with healthy controls\textsuperscript{54, 209}. Phagocyte function has been reported to be normal in APECED patients\textsuperscript{364}. Thus AIRE-related defects in innate immunity signaling appear to be limited to monocytes and more specifically to DCs which normally express AIRE\textsuperscript{372}.

3.3.3.5 Aire and B cells

Autoantibodies are an important part of the APECED syndrome even though their role in the pathogenesis is unclear. However, autoantibodies clearly indicate that also B cells are dysregulated when AIRE is absent. Interestingly there is one study that found AIRE expression in human B lymphocytes indicating a potentially direct role of AIRE in humoral immunity\textsuperscript{444}. Two different mouse studies have been published on the importance of B cells in Aire-deficient mice but their results are conflicting. In DeVoss model\textsuperscript{104} the T
cells were indispensable for the autoimmune syndrome in Aire-deficient mice, but B cells had a limited role in the disease progression. Gavanescu et al.\textsuperscript{145} challenged these results and claimed that without B cells the Aire-deficient mice remained healthy.

Hässler et al.\textsuperscript{195} reported a significant increase in the incidence of marginal zone B-cell lymphoma in aged Aire-deficient mice. The authors speculated that this could be a result of increased and prolonged B cell activation. They also found that the lymphocyte infiltrates in the liver of Aire-deficient mice were mostly composed of B cells\textsuperscript{195}. This was further supported by the finding of DeVoss et al. that the lacrimal gland lymphocyte infiltration in Aire-deficient mice contained a significant proportion of B cells\textsuperscript{103}. Increased B cell activation has been suggested to result from an increased concentration of B cell activating factor of the TNF family (BAFF) in both APECED patients and Aire-deficient mice\textsuperscript{277}. Increased B cell activation appears to be partly T cell independent, even though a T cell dependent mechanism has been reported\textsuperscript{59}.

The relative importance of B cell-mediated immune mechanisms in the pathogenesis of APECED has not been established. Considering the direct immuno-suppressive role of the Th17 cytokine-targeted autoantibodies, the B cell selective immunosuppressive mAb rituximab could be paradoxically used to treat chronic Candida infection in APECED patients. As rituximab has proven effective in the treatment of some autoimmune diseases, especially those with a relatively strong B cell-mediated pathology, it has been suggested as a therapeutical agent for APECED\textsuperscript{145} together with other immunosuppressive agents\textsuperscript{241}. However, without proper clinical trials, its effect is purely speculative.
Table 8: The many roles of Aire. This table summarizes yet published Aire-associated or Aire-controlled immunological phenomena.

<table>
<thead>
<tr>
<th>THE THYMUS</th>
<th>SECONDARY LYMPHOID ORGANS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>The thymus tissue organization\textsuperscript{113}</td>
<td>DC maturation\textsuperscript{373, 421}</td>
</tr>
<tr>
<td>mTEC development\textsuperscript{154, 297, 311, 336, 487}</td>
<td>DC - driven deletional tolerance\textsuperscript{143}</td>
</tr>
<tr>
<td>Ectopic TSA expression in mTECs\textsuperscript{20, 102, 103, 447}</td>
<td>Chemokine production\textsuperscript{383}</td>
</tr>
<tr>
<td>Deletion of autoreactive thymocytes\textsuperscript{280, 281}</td>
<td>Expression of homing receptors on APCs\textsuperscript{421}</td>
</tr>
<tr>
<td>MHC expression on mTECs\textsuperscript{20, 456}</td>
<td>Increased B cell activation\textsuperscript{59, 195}</td>
</tr>
<tr>
<td>Thymocyte migration\textsuperscript{262}</td>
<td></td>
</tr>
<tr>
<td>Increased apoptosis of mTECs\textsuperscript{124, 167}</td>
<td></td>
</tr>
<tr>
<td>Migration of DCs to the thymus medulla\textsuperscript{272}</td>
<td></td>
</tr>
<tr>
<td>Antigen transfer to thymic DCs\textsuperscript{219}</td>
<td></td>
</tr>
<tr>
<td>Proportion of single-positive thymocytes\textsuperscript{274}</td>
<td></td>
</tr>
<tr>
<td>Development of thymus originating nTregs\textsuperscript{22, 272}</td>
<td></td>
</tr>
<tr>
<td>Development of INKT-cells\textsuperscript{276, 369}</td>
<td></td>
</tr>
</tbody>
</table>
AIMS OF THE STUDY

AIRE is an important transcription factor controlling the negative selection of T cells. The aim of this study was to study the role of AIRE in the generation and maintenance of peripheral tolerance. More specifically the aims of the present study were:

I  To study the functions of regulatory T cells in APECED patients that lack AIRE expression.

II  To establish an experimental model where Aire expression in the thymus and peripheral immune system could be separated.

III To study the relative importance of defective central or peripheral Aire expression in an experimental system that is strongly biased towards autoreactivity and autoimmunity.
SUMMARY OF THE MATERIALS AND METHODS

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

1 Sample material

1.1 APECED Patients and Healthy Controls (I)

26 patients were analyzed (14 females). The mean age of patients was 39.8 years (range 26-60). 26 (15 females) healthy volunteers were used as controls and their mean age was 40.2 years (range 23-65). The disease components of the patients are summarized in Table 9. All patients had APECED diagnosis that was verified by sequencing the 14 exons and the exon-intron boundaries of the \textit{AIRE} gene. The majority (21 / 26) of patients were homozygous for the Finn major mutation R257X in \textit{AIRE}. The HLA typing of the patients has been carried out previously\textsuperscript{183}. The HLA-matched control subject used in the TCR repertoire analysis was identified among donors studied in 1996 for acute viral infection\textsuperscript{327}. The control subject was healthy at the time of the sampling for the current study.

Table 9: Summary of the most common disease components of the APECED patients studied.

<table>
<thead>
<tr>
<th>Disease component</th>
<th>No. of patients affected</th>
<th>% of patients affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucocutaneous candidiasias</td>
<td>26 of 26</td>
<td>100%</td>
</tr>
<tr>
<td>Addison's disease</td>
<td>22 of 26</td>
<td>85%</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>19 of 26</td>
<td>73%</td>
</tr>
<tr>
<td>Hypogogdanism</td>
<td>13 of 26</td>
<td>50%</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>9 of 26</td>
<td>35%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 of 26</td>
<td>23%</td>
</tr>
</tbody>
</table>

None of the patients or controls received systemic immunosuppressive treatment at the time of the sampling, were pregnant, suffered from infections, or had any vaccinations at the time of sampling. Patients received appropriate hormone replacement therapy for endocrine deficiencies at physiological doses, and dihydrotachysterol and calcium treatment for hypoparathyroidism. These treatments should not have any significant immunomodulatory effects.

1.2 Mice (II-IV)

NOD and NOD/Scid mice were maintained in the specific pathogen free environment in the animal facilities of the National Institute for Health and
Welfare (THL), Turku. Aire-KO, Rag1-KO, and AireRag1-KO breeding was done in barrier environment of the THL animal facility in Helsinki. Cell transfers were carried out in the specific-pathogen free animal facility of the same institute.

Aire<sup>−/−</sup>C57BL/6 mice were produced as described by Ramsey et al. and maintained by heterozygous sibling breeding with standard backcrossing into the C57BL/6 background. NOD, NOD/Scid and Rag1<sup>−/−</sup> C57BL/6 mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained by homozygous sibling breeding.

In order to generate lymphopenic Aire-deficient mice, Aire<sup>−/−</sup>C57BL/6 mice were crossed with lymphopenic Rag1<sup>−/−</sup> mice in the C57BL/6 background. The progeny was tested for the Aire mutation as described by Ramsey et al. and heterozygotes were selected for further breeding. The Rag1<sup>−/−</sup> phenotype was tested from F2 generation onwards by flow cytometry with staining for CD3<sup>+</sup> cells from PBMCs. After identifying Rag<sup>−/−</sup>Aire<sup>+/−</sup> animals the strain was maintained by heterozygous sibling breeding, together with genotypic and phenotypic analysis as described above.

In all experiments using Aire<sup>−/−</sup> mice the controls were parental, syngeneic wild-type mice. Littermates were used as often as possible.

1.3 Sample Collection and Cell Isolation (I-IV)

Animals were terminated by CO<sub>2</sub> suffocation and cervical dislocation at selected time points and ages. Selected organs were dissected and examined macroscopically. Organs were frozen fresh in liquid nitrogen for RNA extraction. For histological analysis the tissues were fixed in 10% formalin. Spleens and lymph nodes were collected in phosphate buffered saline and mechanically homogenized under sterile conditions. Released cells were collected and used for future analysis.

In live animal experiments, blood samples were collected from the tail vein using heparinized BD Mictotainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). At termination, blood was collected into heparinized syringes by heart puncture. Blood samples were centrifuged and plasma collected. Leukocytes were isolated by lysing erythrocytes with sterile water or by Ficoll-Paque gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden).

From human patients, blood samples were obtained with a vacuum venous sample collection system into heparinized tubes. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation and cryopreserved in 90% fetal calf serum and 10% dimethyl sulfoxide, if not used directly for experiments. For in vitro suppression assays cells were isolated from buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Service, Helsinki, Finland).
Thymic tissue was obtained from children undergoing cardiac surgery. The removed tissue was immediately frozen in liquid nitrogen or fixed in formalin.

If not analyzed immediately, the tissue and plasma samples were stored before analysis at -70°C. Frozen PBMC samples from APECED patients were stored before analysis in -154°C.

2 Magnetic Bead Cell Separation and In Vitro Suppression Assay (I)

Anti-CD25 mAb-coated Dynabeads (Dynal, Oslo, Norway) were used to isolate CD25^hi cells for in vitro suppression assays. The cells attached to the beads were detached by an overnight incubation at +37°C. The purity of the CD4^+CD25^hi cells was determined by flow cytometry and it typically ranged between 70 to 90% (I).

Human PBMC were cultured in duplicate in a 96-well plate for 5 days at 2 x 10^5 cells per 200 μl RPMI medium (Life Technologies, Paisley, United Kingdom), supplemented with 10% heat-inactivated human AB serum (Finnish Red Cross Blood Service), 20 mM HEPES, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin (supplements from Sigma Aldrich, St. Louis, MO, USA). The culture was pulsed for the last 6 hours with ³H-thymidine (1 μCi per well; GE Healthcare Bio-Sciences), harvested with a Skatron harvester (Newington, NH, USA), and analyzed with a liquid scintillation counter (Wallac, Turku, Finland). The background in non-stimulated wells was reduced from the results. Immobilized anti-CD3 mAb, heat-killed Candida albicans, phytohemagglutinin (Sigma Aldrich), and human insulin (Orion Pharma, Espoo, Finland) were used as mitogens in different concentrations.

3 Adoptive Cell Transfers (II-IV) and Colitis Clinical Score (III-IV)

Donor mice for adoptive cell transfer experiments were selected from the animal colonies. For each individual experiment KO and wild-type donor pairs were littermates. Cervical, para-aortic, and axillary lymph nodes were dissected aseptically and homogenized mechanically. Released cells were collected, pooled, washed, and counted. In NOD transfer experiments (I) the cells were stained with monoclonal antibodies and the transferred population was sorted using flow cytometry. In other transfer experiments (II-III), non-
selected bulk lymphocytes were transferred. A small representative fraction of the transfer was stained and analyzed by flow cytometry.

All recipients were lymphopenic female mice. The animals were sedated before intravenous injections with a fentanyl-fluanisone drug combination (Hypnorm, VetaPharma, Sherburn-in-Elmet, UK). Cell amounts from $10^4$ to $10^5$ were injected to tail vein in sterile phosphate buffered saline (PBS). All transfer experiments were performed independently for two times. All donors and recipients were housed in the same animal facility in the same rooms in order to maintain a comparable environment during the experiment.

After the cell transfer the mice were monitored daily and weighed weekly. For NOD mice, the blood glucose level was monitored weekly. For cell transfers in Aire-deficient animals (III-IV) clinical scores for monitoring the symptoms were adopted from Cooper et al. and Simpson et al. with some modifications. The following symptoms or signs at the time of termination were scored according to their severity: wasting (weight loss over 10%, score 0-1), hunching (score 0-1), thickening of the intestinal wall (score 0-1) and stool consistency (score 0-2, 2 = grossly bloody diarrhea).

4 Polymerase Chain Reaction (PCR) and Sequencing

4.1 RNA and DNA Isolation, cDNA Synthesis, and Real-time PCR (I-IV)

Frozen tissues were homogenized in Tripure reagent (Roche Applied Science, Indianapolis, IN, USA) using the Ultra-Turrax apparatus (Janke&Kunkel, Staufen, Germany) or VWR Pellet Mixer (VWR, West Chester, PA, USA). Total cellular RNA was extracted using chloroform phase separation and the RNeasy mini kit (Qiagen, Crawley, UK). If the downstream real-time PCR assay detected genomic DNA, it was removed with DNase I (Sigma-Aldrich) treatment if needed, and decontamination was verified using real-time PCR. First-strand cDNA was synthesized using AMV Reverse Transcriptase (Finnzymes, Helsinki, Finland) and an oligo-dT primer (Sigma-Aldrich). DNA was isolated using the QIAamp Blood kit (Qiagen) or DNA isolation was continued from the Tripure phenol phase as instructed by the manufacturer (Roche).

Real-time PCR was done using Taqman Universal Master Mix and Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA). The ABI 7900HT instrument (Applied Biosystems) and iCycler iQ instrument (Bio-Rad, Hercules, CA, USA) were used for the real-time reaction. Custom primer-probe sets for mouse TCR Cα and T cell receptor excision circles...
(TRECs) were synthesized by Applied Biosystems. For other genes, commercially available TaqMan assays were used (Applied Biosystems) and all assays were intron spanning. mRNA expression data were normalized against expression levels of TCR Cα or house-keeping genes Hypoxanthine phosphoribosyl-transferase (Hprt, mouse), Glyceraldehyde-3-phosphate dehydrogenase (mouse), and β-actin (human). All samples were run in duplicate. All real-time PCR analyses included a non-template control to exclude non-specific amplification due to contamination.

4.2 TCR Repertoire Analysis (I-II)

The TCR repertoire analysis was performed with a method that is called both immunoscope and spectratyping. Selected Vα- or Vβ-specific primers were used with a Cα or Cβ-specific primer to amplify the CDR3 region. The amplified V gene product was labelled in a run-off reaction with a fluorochrome FAM-labeled internal Cα or Cβ primer. Primer sequences used have been published by Pannetier et al. and Casanova et al. All primers were synthesized by Sigma-Aldrich and the AmpliTaq Gold enzyme was used for the PCR reactions (Applied Biosystems). The amplification products were separated and visualized on a 5% acrylamide gel by using an ABI377 sequencer or ABI3730 Automatic DNA Sequencer instrument (Applied Biosystems). Fluorescence data was analyzed with AbiPRISM or GeneMapper (Applied Biosystems) softwares respectively.

The quantitative analysis of changes in the TCR repertoire has been described previously. Areas of individual peaks in a CDR3 length profile were expressed as a percentage of the combined areas of all the peaks in the profile. Comparison of two profiles was done by extracting the relative peak areas of one profile from the other. The absolute values of differences were summed and, because the differences were counted as both increases at one place and decreases elsewhere, the sum was divided by two, putting the values within the range of 0 – 100 %. For human samples, cord blood (samples acquired from Finnish Red Cross Blood Service) TCR repertoire was used as a reference for naïve TCR repertoire. For establishment of mouse naïve repertoires, cDNA extracted from neonatal mice thymuses was used.

4.3 Sequencing (II)

Recipient mouse splenocyte cDNA was amplified with the selected Vα and Cα primers used for TCR repertoire analysis. If the original CDR3 region was not oligoclonal enough, the amplification products were further separated on a 9% acrylamide gel with electrophoresis. The gel was stained using the Silver Sequence DNA Sequencing system (Promega, Madison, WI, USA). The
chosen bands were excised and used as a template for further 40 cycles of PCR with the same primers as in the original amplification. The PCR products were cloned using TOPO TA Cloning Kit and One Shot® TOP10 chemically competent \textit{E. coli} cells (Invitrogen, Carlsbad, CA, USA). The inserts were amplified with an insert spanning plasmid specific primer-set. The PCR product was purified with exonuclease I and shrimp alkaline phosphatase (both from USB, Cleveland, OH, USA), and sequenced using the BigDye® Terminator v3.1 Kit and ABI3730 Automatic DNA Sequencer (Applied Biosystems).

5 Flow Cytometry and Cell Sorting with Flow Cytometry (I-IV)

Cells isolated from peripheral venous blood, spleen, lymph nodes, and mesenteric lymph nodes (MLNs) were stained with mAbs directly conjugated to a fluorochrome (see Table 10). mAbs were purchased from BD Biosciences, eBioscience (San Diego, CA, USA), Sigma-Aldrich, and Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAbs used in these studies are summarised in Table 10. Intracellular detection of FoxP3 and Ki67 required cell fixation and permeabilization that was done using the FoxP3 Fix&Perm kit (eBioscience), according to the manufacturer's instructions. Flow cytometry was performed using the FACScan and FACSaria instruments (BD Biosciences) and the data were analyzed using the CellQuest and Diva softwares (BD Biosciences).

NOD donor cells for adoptive cell transfer (II) were sorted after staining with mAbs against CD44, CD3, TCR \textit{V}D\textit{2} and CD34 (purchased from Becton Dickinson and Sigma Aldrich). Sorting of cells was done using a FACS Vantage instrument (Becton Dickinson). Human CD4^+CD25^{hi} Treg sorting for mRNA extraction (I) was done using a FACStar instrument (Becton Dickinson).

6 Histological Analysis (II-IV)

Tissue samples fixed in 10% formalin were embedded in paraffin, cut into 5 \textmu m sections, and placed on glass slides. The tissue sections were stained with the standard hematoxylin & eosin protocol. The stained slides were randomized and examined independently by two examiners in a blinded fashion. Inflammation of solid organs was evaluated on the basis of mononuclear cell infiltration and changes to the tissue morphology. Inflammation was scored according to the reported severity of inflammation. For example insulitis scoring was done as follows: intact: no mononuclear cell
infiltration; mild to moderate insulitis: infiltration in islet periphery (peri-insulitis) or intra-islet infiltration in less than half of the islet area; severe insulitis: infiltrating cells covering in more than half of the islet areas.

Table 10: Summary of the mAbs used in these studies.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Manufacturer</th>
<th>Original publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>human CD3</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human CD4</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human CD25</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human CD62L</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human CD45RO</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human CD45RA</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human HLA-DR</td>
<td>BD Biosciences</td>
<td>I</td>
</tr>
<tr>
<td>human FOXP3 (150D/E4)</td>
<td>a gift from A. Banham, Oxford, UK</td>
<td>I</td>
</tr>
<tr>
<td>human AIRE</td>
<td>a gift from P. Petterson, Tartu, Estonia</td>
<td>I</td>
</tr>
<tr>
<td>human AIRE</td>
<td>Santa Cruz Biotechnology</td>
<td>I</td>
</tr>
<tr>
<td>human FOXP3 (236A/E7)</td>
<td>eBioscience</td>
<td>I</td>
</tr>
<tr>
<td>mouse CD3</td>
<td>BD Biosciences</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>mouse CD44</td>
<td>BD Biosciences</td>
<td>II, III</td>
</tr>
<tr>
<td>mouse CD34</td>
<td>BD Biosciences</td>
<td>II</td>
</tr>
<tr>
<td>mouse TCR Va2</td>
<td>Sigma-Aldrich</td>
<td>II</td>
</tr>
<tr>
<td>mouse CD4</td>
<td>BD Biosciences and eBioscience</td>
<td>III, IV</td>
</tr>
<tr>
<td>mouse CD8</td>
<td>BD Biosciences and eBioscience</td>
<td>III, IV</td>
</tr>
<tr>
<td>mouse CD19</td>
<td>eBioscience</td>
<td>III</td>
</tr>
<tr>
<td>mouse FoxP3</td>
<td>eBioscience</td>
<td>III, IV</td>
</tr>
<tr>
<td>mouse Ki67</td>
<td>BD Biosciences</td>
<td>III, IV</td>
</tr>
</tbody>
</table>

7 Immunohistochemistry Staining (I, III)

For immunohistochemistry of the thymus tissue, 5 μm sections of the paraffin embedded formalin-fixed tissues were used. The slides were dewaxed and heated in a microwave oven for 10 minutes in a buffer containing 1 mM EDTA in 10 mM Tris-HCl, pH 9.0 for antigen retrieval. Then slides were stained with the monoclonal anti-AIRE antibodies and a secondary horseradish peroxidase conjugated antibody. The reaction was developed with the EnVision+ protocol (DAKO, Glostrup, Denmark).

Frozen sections (5 μm) of organs dissected from Rag1$^{-/-}$ mice or human thymus tissue were used for immunofluorescence (IF) stainings. The tissues used were freshly frozen in liquid nitrogen before cutting into sections. Thymic sections were stained with an anti-AIRE mAb and an appropriate fluorochrome-conjugated secondary antibody. In order to screen mouse recipients of the adoptive cells transfers for autoantibodies, plasma samples were diluted 1:5 and incubated on the frozen sections from selected organs. Autoantibodies bound to the sections were detected using fluorescein
isothiocyanate-conjugated polyclonal rabbit anti-mouse IgG+IgM antibody (DAKO) diluted in 1:50.

The sections were analyzed independently by two examiners using the fluorescence microscope Olympus BX51 (Olympus, Hamburg, Germany) equipped with an Orca IIIm CCD-camera (Hamamatsu Photonics, Hamamatsu, Japan). For positive samples, the quantification of the autoantibody amount was done by analyzing serial plasma dilutions.

8 Enzyme-linked Immunosorbent Assay (ELISA) (I, III-IV)

The acute phase protein serum amyloid P component (SAP) and BAFF concentrations were measured using commercial ELISA kits (SAP: Immunology Consultants Laboratory Inc., Newberg, OR, USA; BAFF: Axxora, San Diego, CA, USA), according to the manufacturer's instructions. Plasma samples were diluted 1:2000 (SAP) or 1:5 (BAFF), analyzed in duplicates, and absolute concentrations were calculated from a control dilution curve with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Absorbances were measured with the Labsystems iEMS Reader MF instrument (Thermo Fisher Scientific Inc., Loughborough, UK).

For total immunoglobulin G measurement, a commercial ELISA kit was used (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer's instructions, with the following dilutions: coating antibody 1:100, samples 1:2000 (run in duplicates) and conjugated secondary antibody 1:70 000.

For bacterial antibody measurement an in-house ELISA protocol was used. Bacterial strains of *E. coli* and *E. faecalis* were isolated from AireRAG mouse feces and grown in LB broth on a shaker at +37°C until mid-log phase. Bacteria were then washed twice with PBS and the concentration was adjusted to optical density of 0.6 at 600 nm in PBS. This suspension was used 60 μl/well for coating of a MaxiSorp microtitre plate (Nunc Thermo Fisher Scientific, Roskilde, Denmark), which was let to dry over night at +37°C before use. Heat-inactivated serum samples were diluted 1:30. Mouse serum IgG and IgA specific for *E. coli* and *E. faecalis* were detected by subsequent incubation with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch laboratories, West Grove, PA, USA) or biotinylated rabbit anti-mouse IgA (Zymed, San Francisco, CA, USA) followed by HRP-conjugated streptavidin (Jackson ImmunoResearch laboratories) (all diluted 1:10 000). OPD substrate was purchased from Dako. The amount of bound antibody was measured as optical density at 492 nm.

82
Human peripheral blood TREC concentration was estimated by a quantitative PCR-ELISA assay, as described by Al-Harthi et al.\textsuperscript{11}.

9 Statistical Analysis (I-IV)

Statistical significances of the differences or for the change were calculated with two-tailed Student’s t-test with $P < 0.05$ as the limit for statistical significance. Correlations were calculated with Pearson’s correlation coefficient, with a limit of $P < 0.05$ for significance. For $P$ values of AIRE$^+$ cell densities in the different areas of the thymus Mann-Whitney U test was used.

10 Ethical Considerations (I-IV)

The animal work was approved by the Animal Care Committee of the University of Turku (II), Animal Care Committee of the University of Helsinki, and State Provincial Offices of Southern Finland (III-IV). All work with animals was executed following guidelines provided in the EU directive 86/609/EEC. All human patients and controls gave written informed consent before sampling. The human studies were reviewed and approved by the ethical committee of the Helsinki University Central Hospital.
RESULTS AND DISCUSSION

1 Defective Regulatory T Cells in APECED Patients (I)

1.1 AIRE Expression in the Human Thymus Is Concentrated around Hassall’s Corpuscles

The involvement of the thymus in the generation of T cell central tolerance is indisputable, and the role of Aire role in negative selection has been established in mouse models\textsuperscript{20, 280, 281}. Human AIRE is assumed to function similarly as its murine counterpart, even though formal experimental evidence is mostly missing\textsuperscript{295}. APECED patients are rare and samples from their thymus are practically inaccessible. By analyzing the expression of AIRE in healthy individuals and human cell lines it is possible to study AIRE’s function in the human thymus and immune system\textsuperscript{258, 444}. In mice, different thymic cell populations have been shown to have distinctive roles in the generation of tolerance, especially when it comes to nTreg differentiation\textsuperscript{187, 189, 482}. Therefore, we wanted to study the spatial distribution of AIRE expression in the normal human thymus in detail.

The thymus tissue was obtained from immunologically healthy children undergoing cardiac corrective surgery. In immunohistochemical staining with an AIRE specific mAb I could show that AIRE-expressing cells are concentrated around Hassall's corpuscles. The median concentration of AIRE$^+$ cells was 68.4 cells/mm\textsuperscript{2} (range 19.5–146.5 cells/mm\textsuperscript{2}) around Hassall’s corpuscles. In comparison, the median concentration of AIRE$^+$ cells in the medulla areas devoid of Hassall’s corpuscles was 26.1 cells/mm\textsuperscript{2} (range, 13.0–78.1 cells/mm\textsuperscript{2}). This difference was statistically significant (P=0.003). The corpuscles themselves were mainly negative for AIRE staining (Original publication I, figure 4).

Hassall's corpuscles were originally considered to be less prominent in the mouse thymus\textsuperscript{122}. However, a recent study confirmed the existence of Hassall’s corpuscles as keratinized swirled structures in the mouse thymus by using a specific marker for human Hassall's corpuscle\textsuperscript{181} that also stained similar structures in the mouse thymus\textsuperscript{486}. The authors of this study also confirmed our findings and showed preferential localization of AIRE$^+$ epithelial cells near Hassall’s corpuscles in human paediatric thymuses\textsuperscript{486}. Three other groups have reported similar findings from adult samples\textsuperscript{342, 407, 438}. The preferential localization of Aire$^+$ mTECs around the Hassall's corpuscle-like structures has also been found in the mouse thymus\textsuperscript{336}. 

84
Yano et al.\textsuperscript{500} used transgenic green fluorescent protein as a marker linked to the Aire loci expression. Using this construct, they were able to show that Hassall’s corpuscle-like structures were almost completely absent in the Aire-deficient murine thymus, together with various other morphological changes. Thymus morphology of APECED patients has not been studied, so we do not know what kind of changes primary AIRE deficiency causes in the human thymus. From the mouse data it is, however, possible to speculate that primary AIRE deficiency distorts the morphology of the human thymus as well, possibly resulting in a loss of Hassall’s corpuscles.

There are some clinical conditions where thymic AIRE expression is lost secondary to some other processes. Thymus morphology can be studied in detail in these diseases. For example a secondary loss of AIRE is reported in Omenn’s syndrome\textsuperscript{63}. In this syndrome the thymic histology is largely distorted most likely due to a mutation in the RAG gene that is responsible for the syndrome\textsuperscript{63, 430}. Also the majority of human thymomas fail to express AIRE as discussed previously\textsuperscript{407, 445}. However, results obtained from thymomas must be analyzed with caution since the malignant process itself is capable of changing the morphology of the thymus. Interestingly, Hassall’s corpuscles are generally rare in thymomas, but they are more often seen in the AIRE-expressing tumors\textsuperscript{407}. This can be interpreted so that AIRE expression and Hassall’s corpuscles in thymomas are associated with more differentiated tumors, and only tumors of undifferentiated origin fail to express AIRE and lack Hassall’s corpuscles. It should be noted that the loss of AIRE expression in thymoma can result in an APECED-like syndrome even though this is extremely rare\textsuperscript{73, 438}. Therefore, it can be concluded that a secondary loss of AIRE expression is associated with changes in the thymic morphology. At present there is not enough data from human patients to draw any conclusions of the possible morphological changes in thymuses of APECED patients.

In summary, AIRE expressing cells are spatially linked to Hassall’s corpuscles both in mice and humans. The preferential localization of AIRE\textsuperscript{+} cells around these structures is likely not a coincidence, but the reason for this connection is not clear. AIRE\textsuperscript{+} mTECs are considered to be terminally differentiated, and thus their co-localization with Hassall’s corpuscles could indicate that these keratinized structures somehow drive the maturation of mTECs. This would mean that the closer the mTEC is to the corpuscle, the more mature it is. This is supported by the hypothesis that Hassall’s corpuscles could also function as a “graveyard” for apoptotic mTECs\textsuperscript{114}. When the dying mTECs ends up in Hassall’s corpuscles, the corpuscles can acquire TSAs and be involved in the negative selection. Therefore, the loss of AIRE\textsuperscript{+} mTECs from the thymus can also regulate the negative selection additionally through Hassall’s corpuscles.
1.2 Phenotype and TCR Repertoire of Tregs in APECED Patients

The co-localization of AIRE+ mTECs and Hassall’s corpuscles could also indicate a link in nTreg development and AIRE expression. Thymic stromal lymphopoietin produced by Hassall’s corpuscles has been shown to drive nTreg development\(^{187, 285, 482}\). Developing nTregs have also been found to localize around Hassall’s corpuscles\(^{407, 482}\). A pathogenetic role for defective Hassall’s corpuscles through for example defective Treg development has been proposed in rheumatoid arthritis\(^{36}\). It thus appears that Hassall’s corpuscles potentially create a “tolerizing” environment around them by producing specific cytokines. This effect is not necessarily limited only to Treg development since also thymic B cells have been found to preferentially accumulate around Hassall’s corpuscles\(^{432}\).

Hassall’s corpuscles are thus clearly involved in the development of Tregs originating from the thymus. Loss of AIRE expression could, therefore, also affect Treg development. In human thymomas, the loss of AIRE expression was associated with a reduced number of Tregs and a decreased expression of FOXP3 in the tumor tissue\(^{342, 407}\). Murine Aire has also been linked to thymic nTreg development through Aire-dependent antigen expression in mTECs\(^{25}\) and chemokine production that drives the accumulation of medullary DCs\(^{272}\). However, these studies did not examine the role of AIRE in the development of human nTregs. This motivated us to study Tregs in APECED patients.

1.2.1 Reduced Expression of FOXP3 in Tregs from APECED Patients

We analyzed the phenotype of Tregs in APECED patients and compared that with healthy controls. As measured by flow cytometry, there was no difference in the expression of T cell activation and memory markers CD25, HLA-DR, CD62L, CD45RA and CD45RO between patients and controls (Original publication I, table I). When the FOXP3+ population was analyzed, there was a marked decrease in the frequency of FOXP3+ cells in APECED patients (Original publication I, figure 2C). In a more detailed analysis, the numbers of CD4+CD25\(^{\text{high}}\)FOXP3+ cells were comparable between the patients and controls, but the mean fluorescence intensity of FOXP3-positive cells was significantly lower in the patients’ CD4+CD25\(^{\text{high}}\) cells (Original publication I, figure 2D-F). The lower protein expression level of FOXP3 in APECED patients’ Tregs was confirmed at a mRNA level also by real-time PCR. This was done by analyzing the expression of FOXP3 mRNA in sorted CD4+CD25\(^{\text{high}}\) cells in both patients and controls, and the patients had a
significantly decreased expression level of FOXP3 mRNA (Original publication I, figure 2A-B).

1.2.2 Tregs of APECED Patients Have a Naïve-like TCR Repertoire

The TCR repertoire reflects changes on a clonal level in peripheral T cells. Next we analyzed the TCR Vβ repertoire in 50 000 sorted CD25<sup>high</sup> cells from an APECED patient and a HLA class II- and sex- matched healthy control. The TCR repertoire analysis was carried out with the spectratyping method, where the lengths of CDR3 regions of a given TCR V-C gene combination are visualized on a polyacrylamide gel<sup>89</sup>. The fluorescence intensity of the bands in the gel is measured, and the analysis gives the CDR3 length profile of the gene studied. It has been shown that the relative areas of two peaks in a CDR3 length distribution profile correlate with the difference in sequence diversity within them<sup>23</sup>. This method gives a more accurate estimate of the clonality spectrum of a given T cell population than a mere analysis of the TCR gene expression. Clonal expansion that can be identified by a biased TCR gene usage profile in peripheral blood must be very large, and such expansions are rarely seen except in acute infections or hematological malignancies<sup>166</sup>. By spectratyping it is possible to identify expanded T cell clones within the same TCR V gene. It is also possible to estimate the poly- or oligoclonality of the total TCR repertoire<sup>370</sup>. Thus spectratyping was well-suited for the detailed analysis of Tregs in APECED.

The CD25<sup>high</sup> cells from a healthy control expressed a biased but polyclonal TCR repertoire; a finding that is well in line with published results of Treg TCR repertoires<sup>354</sup>. The patient TCR repertoire was, however, markedly different. Clonal expansions of single CDR3 lengths were fewer in the patient repertoire (Original publication I, figure 3A). Also the overall number of CDR3 length differences was higher indicating that no significant loss of individual TCR clones had taken place in peripheral competition between lymphocyte clones (Original publication I, figure 3B). Generally, the Treg TCR repertoire of the patient resembled more a naïve TCR repertoire than the TCR repertoire of the control subject. The observation was further verified by comparing the TCR repertoires of the control and the patient to a mean TCR repertoire acquired from cord blood samples (Original publication I, figure 3C). Cord blood T cells posses the most naïve TCR repertoire that can be obtained from humans as the T lymphocytes isolated from the cord blood have encountered only antigens that are present <i>in utero</i>. The TCR repertoire in CD25<sup>high</sup> cells from APECED patients was more similar to the mean naïve TCR Vβ repertoire of 11 cord blood samples (Original publication I, figure 3D). In summary, the TCR repertoire of the APECED patient’s Tregs was naïve-like while the repertoire of Tregs from a healthy individual had signs of clonal expansions
indicating past activation and proliferation of certain clones. Similar results were obtained from four non-HLA matched patient-control pairs.

The data presented in publication I indicate several abnormalities in the Treg population of APECED patients. The most prominent defect is the lower expression level of FOXP3. Constitutively high expression of FOXP3 is indispensable for the stable Treg phenotype and suppressive function in humans\textsuperscript{399}. Our data suggest that Tregs are dysfunctional in APECED because they fail to express high levels of FOXP3. The naïve-like TCR repertoire of Tregs in APECED patients also supports this conclusion. In APECED patients Tregs have not undergone as many clonal expansions as healthy controls do. A clonal expansion in the TCR repertoire requires that the particular clone has proliferated considerably. It is thus possible that APECED patient Tregs fail to proliferate as efficiently as they should in the peripheral immune system. Even though Tregs were originally described to be anergic \textit{in vitro}, the current view is that TCR-mediated Treg activation results in a rapid proliferation and effector maturation \textit{in vivo}\textsuperscript{316}. Treg activation, which precedes the proliferation, is required for suppressive functions of Tregs. Our results show that the prominent clonal expansions indicating strong activation and proliferation are missing from the Treg repertoire that has developed in the absence of AIRE.

However, our results do not show at which level this AIRE-dependent defect in Treg activation takes place. AIRE is expressed also in secondary lymphoid organs, so it is possible that extra-thymic AIRE-expressing cells are involved in peripheral Treg activation. Our group has studied the phenotypic defects in APECED patients' Tregs further. Laakso \textit{et al.}\textsuperscript{261} were able to show that the decreased FOXP3 expression was less pronounced in Tregs with the recent thymic emigrant phenotype. On the contrary, activated Tregs in APECED patients showed a significantly lower expression level of FOXP3 when compared to healthy controls. This indicates a failure in FOXP3 expression upon peripheral activation in APECED, which is essential for proper Treg functions\textsuperscript{316}. Thus, taken together, our data and the reported dysfunction of the activated Tregs in APECED patients\textsuperscript{261} suggest that extra-thymic AIRE expression could be involved in the peripheral Treg activation and clonal expansion.

\textbf{1.3 Defective Suppressive Functions of APECED Patient Tregs}

To ascertain a functional defect in APECED patient Tregs implied by the findings of altered phenotype and TCR repertoire, CD4\textsuperscript{+}CD25\textsuperscript{high} cells were isolated from APECED patients and an \textit{in vitro} suppression assay was carried out. Tregs isolated from patients used in a 1:3 ratio to CD25\textsuperscript{−} cells failed to suppress T cell proliferation in response to a polyclonal stimulus (Original
Immobilized mAbs against human CD3 and phytohaemagglutinin were used as polyclonal mitogens. A failure to suppress proliferation was evident in the patient Tregs even using a 1:1 CD4⁺CD25<sup>high</sup> to CD25<sup>-</sup> cell ratio.

Effector cell resistance to Treg-mediated suppression has been proposed to be an important mechanism in some human autoimmune diseases<sup>55</sup>. Thus, we wanted to test whether the effector cell resistance in APECED patients would explain the failed Treg suppression in the <em>in vitro</em> suppression assay. CD4⁺CD25<sup>high</sup> cells isolated from a healthy blood donor were able to suppress anti-CD3 mAb-driven proliferation equally well in patients and controls. Therefore, the effector T cells isolated from APECED patients are not resistant to normally functional Treg-mediated suppression.

When heat-inactivated <i>Candida albicans</i> was used as a mitogen in the <em>in vitro</em> suppression assay, the patient-derived Tregs suppressed the proliferation as well as the controls did (Original publication I, figure 1). This could have resulted from the increased amount of Candida-specific activated effector Tregs in the CD25<sup>high</sup> population, since the patients have chronic Candida infections. Thus the pre-existing numerous Candida-specific Tregs in APECED patients could compensate for the otherwise defective <em>in vitro</em> Treg activation and suppression, and no overt difference with healthy controls could be seen. Tregs isolated from the patients suppressed Candida-specific immune responses <em>in vitro</em> as efficiently as control subjects Tregs even though they failed to suppress the proliferation induced by strong polyclonal mitogens. This indicates that there is a difference between polyclonal and antigen-specific Treg-mediated suppression, and these mechanisms are affected by AIRE differently. Another possible explanation for this difference is that Tregs express also TLRs and can be activated through them<sup>442</sup>. In our experimental conditions we did not differentiate between TLR- or TCR-mediated activation. It is thus possible that the heat-inactivated Candida used could have bypassed the TCR and activated Tregs indirectly through TLRs. Murine Aire has been associated with TLR expression in a monocyte cell-line <em>in vitro</em><sup>514</sup>, but in human APECED patients the TLR expression <em>ex vivo</em> has been reported to be normal<sup>209</sup>.

Our data confirm that Tregs in APECED patients fail to suppress polyclonal lymphocyte proliferation <em>in vitro</em>. Similar Treg defects have been reported for other human autoimmune diseases as well<sup>55</sup>. Treg defects in human autoimmune diseases can be divided into three main categories: inadequate number of Treg cells, defect in Treg-mediated suppression, and effector cell resistance to Treg-mediated suppression<sup>55</sup>. APECED patients have normal numbers of Tregs cells and their effector T cells are normally suppressed by Tregs. Our data show that the Tregs that have developed without AIRE are functionally defective.
Considering the role of AIRE in the thymus driving ectopic TSA expression, it is possible that the nTreg repertoire, which developed without AIRE, has specific inadequacies for certain self-antigens that are expressed in the thymus in an AIRE-dependent manner. This theory could explain the relatively limited number of tissues affected in APECED. The exact mechanism of how nTreg generation is affected without AIRE could also be explained by defective development or function of Hassall’s corpuscles. The results obtained using Aire-deficient mice support this view, but this mechanism remains to be validated using thymic samples acquired from APECED patients.

Even though the suppression assay can be criticized, it is currently the method of choice for testing human Treg functional activity. However, it remains unclear how well the *in vitro* data reflect the suppressive activity *in vivo*. In mice studies where Aire-deficient animals were crossed with FoxP3-deficient Scurfy mice, the offspring developed a more severe disease that either one of the parental strains alone. This finding indicates that the normal function of Tregs is important in maintaining at least some level of self-tolerance in the murine autoimmune syndrome evoked by Aire deficiency. Thus, it is possible that the dysfunctionality in Tregs caused by the loss of AIRE has a role in the pathophysiology of human APECED.

### 1.4 FoxP3 Expression in Aire<sup>-/-</sup> Mice

APECED patients consistently express lower levels of FOXP3 than healthy controls. We wanted to analyze whether the same finding would also apply to Aire-deficient mice as well. mRNA expression levels of FoxP3 in the spleen and thymus lysates of six Aire<sup>-/-</sup> mice and 6 wild-type mice in the C576/BL background were quantitated by real-time PCR. There was no significant difference between Aire-deficient and control mice. When the salivary glands of the same mice were analyzed, the expression level of the TCR gene C<sub>\alpha</sub> was used to normalize the FoxP3 expression level to the total level of T cell infiltration. Since each mature αβ T cell also expresses this gene segment, it is a reliable way to normalize the specific gene expression to the total amount of T cells in the sample. The salivary gland is a common target of lymphocyte infiltration in the different Aire-deficient mouse strains (Table 4). We were able to detect clearly more TCR C<sub>\alpha</sub> mRNA expression in the knockout animals as an indication of an increased amount of T cells infiltrating the tissue compared with the wildtype controls. Also FoxP3 expression was increased in the salivary glands and it correlated significantly (P=0.0005) with the TCR C<sub>\alpha</sub> expression (Original publication I, figure 5). This finding clearly shows that in Aire<sup>-/-</sup> mice the lymphocyte infiltration to a target organ is accompanied with increased Treg accumulation as well.
These data indicate an active ongoing local suppression of inflammation in the Aire-deficient mice. However, the analysis was done using relatively young animals, which could affect the interpretation. Indeed, Hässler et al.\textsuperscript{195} found that when Aire-deficient mice were aged from 15 to 24 months, the autoimmune manifestations were more pronounced. Aricha et al.\textsuperscript{22} reported that the number of peripheral FoxP3\textsuperscript{+} Tregs was normal in animals aged 2 months but it was reduced in 6-month-olds (when analysed by flowcytometry). It is, therefore, possible that also in Aire\textsuperscript{−/-} mice the Treg defect is not in the amount of Tregs, but instead it is a question of a functional impairment that becomes pronounced only when the animals get older, or when their immune system is put under extensive stress. Aire\textsuperscript{−/-} mice used in this experiment were kept under barrier circumstances in a specific pathogen-free – facility so the external antigen load in these animals was minimal.

2 Lymphopenia-induced Proliferation as a Model for Studying Polyclonal T Cell Responses in Autoreactivity-prone Experimental Conditions (II)

Our initial work with the APECED patients and Aire-deficient mice showed conflicting results regarding the Tregs function and FoxP3 expression. The Peltonen’s Aire\textsuperscript{−/-}C57BL/6 mice used in our experiments were actually overtly healthy, even though autoantibodies and lymphocyte infiltration in solid organs found in the mice can be considered a sign of autoimmunity\textsuperscript{236}. In addition to genetic differences between humans and mice, this difference in phenotypes could result from different environmental factors. The only study so far focused on the general environmental factors affecting the Aire-deficient mice phenotype has been conducted by Gray et al.\textsuperscript{168}. They reported that germ-free Aire-deficient mice, i.e. mice that have lived in strictly sterile environment, had similar disease progression as their specific pathogen-free kept counterparts. They also tested whether TLR-stimulation would affect the disease progression, however, no difference was found\textsuperscript{168}. The reliability of this study can be criticised, since the study groups were very small; in some treatment groups only 2 or 3 mice were used.

Treatment or cells and animals with TLR-ligands can mimic certain aspects of microbial infection but it will not fully reproduce e.g. real viral infection. One hallmark of some viral infections is transient lymphopenia\textsuperscript{27} that has been associated with the onset of autoimmunity in humans\textsuperscript{94}. We wanted to study the phenomenon of LIP in more detail since it has been proposed to increase the risk of autoimmunity in both mouse models and in clinical cases\textsuperscript{254}. Also Tregs have been reported to have a major role in controlling
the lymphopenia-initiated proliferation. Therefore LIP appeared to be a good model for studying T cell autoreactivity in a polyclonal, non-transgenic experimental system.

2.1 Lymphopenia-induced Proliferation as a Model to Enrich Self- or Normal Flora- Reactive Clones from Polyclonal Population

T cells that express two different TCRs with separate antigen specificities are consistently found in peripheral blood both in mice and humans\textsuperscript{199, 357}. Their true significance in the function of the adaptive immune system is still unknown. The dual antigen specificity of T cells has been suggested to increase the risk for autoimmunity through faulty negative selection\textsuperscript{504}, but dual-specific TCRs are also preferentially expressed among human Tregs\textsuperscript{462}. Dual antigen specificity has also been proposed to be beneficial by expanding the foreign antigen recognition capacity of a single cell\textsuperscript{198}. We wanted to elucidate these conflicting findings of dual-specific TCRs by using LIP as a model to enrich autoreactive TCR clones from a polyclonal repertoire. We selected the NOD mouse strain for this experiment since it is a well-established mouse model for autoimmunity\textsuperscript{18}.

NOD mice were generated in 1974 from inbreeding cataract-model mice that developed autoimmune diabetes\textsuperscript{286}. NOD mice, especially female mice, spontaneously develop autoimmune diabetes with a high frequency. Over 20 gene loci have been associated with predisposition or protection of diabetes in NOD animals\textsuperscript{18}. When NOD mice are crossed to lymphopenic backgrounds such as nude, i.e. athymic or Scid mice, they are saved from insulitis and diabetes. This strongly suggests that diabetes in NOD mice is caused by the adaptive immune system\textsuperscript{418}. The diabetes in NOD animals is a multifactorial disease similarly to autoimmune diabetes in humans\textsuperscript{153}. In addition to diabetes, the mice often acquire also other autoimmune manifestations, which indicate a severe multilayered immune dysregulation\textsuperscript{56, 151, 431, 465}. NOD is widely used as a model for human tissue-specific autoimmune diseases\textsuperscript{115}.

In order to study how the T cell clones that express two different TCR V\textalpha chains behave in LIP, we sorted 50 000 lymphocytes isolated from the lymph nodes of a NOD donor according to the surface expression of TCR V\textalpha2 gene, and transferred them to lymphopenic NOD/Scid recipients using an i.v. injection. As it has been estimated that 15% of peripheral T cells express two V\textalpha chains\textsuperscript{199}, approximately 7 500 of the transferred cells also expressed another V\textalpha chain. In this experimental system we were able to follow the changes in both single- and dual-specific TCR repertoires during LIP in exactly the same circumstances.
2.2 Dual-specific TCR Repertoire Grows more Oligoclonal in Lymphopenia-induced Proliferation than Single-specific TCR Repertoire

TRECs are loops of un-translated genomic DNAs that are spliced out during the TCR recombination process in the thymus\textsuperscript{197}. After the TCR recombination during each cell division, the TREC concentration is halved because extra-chromosomal DNA is not replicated\textsuperscript{387}. Thus the TREC concentration can be used to estimate the overall proliferation history in a given T cell population. We confirmed the vigorous T cell proliferation in the recipients that received the sorted T cell transfer by measuring the TREC concentration from recipients spleen samples after their termination. The all NOD/Scid recipients were terminated 2 months after the sorted T cell transfer. During the transfer experiment no clinical symptoms of autoimmunity were noticed and at the time of termination the proliferation had most likely reached a plateau phase since all tested recipient cells were positive for memory surface markers as analyzed by flowcytometry (data not shown).

All NOD/Scid recipients that received the sorted T cell transfer were negative for TRECs at the time of termination. In order to estimate how many times the transferred cells had divided, the TREC level was measured in a donor sample that represented the TREC level of the transferred cells pre-proliferation. Serial dilutions from a thymic DNA sample was done in order to estimate how many cell divisions are required to reach a negative TREC level in our assay. Then we compared the TREC level in the pre-transfer donor sample with the dilution curve (Figure 6). This comparison put the number of cell divisions in the transferred cells to a minimum of eight divisions. Most proliferating cells probably divided more than eight times.
Figure 6: A schematic representation of the TREC based estimation of cell divisions in the transferred cells.

In order to study the changes in the dual-specific TRC repertoire during lymphopenia-induced proliferation, we decided to analyze the Vα1 and Vα17 genes to represent dual-specific repertoires in transferred cells. As roughly estimated from the number of the different Vα genes in a normal repertoire, approximately 4% of dual-specific TCRs utilised either one of the selected two Vα genes. The expression level of these two genes were measured in recipient samples by real-time PCR, and there was no difference in the overall expression levels of these dual-specific TCRs after T cell proliferation (Original publication II, figure 1).

For a more detailed analysis, we carried out a TCR repertoire analysis from the recipients’ splenocytes on single-specific TCRs, represented by Vα2, and dual-specific TCRs, represented by Vα1 and Vα17, before and after the proliferation. By using the same model for estimating changes in the TCR repertoire as in APECED patients (see pages 87-88), the single- and dual-specific repertoires were compared with both pre-transfer repertoires and naïve polyclonal TCR repertoires that were acquired from 8 neonatal mouse thymuses. The dual-specific repertoire changed significantly more during the proliferation than the single-specific repertoire: the average change compared with the pre-transfer repertoire was 17% in single-specific Vα2 repertoire, while in the dual-specific Vα1 it was 58% and in Vα17 75%. The difference in the repertoire change remained significant when the repertoires were
compared with the naïve polyclonal repertoire (Original publication II, figure 3).

This comparison, however, did not reveal us how the repertoire changed. Therefore, we compared the total number of different CDR3 lengths in the single- and dual-specific repertoire. The dual-specific TCR repertoires became more oligoclonal during LIP (Original publication II, figure 2). The duration of proliferation had no effect on the change in the repertoire in similar experiments with different durations of the proliferation phase, up to 12 months.

Our data here show that the transferred lymphocytes proliferate in LIP, but all clones do not proliferate equally well. The repertoire of the non-sorted TCRs changed significantly more often during the proliferation, and the resulting repertoire was markedly more skewed than the sorted, and thus mostly single-specific TCR repertoire. It can be argued that the small number of transferred non-sorted TCRs would impact the skewing of the repertoire. To exclude this possibility, another series of transfer experiments were conducted. We transferred a small number of non-selected T lymphocytes to RAG1-knockout recipients and compared the pre- and post-transfer repertoires of selected V\(\alpha\) genes. In this setting the repertoire per V\(\alpha\) gene changed. The change, however, was not so pronounced as it was in the non-V\(\alpha\)2 TCRs in the original transfer, even though the proportions of the V genes in the non-selected transfer were a minority of the total repertoire. Also, in the original sorted transfer, the non-V\(\alpha\)2 TCRs were a minority but their repertoire became significantly more skewed during proliferation than in this second non-selected transfer. Therefore, we can conclude that the narrowing of the dual-specific TCR repertoire did not result from the original small number of transferred TCRs, but it is a phenomenon associated with the special characteristics of dual-specific TCRs.

### 2.3 Lymphopenia-induced Proliferation Can Be Used to Enrich Autoreactive Clones from a Polyclonal Repertoire

Next we characterized the effects of LIP on dual-specific V\(\alpha\)2 and V\(\alpha\)1 expressing cells. First we estimated how many non-V\(\alpha\)2 TCRs were transferred to the recipients. This was done by choosing a single spectratyping band representing a single CDR3 length of the V\(\alpha\)1 gene from a pre-transfer donor sample. It has previously been shown that the relative area of a peak in the spectratyping profile correlates with the sequency diversity within it\(^{23}\). When we sequenced the different CDR3 sequences in the chosen band, 21 different CDR3 sequences were found. Based on the estimated frequency of V\(\alpha\)1 genes in the total TCR repertoire the 21 different CDR3 sequences found represented 12.8% of the total transferred V\(\alpha\) repertoire.
(see figure 7 for illustration how the estimation was done). This confirmed that the original literature-based estimation of 15% dual-specific TCRs in the transfer was quite accurate.

Figure 7: An illustration of how the frequency of non-Vα2 TCRs, i.e. dual-specific TCRs, in the transferred cells was calculated.

The Vα1 CDR3 bands, that were of the same length as in the donor sample, were also analyzed from the post-transfer samples of three recipients. The recipient samples each had one or two different CDR3 sequences, indicating that the dual-specific Vα1 repertoire had contracted to 5%, on average, of the original (Original publication II, figure 4).

One CDR3 sequence was identical both in nucleotide and amino acid sequence in two out of the three recipient mice analyzed. This finding indicates that this particular sequence is targeted to some common self-, or commensal antigen driving LIP, and this antigen and the TCR clone specific to it were shared with different recipients. It highlights how effective the LIP is in enriching autoreactive clones from a polyclonal repertoire. Quiel et al. used TG-TCR T cells diluted with non-specific T cell clones in order to study how the frequency of an antigen-specific TCR affects the proliferative response of T cells. They found that after immunization with the model antigen, the more infrequent the antigen specific clones were in the original T cell repertoire, the more they proliferated. Considering our data with the dual-specific TCRs, it appears that the dual-specific repertoire contains rare autoreactive or commensal flora reactive clones that efficiently proliferate in response to self-antigen-derived activation signals and finally dominate the dual-specific TCR repertoire.

We did not notice any clinical signs of autoimmunity, for example hyperglycemia, in our recipient mice. In histological analysis we found that all
recipients had a mild insulitis at the time of termination (Original publication II, figure 5A). TCR repertoire analysis from pancreas draining lymph nodes showed a similar contraction of the dual-specific Vα1 repertoire as the one we had seen in the splenocytes. The small number of the transferred cells may explain why insulitis did not progress to diabetes in the recipients. It was also possible that the transfer period was too short for the development of diabetes\textsuperscript{269}, or that the sorting affected the CD4 / CD8 ratio of the transfer, as both of the main T cell populations are needed for the development of diabetes in NOD mice\textsuperscript{18}.

One further intriguing possibility is that the sorted transfer would contain a population of Tregs that could have suppressed the disease progression. Indeed, it has been reported that an unusually high percentage of human Tregs have two functional Vα-chains\textsuperscript{462}. It has to be pointed out that our experimental system was not originally designed to study Tregs as such, and thus we could only measure the expression level of FoxP3 in pre- and post-transfer splenocytes. The expression level increased significantly, indicating that Tregs in our transfer expanded during LIP (Original publication II, figure 5B).

These data obtained from the NOD model clearly indicate that dual-specific TCRs behave differently in LIP than the single-specific TCRs. This indicates that dual-specific TCRs contain autoreactive clones that dominate the repertoire after proliferation. The autoreactivity of the dual-specific repertoire could be a result of faulty negative selection, or an increased proportion of Tregs within the dual-specific population. The Treg population has been suggested to contain highly autoreactive clones more often than the normal effector cells even though the Treg repertoire contains also TCRs that are specific for foreign antigens\textsuperscript{354}. If Tregs would have a dual-specific TCR, it could explain some discrepancies seen in the Treg TCR repertoires in different studies\textsuperscript{354}. Tregs proliferate effectively during LIP and are important in controlling the autoreactivity as discussed previously\textsuperscript{49, 269, 492}, so in our experimental LIP system Treg proliferation may be favored.

Even though our results are not complete to answer the question of whether or not dual-specific TCRs are harmful or beneficial to the immune system, this study confirms that dual-specific TCRs contain functional clones. If all dual-specific TCRs would be dysfunctional, there would not have been any detectable non-Vα2 TCRs in the recipients after the proliferation. The different outcome of LIP for single- and dual-specific TCRs indicates that dual-specific TCRs form a separate biological niche in the total TCR repertoire.
APECED patients show a clear functional defect in Tregs but the analysis of the mechanistic of this defect was not possible to be carried out in human patients. Emerging evidence supports a role for AIRE also outside the thymus\textsuperscript{119, 308}. Our data indicated that the Treg defect in APECED could be an extra-thymic AIRE-dependent phenomenon. Next we wanted to study the impact of AIRE on Tregs separately in the thymus and in secondary lymphoid organs. The overall functional defect seen in in the Tregs isolated from APECED patients could result from defects at various stages of the Treg lifecycle: 1) qualitatively defective nTreg development in the thymus, 2) errors in Treg homing to the target tissues followed by inadequate activation signals, 3) failure in the induction of the Treg phenotype in naïve cells (iTregs), 4) defects in Treg activation, proliferation, and maintenance of the suppressive phenotype, and 5) defective cell-to-cell suppression, for example by inefficient production of suppressive cytokines. We reasoned that AIRE expression in the thymus has an impact on stage 1, but peripherally expressed AIRE could be important in the remaining stages. In order to pinpoint the level in which the loss of AIRE causes Treg defects, it was necessary to separate AIRE’s expression in the thymus and in the peripheral immune system.

Our data in the NOD model showed that LIP enriches rare autoreactive T cell clones so that they can eventually dominate the repertoire. We further analyzed whether Aire expression in the thymus during T cell development and in the peripheral immune system have different effects on lymphocytes undergoing LIP. Our findings in the NOD transfer model confirmed that LIP is a good model for studying general autoreactivity of the transferred population and Tregs controlling the proliferation\textsuperscript{269}.

We chose to use Aire-deficient mouse model in the C57BL/6 background that is relatively resistant for the development of autoimmunity. The autoimmune syndrome in this background is spontaneously mild, so it would be relatively easy to see the impact that LIP has on Aire-dependent autoimmune manifestations. The C57BL/6 background also provided a good control group since no autoimmunity has been reported in adoptive cell transfers using un-selected cells from C57BL/6 mice\textsuperscript{269, 491}.

### 3.1 Lymphopenia and Aire – Possible Synergistic Factors in the Breakdown of Tolerance?

We considered that lymphopenia could also be important in the context of Aire as some autoimmune components reported in Aire-deficient mice are shared
with other lymphopenia-associated autoimmune mouse models. A good example of this is gastritis that is a common autoimmune manifestation in Aire-deficient mice in the BALB/c background. BALB/c animals also develop autoimmune gastritis with high prevalence after thymectomy that is carried out on the third post-natal day. The post-thymectomy gastritis in BALB/c mice is strongly associated with the expression level of gastric autoantigens in the thymus but overt autoimmunity also requires LIP. It should be noted that the recognized gastric autoantigens in post-thymectomy and in Aire deficiency associated autoimmune gastritis are different; gastric H/K ATPase and Mucin 6 respectively. The clinical pictures are otherwise similar: lymphocyte infiltration to the gastric mucosa and production of autoantibodies.

In mice, neonatal thymectomy results in a severe T cell lymphopenia and a high rate of LIP, which can cause autoimmunity in selected genetic backgrounds. Results in the conditional Aire-expressing mouse model indicate that Aire expression in the perinatal period is more important in the maintenance of tolerance than later in life. It appears that Aire-dependent autoimmunity shares a strong resemblance with the third post-natal day thymectomy model. In the conditional Aire expression model, autoimmunity following the perinatal loss of Aire expression can be prevented by blocking LIP indicating that the proliferation has an impact on the Aire-related autoimmunity.

Aire deficiency results in a severe autoimmunity in Scurfy and NOD backgrounds. NOD mice have been reported to have defective central tolerance and thus the synergistic effects on the central tolerance following the loss of Aire are believed to explain the severe phenotype of Aire−/−NOD mice. Accelerated autoimmunity due to the loss of Aire in the Scurfy background has been suggested to reflect the importance of fully functional peripheral tolerance in other Aire-deficient mice models. Interestingly both NOD and Scurfy mice have also been suggested to be partially lymphopenic. In both strains, dysregulated LIP is believed to be a co-factor in the generation of autoimmunity.

Scurfy mice spontaneously develop an autoimmune syndrome that is relatively limited in terms of affected organs. This is somewhat strange because the FoxP3 mutation prevents virtually all Tregs’ functions. However, when cells isolated from Scurfy mice were adoptively transferred to RAG-KO mice, the recipient mice developed a strikingly similar autoimmune syndrome to that is seen in the Aire−/−FoxP3sf mice. The autoimmune syndrome of Aire−/−FoxP3sf mice and the disease that developed after adoptive transfer of Scurfy cells to lymphopenic recipients, is different from both original strains with single defects, namely Aire−/− and Scurfy. The syndrome following adoptive transfer of Scurfy cells to lymphopenic recipients has peculiar
components such as inflammation of the skeletal muscles\(^{71,414}\). Therefore it is possible that the severe autoimmunity in Aire\(^{-}\)FoxP3\(^{sf}\) mice results from combined effects of the loss of Aire and FoxP3 expression together with LIP.

In many models of LIP-associated autoimmunity, the disease is believed to result from a reduced Treg/Teffector ratio\(^{138,269,318}\). It appears that in selected genetic backgrounds Tregs fail to act or proliferate as effectively as autoreactive effector T cells do during LIP, and autoimmunity develops. Therefore LIP is a good model for studying Treg function in the context of Aire. Motivated by the Treg findings in APECED patients, we wanted to test whether LIP would be sufficient to break the tolerance in Aire-deficient mice, with special focus on Treg functions during the proliferation. Lymphopenic mice also enabled dissecting the peripheral Aire expression from the Aire expression in the thymus as described in detail below.

### 3.2 Lymphocytes Adoptively Transferred from Aire\(^{-}\) Donors to Normal Lymphopenic Hosts Show Signs of Immune Dysregulation

We took advantage of LIP in order to analyze whether the proliferation would enrich autoreactive clones from the T cell repertoire that had developed without the presence of Aire. A similar approach has been used for example in NOD mice\(^{269}\). We reasoned that this process could worsen the relatively mild spontaneous autoimmunity in Aire-deficient mice without any other genetic defects. One million non-selected peripheral mature lymphocytes from an Aire-deficient donor were transferred to lymphopenic recipients in the same background strain. In order to avoid the leakiness of some lymphopenic strains\(^{1,264}\) RAG1 KO mice were chosen as the recipients. In the control group we transferred the same amount of unselected lymphocytes from a wildtype littermate to lymphopenic recipients (Figure 8).

The donor mice were born and maintained in a barrier animal facility before the cell transfer in order to minimize the external antigen load on the animals. The transferred lymphocytes were isolated and pooled from cervical, para-aortic, and axillary lymph nodes aseptically and injected into the tail-veins of the recipient animals. The transfer experiment was done twice independently so that the total number of donor animals was 4 (Aire-deficient donor n=2, wildtype donor n=2). We analyzed the transferred cells pre-transfer by flow cytometry and saw no significant difference between Aire-deficient and wildtype donors in the major T cell populations. Also the amount of B cells, the proportion of cells in the cell cycle (analyzed by expression of the cell cycle marker Ki67), and the proportion of FoxP3\(^{+}\) Tregs were equal in the donors. All donors and recipients were females.

The cells transferred from Aire-deficient donors to RAG1-KO recipients underwent LIP in an Aire-sufficient environment (hereafter named the Aire-
However, the cells had developed without Aire. Considering the role of Aire in the negative selection of developing T cells, the transferred T cells should have contained more autoreactive clones than the cells transferred to the control group.

![Diagram showing lymphocyte maturation and proliferation in Aire-sufficient and Aire-deficient environments.]

**Figure 8:** The transfer rationale. By transferring mature lymphocytes from an Aire-deficient donor to lymphopenic recipients that have normal expression of Aire, I could isolate the impact of Aire in the thymus during T cell development from Aire's peripheral expression. The control group was otherwise similar, but the transferred T cells had developed in a normal Aire-expressing thymus.

The recipients were terminated two months after the transfer, and peripheral blood lymphocytes and various tissues were collected for analysis. As proof of the immune dysregulation in the Aire-group, we observed a significant hyperproliferation of CD3⁺ T cells in the Aire-group at the time of termination (Table 10). Hyperproliferation was evident both in circulating blood cells and in the spleen samples measured by the increased expression of the cell-cycle marker Ki67. The frequency of CD3⁺ cells in the spleen was higher in the Aire-group as compared with the control group. When we compared Ki67 expression between the major T cell populations, the CD8⁺ cytotoxic T cells originating in the Aire-deficient mouse were significantly more often in the active cell cycle and thus mostly accounting for the general T cell hyperproliferation (Original publication III, figure 1).

In the LIP of T cells, the naïve CD8⁺ have been reported to proliferate more actively than naïve CD4⁺ cells⁴⁴¹. As the proliferation is driven by self-antigen-MHC complexes or antigens derived from commensal flora⁴¹⁵ we concluded that this hyperproliferation of CD8⁺ T cells in the Aire-group reflects an increased autoreactivity of the naïve T cell repertoire in Aire-deficient mice. Ramsey et al. also found T cell hyperproliferation in Aire-deficient mice after
immunization. Antigen-specific T cell proliferation has been reported to be significantly higher in those antigen specific clones that were infrequent in the original T cell repertoire. Therefore the global hyperproliferation of CD8+ cells could actually be a result of hyperproliferation of several originally rare autoreactive clones in the original T cell repertoire. Aire regulates the expression of a set of TSAs in the thymus, so it is indeed feasible that the T cell repertoire from an Aire-deficient host contains many different autoreactive TCRs. LIP liberates these clones from homeostatic control mechanisms and they proliferate en masse. The loss of thymic Aire is, therefore, sufficient to cause a significant dysregulation in the T cell proliferative responses.

Table 11: T cell hyperproliferation in the Aire-group

<table>
<thead>
<tr>
<th></th>
<th>Aire-group % (± SD)</th>
<th>Control group % (± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ of PBMC (1 mo after the cell transfer)</td>
<td>27.9 (± 10.8)</td>
<td>23.0 (± 6.4)</td>
<td>0.235</td>
</tr>
<tr>
<td>CD3+ of PBMC</td>
<td>25.6 (± 12.0)</td>
<td>21.9 (± 21.5)</td>
<td>0.652</td>
</tr>
<tr>
<td>CD3+ of splenocytes</td>
<td>43.6 (± 7.0)</td>
<td>31.8 (± 9.6)</td>
<td>0.0068</td>
</tr>
<tr>
<td>Ki67+ of CD3+ splenocytes</td>
<td>17.6 (± 3.1)</td>
<td>12.1 (± 2.9)</td>
<td>0.033</td>
</tr>
<tr>
<td>CD8+ of CD3+ splenocytes</td>
<td>38.5 (± 10.8)</td>
<td>29.0 (± 6.1)</td>
<td>0.026</td>
</tr>
<tr>
<td>Ki67+ of CD3+CD8+ splenocytes</td>
<td>5.5 (± 0.9)</td>
<td>3.0 (± 0.6)</td>
<td>0.0020</td>
</tr>
</tbody>
</table>

Next, we analyzed the existence of autoantibodies in the recipients, another reported common feature of the Aire-deficient mice models. Both study groups had low levels of circulating CD19+ cells, consistent with the reported lower LIP of B cells. Total plasma IgG and BAFF levels were comparable between the groups indicating no significant B cell dysregulation in the Aire-group on a systemic level (Original publication III, figure 3A).

One donor plasma sample stained positive for smooth muscle and retinal antibodies, and this autoantibody production was transferred to all recipients receiving cells from this donor. De novo antibody production targeted against smooth muscle was found also in the recipients that received cells from the donors that stained negative for smooth muscle autoantibodies (Figure 9). No other types of autoantibodies were seen even though we used a wide array of tissues (kidney, ovary, liver, salivary gland, stomach, intestine, pancreas, adrenal gland) to screen autoantibody production in our recipients. In total, all recipients in the Aire-group had either retinal or smooth muscle autoantibodies. 36% of the control group recipients stained positive for...
smooth muscle antibodies even though all control donors were negative for all autoantibodies tested. This could indicate that smooth muscle autoantibodies are a result of the general proliferation process in LIP. The Aire-group had autoantibodies significantly more often (Original publication III, figure 3B).

Smooth muscle autoantibodies are common in autoimmune hepatitis that is not associated with APECED. Smooth muscle autoantibodies have not previously been found in Aire-deficient mice. In the Finnish APECED patient cohort 7.1% of patients devoid of hepatitis and 0% of patients with autoimmune hepatitis were positive for smooth muscle autoantibodies. Our recipient mice did not show signs of increased hepatitis, so our results are in line with the ones obtained from the APECED patients. Retinal autoantibodies, however, are common in Aire-deficient animals (Table 4). We were able to adoptively transfer the autoreactivity against the eye but not the other components of autoimmunity seen in Aire-deficient mice.

Smooth muscle autoantibodies were generated in both study groups indicating that this kind of autoreactivity is common in general in LIP. However, the Aire-group had these autoantibodies more often than the control group, suggesting increased B cell-mediated autoreactivity. The IF assay used did not differentiate between T cell-dependent and -independent antibody production. Aire-deficient mice have higher levels of BAFF indicating T cell-independent B-cell dysregulation. The study groups did not have significant differences in the plasma concentration of BAFF, so it is safe to assume that the autoantibody production in this transfer setting followed the dysregulated T cell immune response in LIP. During LIP T cells home to the T cell areas in secondary lymphoid organs and proliferate there irrespective of the presence of B cells. Thus the importance of B cells in LIP-associated autoimmunity is insignificant. The significance of B cells in the generation of autoimmunity in Aire-deficient mice has been suggested to be limited, and the Th1 type T cell response has been concluded to be the major effector population driving the autoimmunity.

We conclude that the autoantibodies found in the Aire-group reflect the increased T cell autoreactivity, and do not cause autoimmunity per se since all animals stayed clinically healthy during the follow-up period. However, it was evident that the transfer contained B cells with the capacity to produce autoantibodies and these cells remained functional in lymphopenic recipients. LIP did not amplify humoral autoreactivity as no organ-specific de novo autoantibody production was found.
Figure 9: Immunofluorescence staining of a section of mouse small intestine. A plasma sample from an Aire-group recipient was used for staining. The outer muscular layer of the intestine, that is composed of smooth muscle, is stained positive, but the intestinal epithelium is negative.

3.3 Aire-group Show a Th1-biased T Cell Response

A significant proportion of antigens driving the fast lymphocyte proliferation after adoptive cell transfer to RAG1-KO recipients originates from the gut commensal flora. The rapid burst of proliferation is reduced in animals maintained in a germ-free environment. Adoptive cell transfer of naïve T cells to a lymphopenic recipient often results in colitis. Considering this background, we decided to carry out a detailed analysis of T cells in the gut and gut draining lymphoid tissue of the recipients.

T cell hyperproliferation, which was apparent also systemically, was more pronounced in the MLNs in the Aire-groups (original publication III, figure 4A), but no clinical colitis was evident in the histological sections from colon. Real-time PCR from colon homogenates showed no significant difference in the TCR Cα mRNA expression level in the Aire-group compared with the control group. Thus there was no difference between the study groups in T cell infiltration into the colon tissue. When we measured the expression of different Th subtype-associated master regulator genes normalized to TCR Cα expression level, we noted a significant increase in the expression of Tbet in the colons isolated from the Aire-group (Original publication III, figure 4B). Tbet is an important regulatory gene of the Th1 type cells that are important in cell-mediated immune responses. In the Aire-group the colonic T cell infiltrates were dominated by the Th1 type cells, which was not the case in the control group. DeVoss et al. found a similar Th1-dominated lymphocyte infiltration to solid target organs in lymphoreplete Aire-deficient mice.

It has been shown that CD8+ cell do not proliferate in LIP efficiently without the help from CD4+ T cells, nor are they alone sufficient to cause
In autoimmune pathology\textsuperscript{186, 270}. In order to cause tissue destruction, the CD8\(^+\) cells need additional help from Th1 type CD4\(^+\) cells. Also in Aire-deficient mice, CD8\(^+\) cells failed to cause autoimmunity without CD4\(^+\) cells\textsuperscript{104}. In our model, the CD8\(^+\) T cells hyperproliferated in the peripheral blood of the Aire-group. It is possible that the Th1 dominance in the colon reflects some kind of Aire-dependent dysregulation in the initiation of Th immune responses. This could cause bias in the downstream immune responses. For example, the DCs from APECED patients have been shown to produce more Th1 type cytokines in response to endotoxin stimulation \textit{in vitro}\textsuperscript{396}. This dysregulation was, however, not sufficient to cause autoimmunity in our lymphocyte transfer model.

In APECED patients, neutralizing autoantibodies against Th17 effector cytokines are common\textsuperscript{241}. It has been speculated that these autoantibodies could be a secondary event caused by a chronic \textit{Candida} infection resulting in an increased production of these cytokines followed by autoantibody formation against them. Supporting this view an enhanced Th17 type response towards \textit{Candida} has been found in APECED patients\textsuperscript{4}. In mice, no cytokine autoantibodies have been published so far, but it is possible that the aseptic environment of mice facilities and the absence of chronic candidiasis explains this difference. Only one group has been able to show that when Aire\(^{−/−}\) mice were exposed to \textit{Candida}, the mice showed increased susceptibility to the pathogen\textsuperscript{4}. The effector T cells isolated from the gut draining lymph nodes of the intragastrically infected Aire\(^{−/−}\) mice showed a more pronounced Th1 and Th17 type cytokine response to \textit{Candida} antigens \textit{in vitro} when compared with infected Aire\(^{+/+}\) controls\textsuperscript{4}. This finding could indicate that the biased Th response in the absence of Aire could have a pathogenetic role in the failure to clear \textit{Candida} infection. Our results also suggest that Aire deficiency causes a bias in Th response. This Th response dysregulation is also evident in responses to both self and commensal antigens.

3.4 Hyperproliferation of Aire\(^{−/−}\) Donor Originating Tregs in Gut-draining Lymphoid Tissue

The recipients in the Aire-groups stayed clinically healthy even though signs of immune dysregulation and hyperproliferation of autoreactive T cells were evident. We wanted to study whether the suppressive functions of peripheral tolerance would prevent the hyperproliferating autoreactive T cell clones from causing tissue damage. During proliferation Tregs are important in controlling the fast LIP\textsuperscript{492}, but under a steady-state environment they appear to function and develop relatively independently of the presence of gut microbiota\textsuperscript{312}. iTregs, however, are generated especially in the intestine, and gut microbial antigens are important in the process\textsuperscript{165}. The importance of Tregs in
controlling LIP is highlighted by T cell transfer colitis, which can be prevented by the co-transfer of Tregs\(^227\).

Initially one month after the transfer, the proportion of FoxP3\(^+\) Tregs in the circulation was comparable between the study groups. At the time of termination, CD4\(^+\) cells in the Aire-group did not hyperproliferate systemically, but when we analyzed the subpopulation of FoxP3\(^+\) expressing Tregs, a significant difference between the groups was found. The proportion of Tregs in the cell cycle was higher in the Aire-group both in the circulation and in the gut draining lymphoid tissue than in the Control group (Original publication III, figure 5B). In MLNs, the frequency of Tregs was also significantly elevated, indicating an accumulation of Tregs to the site of active proliferation (Original publication III, figure 5C-D). Also in the colon tissue the amount of FoxP3 mRNA normalized to the expression level of TCR C\(\alpha\) was significantly higher in the Aire-group (Original publication III, figure 5E).

An increased proportion of FoxP3\(^+\) cells does not fully reflect the suppressive function of the Tregs. For a more detailed analysis of the increased Tregs in the Aire group, we measured the relative expression of a suppressive cytokine TGF-\(\beta\) in MLNs. TGF-\(\beta\) mRNA expression was significantly higher in the Aire-group than in the control group (Figure 10). The increase was evident when the TGF-\(\beta\) mRNA level was normalized to C\(\alpha\) mRNA expression. Thus, there were significantly more TGF-\(\beta\) producing T cells in the MLNs of the Aire-group. This increase in TGF-\(\beta\) production is most likely due to the increased amount of Tregs in the MLNs. In contrast to humans, in mice the transient non-suppressive FoxP3 up-regulation in activated T effector cells has not been reported\(^517\). Thus it is unlikely that the increased proportion of FoxP3\(^+\) cells would be non-suppressive cells. The increase in the production of the suppressive cytokine TGF-\(\beta\) also indicates that FoxP3\(^+\) Tregs, which accumulated in MLNs, were indeed true suppressive regulatory cells.

![Figure 10: Relative TGF-\(\beta\) mRNA expression normalized to C\(\alpha\) expression in total lymphocytes isolated from mesenterial lymph nodes.](image-url)
Taken together, we can conclude that the dysregulated lymphocyte proliferation in the Aire-group did not result in autoimmunity because of increased Treg-mediated suppression at the sites of active T cell proliferation. Correct homing and accumulation of Tregs to the site of inflammation has been shown to be indispensable for their suppressive activity. Our findings indicate that the loss of Aire during T cell development does not affect Treg functions during LIP, or the accumulation of Tregs to the site of inflammation.

One interesting explanation for the increase in Treg frequency could be that the high affinity autoreactive clones that had escaped negative selection in the Aire-deficient thymus, would have acquired a Treg phenotype in the periphery during LIP in an Aire-sufficient environment. Peripheral iTreg conversion has been reported to take place especially in the gut draining lymphoid tissue and during LIP. TCR signaling and TGF-β are needed for the conversion. Originally it was believed that low-affinity TCR engagement up-regulates FoxP3 in the periphery, but recent evidence shows that high-affinity peptide-TCR interactions are needed for a stable iTreg phenotype. iTreg conversion appears to be antigen-specific in the generation of oral tolerance and under lymphopenic conditions. The iTreg and nTreg repertoires have been reported to be overlapping and, therefore, it is hypothesized that the peripheral conversion to the Treg phenotype could give certain TCR specificities “a second chance” to become Tregs after thymic selections.

Our model could not distinguish between iTreg conversion and the expansion of nTregs from the original transfer. The elevated TGF-β expression level could result from an increased amount of active Tregs in the mesentery. However, these two explanations need not be exclusionary. Haribhai et al. reported that the collaboration between iTregs and nTregs is needed for effective protection from T cell transfer colitis. nTregs can enhance iTreg conversion via TGF-β production. Thus, the increased proportion of Tregs in the Aire-group MLNs could result from both increased homing and proliferation of nTregs, and increased iTreg conversion in a TGF-β-dependent fashion. The loss of thymic Aire did not affect this process.

4 Loss of Aire Expression in the Peripheral Immune System Causes Symptomatic Autoimmunity (IV)

4.1 Generation of Lymphopenic Aire-deficient Mice (AireRAG1)

In order to study whether Aire expression outside the thymus has an impact on peripheral tolerance, we crossed Aire−/− mice with RAG1-KO mice in the C57BL/6 background. The resulting double-KO mice were completely devoid
of autoimmune manifestations and also produced litters in a comparable amount compared to wildtype mice. This finding proves that the subfertility often seen in Aire-deficient males\textsuperscript{220} is of autoimmune origin and mediated by cells of adaptive immune system. The RAG1-KO genetic construct prevents all B or T cell receptor recombinations, and accordingly no mature B or T cells were found in the periphery of AireRAG1 double-KO mice.

4.2 Adoptive Transfer of Unselected Lymphocytes from a Normal Donor Generates Colitis in AireRAG1 Mice

A similar transfer as in the Aire-group experiment was carried out using the AireRAG1 double-KO mice as recipients. Donors were wildtype mice from the Aire breeding colony. This study group is hereafter called the AireRAG1-group. In the control group recipients were Aire-sufficient RAG1-KO mice (Figure 11). In this setting we were able to follow how the loss of Aire from the peripheral immune system affected the outcome of LIP. Donor cells were chosen to be isolated from wildtype donors, since in this experiment we wanted to exclude the role of Aire in the thymus and in negative selection.

![Figure 11: The transfer rationale. By transferring mature lymphocytes from Aire-sufficient donors to Aire-deficient lymphopenic recipients, that express no Aire in the secondary lymphoid organs, we could follow the role of peripherally expressed Aire during LIP.](image)

We followed the wellbeing of the AireRAG1 and control recipients weekly. The majority of the recipients (11 out of 12) in the AireRAG1-group developed diarrhoea from the second week after transfer onwards (Original publication IV, figure 1A). No recipients in the control group were
symptomatic. Three out of 12 AireRAG1 recipients lost weight or developed general signs of wasting disease (bad fur, hunching) together with the diarrhoea. The disease was so severe in one mouse to necessitate termination before the end of the experiment. The mouse was terminated on the fourth week after the transfer since it had lost over 20% of its body weight. The rest of the recipients were terminated 2 months after the transfer.

At the time of termination a macroscopical analysis of the AireRAG1 recipients showed that the animals were suffering from colitis that was characterized by a grossly enlarged colon (7/11), fibrotic mesentery, mesenterial lymphadenopathy (6/11), and splenomegaly (6/11). These signs of intestinal inflammation were also found in the only mouse that did not develop clinical diarrhoea. In the control group two mice out of 11 lost over 10% of their body weight during the two-month transfer experiment and one had mesenterial lymphadenopathy. A clinical score was used to compare the disease in the AireRAG1- and control groups (modified from Simpson et al.423). In the clinical score animals are given points according to the following symptoms found at autopsy: wasting (weight loss in excess of 10%), hunching, and colon enlargement each gave a maximum of one point. Stool consistency was evaluated on a scale from 0 to 2, watery stools giving the maximum of 2 points. The clinical scores were thus evaluated on a scale of 0-5. The mean clinical score in the AireRAG1-group was 1.8 (SD ± 0.9) while in the control group it was 0.2 (SD ± 0.4). The difference was statistically significant (P < 0.0001).

Colitis was confirmed by a histological analysis of colon sections. The colon wall was thickened and mononuclear basal layer lymphocyte infiltration was evident (Original publication IV, figure 1B). However, epithelial ulcerations were not seen, so the colitis was graded histologically as mild. A more thorough histological analysis was done in one of the two independent transfer experiments. Histological staining revealed gastritis in 4/6 and exocrine pancreatitis in 2/6 of the AireRAG1 mice tested. In other reported target organs in Aire-deficient mice, histological analysis did not reveal any significant difference between the groups.

We used the systemic inflammation marker SAP to measure the overall inflammation in the recipients. One month after the transfer, both recipient groups had elevated serum SAP levels: AireRAG1-group, mean 173 mg/l (SD ± 90), and control group, mean 133 mg/ml (SD ± 69, P=0.27). In the four weeks previous to the termination, the SAP concentration increased in the AireRAG1-group significantly more than in the control group. The mean concentration at the time of termination was 389 mg/l (SD ± 306) in the AireRAG1-group and 224 mg/l (SD ± 188) in the control group (significance for change; AireRAG1 P=0.020, control P=0.14) (Original publication IV, figure 1C). It should be noted that both groups showed high variation in the SAP
concentrations and that explains why, even though the average concentrations were clearly different, the differences did not reach statistical significance.

The loss of Aire could affect the barrier function of the gut epithelium, as its expression has been associated with tight junction molecules and Aire has been suggested to control the expression of many intestine-associated genes. Increased epithelial leakage due to the loss of Aire could explain the colitis in the AireRAG1-group irrespective of LIP, even though this should then be evident in immunocompetent Aire-deficient mice as well. Increased epithelial leakage should manifest as a systemic immune response towards gut flora antigens. Therefore, we analyzed recipients for the production of antibodies targeted against the commensal flora bacteria *E. coli* or *Enterococcus spp.* but found no difference between the groups. In conclusion, the colitis in the AireRAG1 recipients did not result from a significantly increased systemic leakage of intestinal flora antigens.

Next we tested the recipients for autoantibodies targeted to intestinal tissue, since we reasoned that these autoantibodies could reflect an autoimmune inflammation targeted to the gut tissue itself. The loss of Aire could, in theory, for example reveal new antigenic structures in the gut. If this kind of scenario would be true, the colitis seen in our AireRAG1 recipients would actually be the result of the reactivity of a normal T cell repertoire to new antigenic structures. Naturally, this would mean that Aire-deficient animals would spontaneously develop autoimmunity towards intestinal tissue, which is not the case. In IF staining using frozen gut sections, 40% of the control and 50% of AireRAG1-group recipients stained positive for smooth muscle antibodies similarly to the first Aire transfer experiment (III). This time the difference between the groups was not statistically significant, and the staining intensity was generally weak in both groups. No autoantibodies targeted to the intestinal epithelium were found.

In summary, in the AireRAG1-group the intestinal barrier function was overtly normal. A comparable proportion of the recipient mice in both groups had low levels of autoantibodies targeted to the smooth muscle layer of the intestinal wall. Most likely, the smooth muscle autoantibodies reflect common immune processes during LIP and were not associated with the loss of peripherally expressed Aire, since they were as common in the control group. The inflammation marker SAP increased more in the AireRAG1-group indicating that the systemic inflammation was present in both groups, but it was more pronounced when peripheral Aire was missing. Considering the evidence that LIP is strongly dependent on the gut commensal flora antigens, we suggest that the loss of Aire expression from the secondary lymphoid organs caused a local dysregulated inflammatory response to gut microbial antigens. This dysregulation resulted in a mild
colitis. No systemic antibodies targeted to the gut microbiota were found, so the dysregulation appeared to be weak and anatomically restrict to the intestine or, limited only to cellular immune responses.

4.3 Adoptively Transferred Normal Tregs Proliferate Slower in Aire-deficient Lymphopenic Mice

The AireRAG1-group did not show significant systemic hyperproliferation of T cells one month after the transfer, or at the time of termination. The CD3+ frequencies and CD4 / CD8 proportions were comparable between the study groups both in the circulation and the spleen (Figure 12). Therefore, the colitis in the AireRAG1-group was not caused by systemic uncontrolled lymphoproliferation like it is in for example the IL-2 KO mice. Ramsey et al. found that Aire-/- DCs drive T cell hyperproliferation in vitro. Our results do not support this view, at least not systemically. On the contrary, the AireRAG1-group had lower numbers of circulating T cells compared to the control group, even though the difference did not reach statistical significance.

Figure 12: Analysis of T cell populations in the AireRAG1-group. No significant hyperproliferation at the time of termination of A) T cells in general or B) in the CD4+ T cell subset in the AireRAG1 group. The cells analyzed were isolated from the spleen. The differences are not statistically significant.

Consistent with the inflammation in the colon tissue, we noted an increase in the proportion of CD3+ cells in MLNs in the AireRAG1-group. In the AireRAG1-group on average 63% (SD ±17) of lymphocytes isolated from the MLNs were CD3 positive as compared with the control group where the proportion of T cells was 28% (SD ±18, P=0.015). The proportion of CD3+ cells in active cell cycle was comparable in both groups. This could indicate that the accumulation or proliferation of T cells in the AireRAG1-group mice mesenterium had already taken place before termination. The CD4 / CD8 ratio was comparable in both groups. Thus, both of the main T cell populations accounted equally to the increased amount of T cells in the gut draining lymph nodes. The accumulation of CD3+ cells into the gut draining lymph nodes could explain the lower proportion of T cells in the spleen (Figure 12A). This finding, together with the lack of intestinal autoantibodies, strongly
indicates that the colitis found in the AireRAG1 recipients was mediated by T cells.

The AireRAG1-group showed a significant accumulation of T cells into gut draining lymph nodes and had symptomatic colitis. Taken together, these findings indicate that during LIP, the T cell responses were locally dysregulated when peripherally expressed Aire was missing, and the local dysregulation resulted in symptomatic autoimmune disease. As discussed previously, Tregs are important in controlling LIP. Therefore, the symptomatic autoimmune colitis in AireRAG1 animals could have been caused by peripheral Aire-dependent defects in Treg functions.

We measured the amount of FoxP3+ Tregs in the circulation one month after the transfer. The frequency of FoxP3+ positive cells in the total CD4+ population was lower in the AireRAG1-group, but the difference did not reach statistical significance. In the AireRAG1-group 4.2% (SD ± 0.98) CD4+ cells expressed FoxP3 while in the control group 5.8% (SD ± 1.6) of CD4+ cells were positive for FoxP3 (P=0.0944). However, when we analyzed the Ki67+ subpopulation of Tregs one month after the transfer it was evident that in the AireRAG1-group a significantly lower proportion of Tregs was in an active cell cycle. The proportion of FoxP3+Ki67+ cells of total CD4+ cells was 1.8% (SD ± 0.7) in the AireRAG1-group, while it was 3.4% in the control group (SD ± 0.9, P=0.012, original publication IV, figure 2A). The difference was still evident in the spleen at the time of termination (AireRAG 1.6% SD ± 0.8, control 3.3% SD ± 0.5, P=0.0036) but in the circulation the situation had normalized (Figure 13).

![Figure 13: Percentage of Tregs in the cell cycle during the 2-month transfer.](image)

It appeared that the loss of Aire from the peripheral immune system caused a decreased systemic proliferation of Tregs during LIP. This resulted
in a decreased frequency of Tregs in the spleen at the time of termination: the percentage of the FoxP3^+ cells in the total CD4^+ population in the AireRAG1-group was 10.5% (SD ± 6.0), when in the control group it was 18.9% (SD ± 2.1, P=0.0174, original publication IV, figure 2C). It is known that defective Treg activation may cause delayed Treg proliferation. Our data suggest that the peripheral expression of Aire is required for correctly timed and effective Treg activation during LIP. This is illustrated when the data of Tregs in active cell cycle is plotted against time (Figure 14).

If Tregs are not fully activated, it may result in a decreased Treg / T effector cell ratio that has been associated with autoimmunity in mice\textsuperscript{246,318}. It is thus possible that the delayed proliferation of Tregs in the AireRAG1-group explains the locally dysregulated immune response in the colon during LIP. Intestinal lymph nodes have been shown to express intestinal self-antigens together with Aire\textsuperscript{271}. It is possible that an Aire-dependent antigen expression in the gut-associated lymphoid tissue would be associated with Treg activation, or even conversion of iTregs from naive T cells.

5 Summary of the Results (III-IV)
The findings from these two separate transfer experiments (Publications III and IV) indicate that the loss of Aire from the peripheral immune system results in delayed proliferation of Tregs, but that the thymic loss of Aire expression does not affect Treg function in the peripheral immune system.
LIP in chronically lymphopenic animals is largely driven by gut commensal antigens, and shares many features of a normal microbial antigen-specific immune response\textsuperscript{123, 441}. Our experimental model of adoptive cell transfer to RAG1-deficient recipients thus mimics a normal immune response to foreign antigens. We have shown here that peripherally expressed Aire is involved in Treg activation and proliferation during such a response. The loss of Aire from the secondary lymphoid organs can cause autoimmune disease resulting from a defective systemic Treg proliferation. This resulted in a reduced Treg frequency and dysregulated effector T cell response to commensal antigens. In contrast, Tregs that had developed without Aire hyperproliferated in an Aire-sufficient lymphopenic environment and protected the animals from autoimmune pathology, even though signs of immune dysregulation were evident.

FoxP3\textsuperscript{+} Tregs are generated \textit{in vivo} through multiple pathways. nTregs originate from the thymus, but iTregs are also generated from the memory T cell population, or through peripheral iTreg conversion\textsuperscript{88}. There is increasing evidence showing that Tregs are important in controlling the immune responses to infectious agents as well, possibly guiding the immune response to the most effective mechanism of fighting the external microbes\textsuperscript{34}. Antigen-specific Tregs expand very early during the immune response, so it appears that the fast proliferation of Tregs is essential for controlling the immune response. In the light of the results gained from our mouse model, it is possible to speculate that extra-thymic Aire-expressing cells are important in the activation, or conversion of Tregs during immune responses.
Figure 15: Summary of the results of the two independent adoptive cell transfers, isolating Aire’s thymic and peripheral expression. Teff = effector T cell, i.e. non-suppressive T cell.
Concluding remarks and future directions

Originally AIRE was believed to function solely as a transcription factor driving the promiscuous antigen expression in the thymic medulla. By utilizing the Aire-deficient mouse models, the functions and expression of Aire during thymic organogenesis and in peripheral lymphoid organs has been established. Furthermore, recent data also provide evidence for Aire in complex immunological processes, such as lymphocyte trafficking. AIRE has thus evolved from a transcription factor enabling negative selection to a significant controller of T cell development and functions, with the main emphasis still on the development of T cell tolerance. The complete role of AIRE in the immune system as a whole remains to be revealed.

When we published the Treg defects in APECED patients, the article received critical attention; for example it was claimed that there is no evidence that AIRE has an effect on Tregs at a global level. It was also suggested that the decrease in FOXP3 expression in APECED patients could result from a dilution effect, caused by the increased amount of activated effector T cells due to an ongoing immune response against for example Candida. In our work, there were no significant differences between patients and healthy controls in the number of activated CD25+ cells, nor in the expression of other activation-associated markers. None of the patients were suffering from systemic infections during sampling. Considering the fact that activated human lymphocytes up-regulate FOXP3 without suppressive function, the non-suppressive FOXP3 expressing effector cells would have been seen as a pronounced CD25highFOXP3low population. This was, however, not the case. We can thus conclude that the increased effector cells in APECED patients do not explain the decrease in FOXP3 expression.

The observation that Tregs from APECED patients express low levels of FOXP3 has been replicated in a Norwegian APECED-patient cohort, even though the authors only did phenotypical analysis and carried out no functional analyses. Also, our own group has further substantiated that the defective Treg population in APECED patients is actually the activated Tregs that fail to express high levels of FOXP3.

The main argument against an AIRE-related Treg defect has been that our findings in APECED have not been replicated in Aire-deficient mice. Indeed, originally Aire-deficient mice had an overtly normal Treg function. Recent more detailed analyses have, however, shown that murine Tregs are not normal when Aire is missing. Aschenbrenner and Le have shown that Aire-expressing mTECs are required for murine nTreg development. Hubert analyzed the published Treg frequencies in different Aire-deficient animals in detail and suggested that there is a trend towards a decreased proportion of
peripheral Tregs, even though the differences in single studies were not statistically significant. Aricha\textsuperscript{22} analyzed the proportion of Tregs in Aire-deficient mice and found that Aire-deficient mice have significantly less Tregs in the thymus, but that the peripheral proportion of Tregs is decreased only in older animals. In subsequent more detailed analyses, the peripheral Tregs and thymic Treg development were found to be impaired in the Aire-deficient mice.

In the two transfer experiments we showed that the loss of Aire from the periphery during lymphopenia-induced proliferation resulted in autoimmune colitis, while the loss of Aire during lymphocyte maturation did not cause symptomatic autoimmunity in a similar setting. This finding provides experimental support for the role of extra-thymic Aire in the maintenance of peripheral tolerance. Aire-deficient recipient mice suffering from colitis had a lower proportion of Tregs in the cell cycle, and this slower Treg proliferation resulted in a decreased systemic amount of FoxP3. This indicates that extra-thymic Aire is involved either in Treg activation, iTreg conversion, or efficient FoxP3 expression. An intrinsic defect in Tregs is excluded, since the transferred Tregs originated from wildtype donors with unaffected Treg development. In comparison, Tregs that had developed in the absence of Aire hyperproliferated in Aire-sufficient recipients. This difference indicates that Tregs that developed without Aire are functional and capable of suppressing autoreactive proliferation if Aire is present in the secondary lymphoid organs.

Teh \textit{et al.}\textsuperscript{449} studied different molecules associated with the maintenance of peripheral tolerance together with Aire deficiency (as discussed on page 58). They found that Aire-deficient mice crossed with the Cbl-b deficient mice developed a more severe autoimmune syndrome than either one of the parental strains. However, when Aire-deficient mice were crossed with Card11 deficient animals, the disease severity was not affected\textsuperscript{449}. The authors concluded that the reduced need for co-stimulation caused by Cbl-b deficiency would be the causative agent for accelerated disease, but the results can be also interpreted differently. Cbl-b signaling has been shown to mediate FoxP3 up-regulation and iTreg conversion\textsuperscript{158, 191} in the periphery, while Card11 signaling has been shown to be required for thymic development of nTregs\textsuperscript{317} but it not essential in the periphery\textsuperscript{31}. It is thus possible that the severe autoimmune disease in Aire\textsuperscript{−/−}Cbl-b\textsuperscript{−/−} mice is caused by additive defects in the peripheral up-regulation of FoxP3, or the iTreg phenotype conversion.

The APECED patient Tregs failed to suppress T cell proliferation in response to polyclonal stimulus \textit{in vitro}. Also, in Aire-deficient lymphopenic mice, Tregs failed to suppress T cell hyperproliferation in MLNs during an immune response driven by polyclonal microbial antigens\textsuperscript{123}. It thus appears that the Treg defect following the loss of AIRE is not universal but, instead, it
appears only in a situation where several clones of lymphocytes proliferate. If this would be the case, the failures in Treg activation and suppression could explain the episodic disease progression in APECED that is one of the special features of the disease. Without prompt Treg action in the very early phases of the immune response, lymphocyte proliferation and activation is dysregulated and can cause autoimmune tissue damage. In patients, these attacks could follow for example dysregulated immune responses towards viral or bacterial infections.

It has been speculated that iTregs would be more important in the maintenance of tolerance in humans than in mice, because a longer lifespan results in thymic involution that reduces the thymic output of nTregs. This could explain the original differences in the disease severity between Aire-deficient mice and APECED patients; the peripheral loss of AIRE reduces the amount of peripheral functional Tregs and the effect is cumulative with time. Also in Aire-deficient mice the autoimmune syndrome is more severe in aged animals that also have reduced numbers of Tregs\textsuperscript{22}. This is consistent with our theory of serial dysregulated immune responses to foreign antigens that lead to autoimmune episodes.

Future studies should be aimed at identifying the peripheral AIRE-expressing cells that are involved in Treg activation. The mechanisms of the defect in Treg function should be dissected in more detail; our data are not sufficient to differentiate between defective activation, proliferation, or iTreg conversion. If an AIRE-dependent peripheral pathway for Treg function would be identified, it could be utilized for example in antigen-specific induction of Tregs \textit{ex vivo}. The knowledge of how to manipulate Treg responses is important for the development of new therapy options for autoimmune diseases and chronic infections.
ACKNOWLEDGEMENTS

This study was carried out in the Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki. I want to thank the Head of the Institute prof. Seppo Meri for the excellent research facilities and also for the inspiring mentoring that you have given over the years.

Financial support for this study was provided by the Research Foundation of the University of Helsinki, Diabetes Research Foundation, Emil Aaltonen Foundation, Oskar Öflund Foundation, Orion Farmos Foundation, Duodecim Medical Association, Biomedicum Helsinki Foundation, Finnish Cultural Foundation and the Academy of Finland.

Professors Ilkka Julkunen and Jorma Ilonen kindly agreed to examine my thesis. I thank them for their thorough work. Your comments improved the book significantly. I am obliged to prof. Olle Kämpe for accepting the role of official opponent in the thesis dissertation.

I want to thank my supervisor docent Petteri Arstila for giving me the opportunity to become a scientist. You always listened to my ideas and quite often also let me execute them. After I had presented my disorganised plans, I often heard the question “Why?” that forced me to refine my thinking and argumenting – and sometimes the question also fired up my temper, which you endured admirably. Your resistance to new technology also indirectly forced me to solve practical problems often independently, which I believe will be a handy skill in my future scientifical career. You never held me down and were always supporting. I am truly grateful for that.

My warm thanks also go to my unofficial supervisor and ex-neighbour Hanna Jarva. You are a true friend. Hanna has a similar working rythym as I do so we became the late-bloomers of the Haartman Institute. This let to many scientifical, and many more non-scientifical, Balint-discussions. Hanna is also credited for the language revision of my thesis.

The Arstila lab members, past and present, you have made an unforgettable impression to me. Our lab is not only a place to work but it is also a second home for me. Here I can only scratch the surface of the experiences we have shared over the nine years I worked with you.

Heli, I am glad that our friendship has evolved from pipetting together to so much more. I am honored to be Viljami’s kummatäti and thus to be involved in the lives of all your three amazing boys. You and Simo were incredibly supportive during the thesis writing and the many debriefing sessions we had kept me sane. The same applies to Tuisku-Tuulia and Kimmo as well – without you welcoming me to your home over the summer I would have been homeless! I thank Tuisku especially for the designing and
drawing the cover picture. Tuisku and Sara (a.k.a karonkkakaasot) were indispensable in helping with all the karonkka-party organizing.

Laura was of great help during the exhausting mice experiments and we shared some good moments in and out of the lab. Nora thought me all I know of animal handling and we shared some pretty traumatizing, but also hilarious moments in the mouse facility. Nora also helped me with the language corrections of my thesis. Eero and Maija-Katri, you are both obedient, fun, and smart students that I ruthlessly used for pipetting. I hope that this project did not kill all your interest in science! Pirkka, you have been a good addition to our lab; first you balanced the rather biased gender distribution but soon also proved to share my inappropriate taste of humour, which sparked up the lab conversations. Sini and I have had many inspiring discussions in our isolated “T cell homeostasis unit” across the hall. Anni, Iivo, Nelli, and Helga: you all are fun and hard working people, just perfect for the queer late-hour Arstila lab atmosphere. Keep up the good spirit! I am so glad that we found Tamás to be our lab-master. You have been a big help during the final experiments for this book.

I also want to thank the great people of the department of Bacteriology and Immunology: Rauna, Hanna J, Marcel, Annika, Derek, Taru, Markus, Aino, Satu, Karita, Sari and Heidi. You make this department what it is, a great place to work. Nathalie always made me smile, even when the walls were already closing in. Antti L, well, maybe it is enough said that you and I are the honorary members the lower department of the Haartman Institute. I want to acknowledge Tobias for giving me important information about the intestinal immune system and mice models of colitis. Sakari was the computer wizard that saved my thesis by converting it to the correct printing format in the very last moments.

I want to thank my collaborators and co-authors Aaro Miettinen, Ismo Ulmanen, Kimmo Talvensaari, Joonas Joensuu, Rauli Franssila, Mikhail Gylling and Jaakko Perheentupa. The administrative personnel of our department Kirsi Udueze and Marjatta Turunen have always helped me with my many questions. The personnel of the Animal Facility and especially Leena Liesirova have been always there to help with my mouse problems. Marjatta Ahonen helped me with frozen sections. Ilkka Vanhatalo and Marko Hietavuo are indespensable in being the Institute’s technical wizards. I originally started working in the Department of Virology and I want to acknowledge Leena Kostamovaara and Tytti Manni for teaching me the basic principles of laboratory work.

My friends and family have endured my ups and downs with this project, and I am deeply grateful for that. Sara and Juho-Antti helped and housed me with during the tricky in-between phase before moving to Sweden. The lovely ladies, Eeva, Helka, Kanerva, Marianna, Niina and Ulla, supported
me in everything I did. Suvi and Pyry dragged me out of the lab often enough so I did not become a complete Gollum (even though I still find this thesis very precious).

Mom and dad, thank you for everything. Sanna and Richard, thank you for always looking after me. Now I do not only have a big sister but also a big brother too. Teppo, my sweet baby brother, I glad that you have continued the family Kekäläinen tradition in Speksi after me. You do it so much better than I ever did. Auli and Eljas, thank you for welcoming me in the family Kasurinen.

This thesis would not have ever been finished without Mikael. There are no words to describe how deeply grateful I am for all your support. I love you.

Helsinki, September 2011

Eliisa Kekäläinen
REFERENCES

1. Immune deficient model selection: Choosing a nude, scid or Rag1 strain. (2000) JAX Communication - Scientific Publication for Users of JAX Mice (No 2).


