T Cell Homeostasis in Autoimmune Polyendocrinopathy - Candidiasis - Ectodermal Dystrophy (APECED)

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ACADEMIC DISSERTATION

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TABLE OF CONTENTS

ABSTRACT ......................................................................................................................................... 5
TIIVISTELMÄ ...................................................................................................................................... 7
ABBREVIATIONS ............................................................................................................................ 9
ORIGINAL PUBLICATIONS ............................................................................................................. 11
REVIEW OF THE LITERATURE .................................................................................................... 12

1 T CELL ACTIVATION .................................................................................................................. 12
   1.1 Role of T cells in the immune system .................................................................................... 12
   1.2 T cell activation requires the functions of APC ................................................................. 12
   1.3 TCR and MHC interaction as the basis of T cell classification ......................................... 13
   1.4 The intracellular cascade of T cell activation ..................................................................... 13

2 T CELL SUBSETS AND THEIR FUNCTIONS ......................................................................... 15
   2.1 CD4⁺ T cells ...................................................................................................................... 15
      2.1.1 Th1 cells .................................................................................................................. 15
      2.1.2 Th2 cells .................................................................................................................. 15
      2.1.3 Th17 cells ................................................................................................................ 16
      2.1.4 Regulatory T cells: natural and induced .................................................................. 17
      2.1.5 Plasticy of lineage commitment .............................................................................. 17
   2.2 CD8⁺ T cells .................................................................................................................... 18

3 T CELL MEMORY AND HOMEOSTASIS ........................................................................... 19
   3.1 Peripheral maturation and T cell memory ........................................................................ 19
   3.2 T cell homeostasis .......................................................................................................... 21
      3.2.1 TCR signalling as a homeostatic mechanism ......................................................... 22
      3.2.2 IL-7 and other homeostatic cytokines ................................................................. 22
   3.3 Local T cell residency: the skin as an example ............................................................... 23

4 T CELL DEVELOPMENT AND CENTRAL TOLERANCE ....................................................... 24
   4.1 Early stages of T cell development .................................................................................. 25
      4.1.1 Thymus as the site of T cell development ................................................................ 25
      4.1.2 V(D)J recombination .............................................................................................. 26
      4.1.3 IL-7 in the thymus .................................................................................................... 27
      4.1.4 Positive selection ..................................................................................................... 27
   4.2 Negative selection ............................................................................................................ 27
   4.3 Role of AIRE in central tolerance .................................................................................... 28
   4.4 Natural Treg development in the thymus ....................................................................... 31

5 PERIPHERAL TOLERANCE ........................................................................................................ 33
   5.1 Tregs in the periphery ........................................................................................................ 33
      5.1.1 The discovery of Tregs ............................................................................................. 33
      5.1.2 FOXP3 and Treg modes of action .......................................................................... 34
      5.1.3 Peripheral conversion of Treg cells and Treg homeostasis .................................... 35
   5.2 Additional tolerogenic mechanisms inside the lymph nodes ........................................ 38
      5.2.1 T cell anergy .......................................................................................................... 38
      5.2.2 Clonal deletion ...................................................................................................... 39
   5.3 The role of T cell homeostasis in peripheral tolerance ................................................... 39
   5.4 Role of AIRE in peripheral tolerance .............................................................................. 40

6 APEXED ........................................................................................................................................ 43
   6.1 Genetic background: mutations of AIRE ......................................................................... 43
   6.2 Clinical features of APEXED ............................................................................................ 45
      6.2.1 Disease components and autoantibodies .................................................................. 45
      6.2.2 Treatment ............................................................................................................... 48
   6.3 Features as a model for human autoimmunity .................................................................. 49
      6.3.1 Common autoimmune diseases .............................................................................. 49
      6.3.2 Challenges with autoimmune mouse models ....................................................... 50
      6.3.3 Other monogenic autoimmune diseases .................................................................. 50
      6.3.4 AIRE in more common autoimmune diseases ......................................................... 51

AIMS OF THE STUDY .................................................................................................................. 52
SUMMARY OF MATERIALS AND METHODS................................................................. 53
1 SAMPLES ........................................................................................................ 53
  1.1 APECED patients (I-III) ........................................................................ 53
  1.2 Healthy subjects (I-III) .......................................................................... 54
  1.3 Mantoux testing and blister induction (III) ............................................. 54
2 FLOW CYTOMETRIC ANALYSIS (I-III) ......................................................... 55
3 DETECTION OF APOPTOTIC CELLS (I) ....................................................... 55
4 CELL SEPARATION ....................................................................................... 56
  4.1 Sorting (II) ............................................................................................ 56
  4.2 Immunomagnetic cell separation (II) .................................................... 56
5 CELL CULTURE (III) .................................................................................... 56
6 PCR .............................................................................................................. 56
  6.1 RNA/DNA isolation and cDNA synthesis (II-III) ............................... 56
  6.2 Quantitative PCR (II-III) .................................................................... 57
  6.3 TREC analysis (II) .............................................................................. 57
  6.4 TCR repertoire analysis (II) ................................................................. 57
7 ELISA (II) .................................................................................................... 58
8 STATISTICAL ANALYSIS (I-III) ................................................................. 58
9 ETHICAL CONSIDERATIONS (I-III) ............................................................ 59

RESULTS........................................................................................................... 60
1 DEFINITION OF T CELL SUBPOPULATIONS (I-III) ................................. 60
  2.1 Activated Tregs express FOXP3 at a lowered intensity ....................... 61
  2.2 RTE Tregs are decreased in frequency and undergo accelerated proliferation .................................................. 61
  2.3 Defect of Tregs is more severe in the peripherally activated population .................................................. 63
2 CD8 T CELLS ARE PERTURBED BY IL-7 DYSREGULATION (II) ............... 65
  3.1 Increased proliferation of CD8 T cells arising from the CD8na subset .... 65
  3.2 CD127 expression is drastically reduced and is related to elevated IL-7 levels in CD8 T cells and the CD8na subset ...................................................................................................................... 66
  3.3 CD5 expression is decreased and an oligoclonal repertoire is revealed in CD8 T cells and the CD8na subset ...................................................................................................................... 67
  3.4 CD8na and naive CD8 T cells produce increased amounts of perforin, and the IL-7 dysregulation is implicated to begin already in the thymus ...................................................................................................................... 68
3 CUTANEOUS IN VIVO EXPERIMENT REVEALS A STABLE DEFECT OF IL-22 PRODUCTION (III) .............................................................. 70
  4.1 T cell accumulation and phenotype in the skin are unaltered, but pre-existing eye disease is activated in the patients ...................................................................................................................... 70
  4.2 Mantoux testing induces normal Th1 responses .................................... 71
  4.3 IL-22 expression is severely repressed with and without a challenge ...................................................................................................................... 72

DISCUSSION...................................................................................................... 74
1 FAILURE OF PERIPHERAL TOLERANCE IN APECED ............................... 74
  1.1 Treg dysfunction in APECED ............................................................... 75
  1.2 Autoreactive CD8na T cells escape the control of the lymph nodes ........ 75
  1.3 Role of T cell homeostasis in APECED .............................................. 77
    1.3.1 IL-7 in APECED ........................................................................ 77
    1.3.2 Special features of human Treg homeostasis reflected in APECED ........................................................................ 78
2 IS THE PERIPHERAL FAILURE ACTUALLY PERIPHERAL? ......................... 79
3 HOW DOES THE CHRONIC PATHOGEN EXPOSURE ON THE SKIN AFFECT APECED ................................................................. 81
4 KINETIC MODEL OF APECED PATHOGENESIS – IMPLICATIONS FOR MODELLING COMMON AUTOIMMUNE DISEASES ...................................................... 83

CONCLUDING REMARKS .................................................................................. 87
ACKNOWLEDGEMENTS .................................................................................... 88
REFERENCES ...................................................................................................... 91
ABSTRACT

Autoimmune polyendocrinopathy - candidiasis - ectodermal dystrophy (APECED) is a rare but severe, recessively inherited monogenic autoimmune disease that is part of the Finnish disease heritage. Loss-of-function mutations in the Autoimmune regulator (AIRE) gene cause APECED, which thereby provides a unique model for studying human autoimmunity. The patients suffer from chronic mucocutaneous candidiasis (CMC) and organ-specific autoimmunity targeted against especially the endocrine organs; hypoparathyroidism and Addison’s disease are the most common endocrinopathies, but many other disease components frequently occur. AIRE is a transcriptional regulator that is expressed the most in medullary thymic epithelial cells where it controls the expression of peripheral tissue restricted antigens. AIRE is therefore regarded an important tool of negative selection, which is the key step of central tolerance – the process of generating T cells that are tolerant to self. AIRE has been described to have various other functions in the thymus and it is also expressed in the secondary lymphoid organs. Central tolerance is complemented in the immune system by the mechanisms of peripheral tolerance, such as the functions of regulatory T cells (Tregs) that suppress the responses of other T cells. In APECED, Treg functions have been found impaired. With the differences between the clinically healthy remaining Aire−/− mice and the severe human disease, the pathogenesis of clinical APECED is still unclear.

The aim of this study was to discover mechanisms of autoimmunity in human APECED. This was approached first by studying further the population of Tregs in the patients, because understanding the reasons behind their functional defect could bring new knowledge on the impact AIRE has on peripheral tolerance. CD8+ T cell function in APECED was then addressed, because this population is thought to contain the highly autoreactive T cells in autoimmune diseases responsible for the direct tissue damage, and the pre-existing data on CD8+ T cells in the patients was scarce. The third study focused on the skin: the recently discovered autoantibodies against T helper 17 (Th17) class cytokines have implicated autoimmunity as a mechanism even behind CMC, which motivated a study on the in vivo immune response of Th cells in APECED patients.

Flow cytometry was used to study the phenotype of T cells, which were for the most part isolated from peripheral blood samples of APECED patients and healthy controls. To generate an in vivo memory response, intradermal injections of purified protein derivative (PPD, tuberculin) were given to the skin of APECED patients and healthy controls, and the responding cells made retrievable by forming blisters on top of the reactions with a clinical suction pump. The isolated cells from peripheral blood and the skin blisters were analyzed also by quantitative polymerase chain reaction, to determine relative gene expression. This was preceded in some experiments with further separation of T cell subpopulations, performed by sorting or using immunomagnetic cell separation. The T cell receptor repertoire of CD8+ T cells was analyzed by spectratyping. Cell cultures were used to study the effects of polyclonal stimulation on peripheral blood Th cell function.
The defect of Tregs in APECED was found to be more severe in the peripherally activated subpopulation than in the recently from the thymus emigrated (RTE) Tregs, indicating a defect of Treg peripheral homeostasis. This was associated with an increased attrition of RTE Tregs in the patients, most probably leading to insufficient long-term recruitment of functional Tregs in the periphery. The population of CD8+ T cells in the APECED patients was found to be under a severe interleukin 7 (IL-7) dysregulation, which is an important homeostatic cytokine in the immune system. This was associated with a notable skewing of the CD8+CD45RO− T cell subset of the patients, highly suggestive of autoreactivity. Naïve CD8+ T cells were also found severely altered, as well as the CD8+ T cells bearing a marker of recent thymic emigrancy, which suggested that the IL-7 dysregulation had begun already in the thymus. Studying the in vivo immune response, a selective defect of the Th17 class cytokine IL-22 production emerged, which was evident both at the site of antigen challenge as well as on the unexposed skin, and appeared also in stimulated cells of the peripheral blood. Production of the cytokine IL-17 was not significantly altered, which suggested that the Th population affected in APECED might be the emerging Th22 cells, especially involved in maintaining the barrier function and attracting proper immune responses in the skin.

This study reveals multiple defects of T cell homeostasis in APECED that can explain the severity of the disease in humans and the appearance of additional disease components with aging: The defect of Tregs in APECED has a temporal component, which is likely to result from alterations imprinted in them in the thymus, and their peripheral activation fails, possibly relating to the lack of functional AIRE in the periphery. Therefore the autoreactive T cells, which gain further momentum from the IL-7 dysregulation, can cause damage to additional target organs. The IL-7 dysregulation might be the consequence of increased type 1 interferon exposure inside the thymus, which has been suggested to be causing the autoantibodies against these cytokines in APECED. The IL-22 defect, in turn, increases microbial exposure and can imbalance the immune regulation even further.

The findings offer novel insights into the pathogenesis of organ-specific autoimmunity, and may have implications for more common autoimmune diseases where Tregs, IL-7 regulation and the effects of IL-22 are indicated. Furthermore, the study provides support for seeking therapies for APECED that balance the homeostatic milieu of T cells, a strategy perhaps easier to implement than repairing the process of negative selection.
TIIVISTELMÄ


T-solujen ilmiasua tutkittiin virtuaalysytomietrian avulla, jota varten solut eristettiin pääasiassa APECED-potilaiden ja terveiden verrokkien laskimoverinäytteistä. In vivo -koasetelmassa muistihojainen immunivaste aikaansaatiin ihonisissä jälkeen tuberkuliini-pistoksilla. Tähän altistukseen reagoivien solujen keräämisestä pistoskohtien päälle luotiin alipaineen avulla

Säätelijä-T-solujen häiriö oli APECED-potilailla voimakkaampi periferiassa aktivoituneessa populaatiossa kuin juuri kateenkorvasta tulleissa säätelijä-T-soluissa, mikä viittasi perifeeriseen säätelijä-T-solujen ylläpidon häiriöön. Tähän liittyi löydös lisääntyneestä juuri kateenkorvasta tulleiden säätelijä-T-solujen populaation kulumisesta, mikä todennäköisesti johtaa pitkällä aikavälillä toimintakykyisten säätelijä-T-solujen joukon puutteelliseen täydentymiseen periferiassa. CD8⁺ T-solujen populaatio kärsi APECED-potilailla merkittävästä interleukini 7 -välittäjääineen, joka on tärkeä populaatiointa ylläpitävän välittäjäaineen imunijärjestelmässä, sääteilyhäiriöstä, ja tällä oli yhteyds CD8⁺ CD45RO⁻ T-solujen ilmiasun muutoksiin. Näiden solujen joukossa vaikuttikin potilailla olevan kehon oma rakketaite vastaan reagoiva, autoreaktiivinen, solurrayhmä. Myös näivien, periferiassa aktivoitumattomien solujen, ja kateenkorvasta juuri tulleista vaikuttavien CD8⁺ T-solujen todettiin olevan hyvin poikkeavia APECEDissa, minkä perusteella IL-7 sääteilyhäiriön pääteeltiin alkanee jo kateenkorvassa. In vivo -immuunivasteen tutkimuksessa IL-22 välittäjäaineen tuotanto oli häiriintynyt, mikä tuli esiin paitsi immuunivasteessa, myös altistamattomalla iholla, sekä stimuloiduissa laskimoveren soluissa. IL-17 tuotanto ei ollut merkitsevästi alentunut, mikä viittasi siihen että APECEDissa auttaja-T-solujen 22-alaluokka, joka on erityisen tärkeä ihon puolustusrakenteiden ylläpidossa ja oikeanlaisten immuunivasteiden luomisessa iholle, saattaisi olla häiriön ensisijainen kohde.

Tämä tutkimus paljastaa useita T-solujen populaatioiden ylläpidon häiriöitä APECEDissa, jotka voivat selittää taudin vaikeusasteen ihmispotilaissa ja uusien tautikomponenttien ilmenemisen ikääntymisen myötä: Säätelijä-T-solujen häiriöllä on ajallinen osatekijä, joka todennäköisesti johtuu häiriöistä solujen kehityksessä kateenkorvassa, ja säätelijä-T-solujen perifeerinen aktivaatio on puutteellinen, johtuen mahdollisesti perifeerisen AIREn puuttumisesta. Näin ollen autoreaktiiviset T-solut, jotka vahvistuvat IL-7 sääteilyhäiriöstä, pääsevät aiheuttamaan vauriota uusille kohdelle-elimille. IL-7 sääteilyhäiriö saattaisi olla seurausta lisääntyneestä tyypin 1 interferonialtistuksesta kateenkorvassa, mitä on ehdotettu näitä välittäjäaineita vastaan kohdistuvien autovasta-aineiden syynä APECEDissa. IL-22 häiriö johtaa puolestaan lisääntyneeseen mikrobialtistukseen, mikä voi edelleen johtaa immunisäätelyn epätaasapainoon.

Löydökset tuovat uutta kohdelle-elinspesifisen autoimmuunisairauskäseken attainment ymmärrykseen, ja voivat olla hyödyllisiä yleisempiä autoimmuunisairauskäseihin tutkimukselle, missä säätelijä-T-solut, IL-7 sääteily ja IL-22n vaikutukset on alustavasti tunnistettu. Tutkimus myös kannustaa etsimään hoitoja APECED-potilaille T-solujen ylläpitävää perifeeristä ympäristöstä tasapainottavista tekijöistä, mikä saattaa olla helpommin toteutettavissa kuin puutteellisen negatiivisen valinnan korjaaminen.
ABBREVIATIONS

AIRE   autoimmune regulator, gene/protein (human)
Aire   autoimmune regulator, gene/protein (murine)
APC    antigen presenting cell
APECED Autoimmune polyendocrinopathy - candidiasis - ectodermal dystrophy
Cbl-b  Casitas B-cell lymphoma ubiquitin ligase
CCR7   CC-chemokine receptor 7
CD     cluster of differentiation
CDR    complementarity determining region
CD4RA,CD8RA CD45RO- subset of CD4+ T cells or CD8+ T cells
CD4RO,CD8RO CD45RO+ subset of CD4+ T cells or CD8+ T cells
CMC    chronic mucocutaneous candidiasis
cTEC   cortical thymic epithelial cell
CTLA-4 cytotoxic T lymphocyte antigen 4
DC     dendritic cell
DN     double-negative, CD4-CD8-
DNA    deoxyribonucleic acid
DP     double-positive, CD4+CD8+
ELISA  enzyme-linked immunosorbent assay
Foxo1  forkhead box O1
FOXP3  forkhead box P3
GATA3  GATA binding protein 3
GRAIL  gene related to anergy in lymphocytes
HLA    human leukocyte antigen
IDO    indoleamine 2,3-dioxygenase
IFN    interferon
Ig     immunoglobulin
IL     interleukin
IL-7R  interleukin-7 receptor
IPEX   Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
Jak3   Janus kinase 3
LCK    lymphocyte-specific protein tyrosine kinase
mAb    monoclonal antibody
MFI    mean fluorescence intensity
MHC    major histocompatibility complex
mRNA   messenger ribonucleic acid
MS     multiple sclerosis
mTEC   medullary thymic epithelial cell
mTOR  mammalian target of rapamycin
NF-κβ  nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC  peripheral blood mononuclear cell
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PPD  purified protein derivative (tuberculin)
RAG  recombination activating gene
RORC  RAR-related orphan receptor C
RTE  recent thymic emigrant
STAT  signal transducer and activator of transcription
Tbet  T-box 21
T<sub>CM</sub>  central memory T cells
TCR  T cell receptor
T<sub>EM</sub>  effector memory T cells
T<sub>EMRA</sub>  effector memory CD45RA<sup>+</sup> T cells
TGF-β  transforming growth factor beta
Th  T helper cell
TNF-α  tumour necrosis factor alpha
TREC  T cell receptor excision circle
Treg  regulatory T cell
TSLP  thymic stromal lymphopoietin
V(D)J  variable-diversity-joining (recombination of the TCR)
ZAP-70  zeta-chain-associated protein kinase 70
ORIGI NAL PUBLI CATIONS

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


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REVIEW OF THE LITERATURE

1 T CELL ACTIVATION

T cells depend upon the function of immunologic networks that initiate, guide and control them. To understand T cell behaviour, the environment of T cell function must first be introduced.

1.1 Role of T cells in the immune system
The immune system is divided into two systems, the adaptive and the innate immunity, and T cells are essential in the processes of adaptive, or learning, immune responses. From the perspective of T cells, innate immunity is the sentinel to their activation, and is thought to evoke a T cell response when faced by a pathogen overcoming their solitary efforts. The role of T cells is therefore to form a secondary line of defense. (Murphy et al. 2008)

1.2 T cell activation requires the functions of APC
Innate cells that activate naive T cells are called the antigen presenting cells (APC). An important subset of them is the dendritic cells (DC) that process substances encountered in the tissues, and convey these as antigens to the peripheral lymphoid organs. Here the presentation of the antigenic peptide the T cell is specific against, together with an activating cytokine milieu created mainly by the APC, leads to the naive T cell to dedicate to a specified function (discussed further in chapter 2), to proliferate and to eventually migrate from the lymph nodes to the site of inflammation – a process known as T cell activation. (Stockwin et al. 2000)

Full T cell activation requires three separate signals: First, the interaction of a T cell receptor (TCR), a specific antigen, and a surface structure of the APC called the major-histocompatibility-complex (MHC; discussed in detail in the following paragraph). Second, the same APC has to give a co-stimulatory signal, most important of which is the expression of B7-1 or -2 (CD80 or CD86) on its surface that then activates their receptor CD28 on the T cell. The third signal comes from the cytokines. (Andersen et al. 2006)
1.3 TCR and MHC interaction as the basis of T cell classification

The fingerprint of T cells is the TCR on their surface. The unique, varying TCR ensures highly specific – and as a system, most diversified – responses. The TCR comprises a variable TCRαβ heterodimer. Only 5% of human T cells do not have a TCRαβ on their surface, but a γδ TCR instead, thus representing a quite separate unit of immune cells (Aljurf et al. 2002). At a more detailed level, the TCRα chain and β chain both have a constant (C) domain and a variable (V) domain, and the extremities of the V domains is where the specificity of the TCR is created by hypervariable areas called the complementarity determining regions (CDR). CDR3 binds the actual antigen, whilst CDR1 and CDR2 mediate the other key feature of the TCR – MHC restriction – by demanding a simultaneous contact with an MHC. (Andersen et al. 2006, Borg et al. 2005)

The MHC, in humans referred to as the human leukocyte antigen (HLA), is divided into two, namely the MHC class I and class II molecules. This division converses to activation of only either the CD4+ T cells (MHC-II) or CD8+ T cells (MHC-I), which constitutes the basis of T cell classification. (Stockwin et al. 2000; T cell subclasses are discussed in depth in chapter 2). MHC II expression is limited to professional APCs, who use them to present endocytosed antigens to CD4+ T cells in the process of T cell activation. MHC class I molecules present antigens of intracellular origin with a length of 8-10 amino acids, and are used by the APC to activate CD8+ T cells. They are also found on virtually all nucleated cells where they are closely linked to the functions of the already once activated CD8+ T cells. Only an APC can also use the MHC I to present not intracellular but endocytosed antigens to CD8+ T cells, a special activation event termed cross-presentation. (Andersen et al. 2006, Rudolph et al. 2006)

1.4 The intracellular cascade of T cell activation

The TCR is functionally inseparable from a non-variable CD3-signal-transduction-complex, which relays the stimulus to an intracellular activation cascade. The CD3-complex and associated intracytoplasmic TCRζ chains host the immunoreceptor tyrosine-based activation motifs, the phosphorylation of which by kinases lymphocyte-specific protein tyrosine kinase (LCK) and Fyn is the first step of this cascade. This phosphorylation attracts another kinase, the zeta-chain-associated protein kinase 70 (ZAP-70), which converts the stimulus further down and leads to, with the help of other positive inputs such as the CD28-triggered Ca2+-influx and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) recruitment, in a transcriptional activation inside the nucleus. (Smith-Garvin et al. 2009, Figure 1). For the TCR-MHC interaction to amplify into a relevant activation event, there is a considerable threshold to be overcome, where aggregation and conformational change of the TCR-CD3 system
and segregation of inhibitory molecules such as CD45 from their vicinity, have been implicated. (van der Merwe and Dushek 2011)

Figure 1. The TCR-MHC interaction, costimulation, the third signal of activation from cytokines, and the resulting intracellular cascade (modified from Murphy et al. 2008). Here, the activation of a CD4+ T cell is shown, where an APC presents the antigen the TCR is specific against bound to a class II MHC molecule, and the CD4 molecule binds to the MHC II. In CD8+ T cells, the antigen would be bound to a class I MHC molecule. The αβ TCR contains the V and C domains. The αβ TCR is inseparable from the CD3 molecules, and their intracellular tails contain the immunoreceptor tyrosine-based activation motifs (marked here as ITAMs) to be phosphorylated in the first step of the intracellular cascade of activation. Here, the enzymes LCK, Fyn and ZAP-70 are vital. For the activation to be sufficient, costimulatory signals through the CD28 molecule are needed, which must come from B7 molecules on the same APC. The third requirement is the influence of cytokines favouring activation of the T cell. The necessary Ca2+ influx created by the costimulation leads through additional intermediate steps and the recruitment of transcriptional regulator NF-κB into transcriptional activation in the nucleus. This finally alters the T cell phenotype.
2 T CELL SUBSETS AND THEIR FUNCTIONS

The two major subgroups of T cells have clearly separate functions in adaptive immunity, which is evident from their descriptive names, CD4+ helper T cells and CD8+ cytotoxic T cells. Their specialization and functions will now be explored.

2.1 CD4+ T cells

CD4+ T cells form the majority of T cells. They carry the cell surface molecule CD4 that interacts with the MHC class II molecules. Activated CD4+ T cells are functionally divided into lineages, which have different response types. As only recently understood and discussed below, this commitment can change. (O’Shea and Paul 2010)

2.1.1 Th1 cells

T helper 1 (Th1) cells promote immune responses against intracellular pathogens, such as Mycobacterium tuberculosis. They are formed in the presence of the cytokines interleukin 12 (IL-12), IL-18 and IL-27, the most critical being IL-12. These cytokines cause, through activation of signal transducer and activator of transcription 4 (STAT-4), the production of the master regulator T-box 21 (Tbet) inside the naive CD4+ T cell. This production blocks alternative differentiation pathways. Tbet elicits interferon (IFN) -γ production, which is the signature cytokine of Th1 cells. (Commins et al. 2010)

In the tissues, Th1 cells then stimulate, through secretion of IFN-γ, macrophages, cytotoxic CD8+ cells, neutrophils and natural killer cells to accumulate and perform actions against intracellular viruses and bacteria. IFN-γ stimulates APCs also in general, and can inhibit viral replication. (Commins et al. 2010). Th1 cells activate B lymphocytes and guide the immunoglobulin (Ig) class switching of their antibody production towards the opsonizing classes IgG2a and IgG3 (Murphy et al. 2008). Unorthodox activity of Th1 cells is a common feature of organ-specific autoimmunity (Korn et al. 2009).

2.1.2 Th2 cells

The main function of Th2 cells is to help the immune responses against helminths and other extracellular parasites. For a naive CD4+ T cell to commit to the Th2 lineage, the presence of both IL-4 and IL-2 is usually required. Also homeostatic cytokines IL-7 and thymic stromal lymphopoietin (TSLP) might be involved here (discussed further in chapter 3.6). These lead through STAT-6 and STAT-5 activation to the functions of transcriptional regulator GATA binding
protein 3 (GATA3), and finally the Th2 cell undertakes its major task, the production of the cytokines IL-4, IL-5, IL-9, IL-13, and IL-25. (Zhu and Paul 2010)

Of these effector molecules, the ones vital for Th2 responses are IL-4 and IL-5. IL-4 interacts with B cells, driving them to produce antibodies such as IgE and IgG1, and with mast cells, which become sensitized to IgE. IL-4 also helps basophils, eosinophils, monocytes and T cells to enter tissues, by inducing an endothelial adhesive molecule, VCAM-1 (vascular cell adhesion molecule 1). IL-5, on the other hand, supports eosinophils and augments IgA production in numerous ways. Through these functions, the Th2 cells are related to the pathogenesis of allergy and asthma. (Commins et al. 2010, Murphy et al. 2008)

2.1.3 Th17 cells
Th17 cells have been recognized as an independent differentiation lineage during the last decade. Detected first in the setting of autoimmune inflammation, they have attracted great attention, and only further studies have indicated a positive role in anti-pathogen responses where a flourishing inflammatory milieu is needed. Such a bridge between the innate and adaptive immunity is essential for example in responses against Candida albicans, Propionibacterium acnes or Borrelia burgdorferi, where the Th17 cytokines IL-17A, IL-17F and IL-22 are needed to stimulate the epithelium to attract more neutrophils on-site and to produce anti-microbial peptides. (Korn et al. 2009)

Th17 lineage induction is currently understood to depend upon the presence of the cytokines transforming growth factor beta (TGF-β) and IL-6. Under certain conditions, IL-1β, IL-21 or IL-23 can replace IL-6 in humans. The requirements were first thought to essentially differ between humans and mice, but later the key role of TGF-β has been proven also in humans. In pair the cytokines induce the phosphorylation of STAT-3 inside the naive cell, which in turn activates the transcription factor RAR-related orphan receptor C (RORC). IL-21 is also involved in an autoamplification loop that helps to sustain the population. (Korn et al. 2009)

In addition to the functions described above, IL-22 is also capable of stimulating tissue repair. This unusual function of an immune effector molecule, together with the isolation of IL-22+/IL-17− cells from the epidermal layer of inflammatory skin diseases such as psoriasis, atopic eczema and allergic contact dermatitis (Eyerich et al. 2009), has raised controversy of the true borders of the Th17 lineage. In fact, the yet to be substantiated Th22 cells have even gained support as the major producers of IL-22 in humans (Sonnenberg et al. 2011). The accumulating evidence of Th17 cell involvement in diseases such as psoriasis, multiple sclerosis (MS) and inflammatory bowel disease, however, holds these cells in the spotlight for autoimmune research (Korn et al. 2009).
2.1.4 Regulatory T cells: natural and induced

Amidst the effector helper T cell paths, yet a quite different role exists for the CD4+ T cell to adopt: the suppressive function. Regulatory T cells (Tregs) are capable of suppressing the in vitro proliferation of antigen-stimulated effector T cells, and can inhibit the development of autoimmune diseases in mouse models. Natural Tregs arise during thymic development, whereas induced Tregs commit to regulation at the point of naive T cell lineage differentiation in the peripheral lymphatic organs. Natural Tregs are recognized by high expression of the cell surface marker CD25 (IL-2 receptor α chain) and most importantly by the intracellular marker forkhead box P3 (FOXP3). FOXP3 is crucial for Treg function and is activated through STAT-5 signalling (Sakaguchi et al. 2009). For the differentiation of a naive CD4+ T cell into a Treg, TCR-mediated signalling is essential (Sakaguchi et al. 2010). Induced Tregs contain an important subset of FOXP3+ Tregs, but also non-FOXP3+ subsets, such as the IL-10-dependent type 1 regulatory T cells (Saurer and Mueller 2009). Tregs function through cell-to-cell contacts and by secreting the cytokines IL-10, IL-35 and TGF-β (Sakaguchi et al. 2009). Tregs will be discussed in more detail in chapter 5.

2.1.5 Plasticity of lineage commitment

Th cell lineage differentiation is influenced by feedback loops: for example in Th1 cells, Tbet upregulates IL-12 receptor, and the newly synthesized IFN-γ upregulates Tbet through STAT-1 signalling (Commins et al. 2010). The strength of TCR signalling is also suggested to affect Th lineage commitment independently of cytokines so that if the signal is strong, IFN-γ production is favoured, and if low, Th2 commitment is induced without the need for the presence of IL-4. In vivo, the overall gradient of the cytokine milieu and TCR signalling determine the outcome of commitment, instead of dependence on the presence of a single factor. (Zhu and Paul 2010, Figure 2). Concordantly, even the functions of differentiated CD4+ T cells can be plastic so that they can secrete cytokines of another lineage, for example a Th17 cell can become an IFN-γ producer, and even the expression of master regulators seems to be flexible. This plasticity is suggested to be the result of epigenetic modifications, such as deoxyribonucleic acid (DNA) methylation, which are heritable and yet environmentally-adapting alterations of gene expression, and cause a naive T cell usually only to lean on one direction instead of permanent differentiation. (O’Shea and Paul 2010)
2.2 CD8\(^+\) T cells

The primary function of cytotoxic CD8\(^+\) T cells is to eliminate viral infections. They also guard the body against tumour cells, and are involved in responses against other intracellularly operating pathogens. Although a professional APC can activate a naive CD8\(^+\) T cell by itself, successful activation often requires a preceding interaction of the APC with an activated CD4\(^+\) helper T cell in the secondary lymphoid organs. (Andersen et al. 2006, Murphy et al. 2008)

Activated CD8\(^+\) T cells lead target cells to apoptosis by three mechanisms: via contact through death receptors, by releasing cytotoxins into the immediate vicinity of target cells, or by expressing the cytokines IFN-\(\gamma\) and tumour necrosis factor alpha (TNF-\(\alpha\)). Death receptor-mediated contact involves the upregulation of Fas ligand (CD95L) on a T cell that then binds to Fas (CD95) on a target cell. This binding triggers a caspase cascade terminating in apoptosis. The cytotoxin perforin forms pores on the cell that is in direct contact with the cytotoxic T cell, and facilitates therefore the entry of granzymes A and B. TNF-\(\alpha\) binding also triggers a caspase cascade, whereas IFN-\(\gamma\) causes cells even at a distance to upregulate their MHC I and Fas expression, which then increases
their susceptibility to CD8+ T cell patrolling (Harty et al. 2000, Andersen et al. 2006).

CD8+ T cells seem to commit to regulatory functions as well. CD8−FOXP3−CD25+ cells have been described to produce IL-10, and suppress activated T cells in cell-to-cell contacts (Smith and Kumar 2008). Another emerging regulatory CD8+ T cell population supposedly monitors autoreactivity of T cells in a unique way: they recognize danger signals such as heat shock proteins connected to MHC I molecule HLA-E, which are preferentially expressed on the surface of autoreactive T cells (Jiang et al. 2010). Although evidence of regulatory CD8+ T cell involvement in autoimmune diseases is rising, an understanding of their significance is yet to be gained.

3 T CELL MEMORY AND HOMEOSTASIS

Normal function of the immune system is dependent on tight regulation of the life cycle of a peripheral T cell. Next this life cycle will be described, followed by a deeper look into how it is controlled – the mechanisms of T cell homeostasis.

3.1 Peripheral maturation and T cell memory

T cell precursors are born in the bone marrow and then undergo developmental phases in the thymus to become actual T cells (discussed further in chapter 4). The fresh T cell then begins its peripheral existence as a naive T cell, is activated in the secondary lymphoid organs as depicted above, and thereafter enters a stage of active function as a dramatically proliferated clone. Most effector T cells migrate to a target site in the tissues, and as the immune response silences, undergo apoptosis. Only a miniature image of this response is kept in the form of a long-lasting memory population. (LaRosa and Orange 2008)

Different stages of T cell life cycle in the periphery are characterized by the expression of cell surface receptors. To enter the secondary lymphoid organs, the naive T cell uses the homing receptors CD62L (L-selectin) and CC-chemokine receptor 7 (CCR7), which facilitate the entry through high endothelial venules and further into the T cell areas of secondary lymphoid organs (Sallustio et al. 2004). Another important cell surface marker of a naive T cell is a long isoform of the tyrosine phosphatase CD45: the more often used CD45RA or the splicing variant CD45RB, which both turn into CD45RO in the process of activation (Carrasco et al. 2006, Hermiston et al. 2003). For CD4+ T cells, an established marker for recent thymic descent is CD31, which functions in cellular adhesion and migration. Recent thymic emigrants are also characterized by a higher
content of T cell receptor excision circles (TRECs) that are DNA by-products of TCR gene recombination, whose amount decreases linearly with cell divisions. (Kohler and Thiel 2009)

The activated, responding effector T cell is detected by the expression of CD45RO and loss of CCR7 expression on its surface. Depending on the site of inflammation, the effector cells display homing markers of various tissues, for example CLA (cutaneous lymphocyte antigen) for the skin (Clark 2010), and this imprinting of addresses on the T cells seems to depend upon the site-specific experiences of the APC (Edele et al. 2008).

Memory is considered the distinguishing characteristic of adaptive immunity. It allows for rapid, antigen-specific recall responses of both T and B cells against introduced pathogens. (Sallusto et al. 2004). Two models have been proposed to explain the path leading to the generation of a persistent T cell memory population: one of descendence and one of early divergence. The conventional view of memory states it as a late stage phenomenon of an effector response, where cells surviving apoptotic circumstances form the memory population (LaRosa and Orange 2008). The challenger model regards memory as an independent fate diverging already at the time of activation of the naive cell (Kalia et al. 2006). This latter model has gained support from the finding that a newly activated T cell undergoes asymmetric division that partitions functional indicators of an effector phenotype to another and memory to the other daughter cell (Chang et al. 2007). The differentiation might also be a mixture of both models (Kalia et al. 2006).

Within T cell memory, subsets with different functions are recognized: The cells of central memory (T_{CM}) that restrict themselves to secondary lymphoid organs, are easily activated but hold limited potential for effector functions, and are regarded as the repository for rapid proliferation under recall challenges. Effector memory T cells (T_{EM}), in contrast, circulate in the peripheral tissues, are fully licensed to participate in active immune responses when they encounter reinvading pathogens, but are strictly controlled as to their reconstitutive capacity (Zielinski et al. 2011). The majority of memory CD4+ T cells in the blood are T_{CM} whereas T_{EM} dominates for CD8+ memory cells (Campbell et al. 2001). Cell surface markers typical for T_{CM} are CCR7, CD62L and CD45RO, whereas T_{EM} show no expression of CCR7 and CD62L (Parish and Kaech 2009). The T_{CM} have been suggested to precede the T_{EM} population as direct ascendants, of which the loss of lymph node homing markers would be an indicator (Lanzavecchia and Sallusto 2000); also, the T_{CM} have been shown to gain T_{EM} features when entering non-lymphoid tissues (Marzo et al. 2007). The network of T cell memory includes also a distinct population of CD8+ memory cells, the effector memory CD45RA+ (T_{EMRA}) that are thought to have regained expression of CD45RA, and are equipped with high amounts of the effector
molecule perforin (Sallusto et al. 2004). CD4+ memory cells might include T_{EMRA} - like cells as well (Libri et al. 2011). Memory against viruses can be T_{EM} or T_{EMRA} - dominated, for example for Cytomegalovirus it seems to be the latter (Champagne et al. 2001).

A plethora of other markers have been used to define T cell populations in the different phases of their life cycle. Of note are the costimulatory molecules CD27 and CD28, which are highly present in naive cells, expressed in some memory cells but are absent from T_{EMRA} (Appay et al. 2002, Table 1).

**Table 1. Features of T cells in the different stages of their life cycle.**

<table>
<thead>
<tr>
<th>T cell subpopulation</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive T cells</td>
<td>CCR7+, CD62L+, CD45RA−, CD45RO−, CD27+, CD28+</td>
</tr>
<tr>
<td>Recent thymic emigrant (RTE) T cells</td>
<td>CD31+, high TREC content</td>
</tr>
<tr>
<td>Central memory T cells (T_{CM})</td>
<td>CCR7+, CD62L+, CD45RA−, CD45RO+</td>
</tr>
<tr>
<td>Effector memory T cells (T_{EM})</td>
<td>CCR7+, CD62L−, CD45RA−, CD45RO+</td>
</tr>
<tr>
<td>Effector memory CD45RA+ T cells (T_{EMRA})</td>
<td>CCR7−, CD62L−, CD45RA+, CD45RO−, CD27−, CD28−</td>
</tr>
</tbody>
</table>

3.2 T cell homeostasis

T cell homeostasis refers to the physiological and functional maintenance of T cell populations. T cell - specific mechanisms for this come from TCR signalling and the influence of certain cytokines (Takada and Jameson 2009). With the human thymus involuting with aging, there is a steady decline in the production of naive RTE T cells from the first year of age on and even in the 80-year-olds, which has been measured by TREC analyses (Lynch et al. 2009). This is accompanied by peripheral clonal expansion of memory cells, with resulting alterations in the composition of the peripheral T cell pool: the ratio of CD4+/CD8+ T cells decreases and the overall TCR repertoire diminishes (Goronzy and Weyand 2005). Homeostatic mechanisms have great importance in balancing these two processes, namely the diminishing amounts of naive RTE T cells and, on the other hand, the expanding population of memory cells.
3.2.1 TCR signalling as a homeostatic mechanism

Homeostasis of naive T cells is especially important for the immune system, because this population preserves the potential to recognize a multitude of new antigens. A special mechanism maintaining the naive T cells is TCR-mediated. (Boyan et al. 2009). As discussed further in chapter 4, thymic development of T cells sustains recognition of specific self-peptide–MHC complexes to a certain extent (Starr et al. 2003), and this feature is in use in giving the naive T cells an initiative to survive in the periphery. In more detail, the secondary lymphoid organs serve as a site where APCs present spontaneously self-peptide–MHC complexes, and the ability to recognize these complexes on a low affinity level gives a survival signal. (Surh and Sprent 2008)

This homeostatic signalling, under normal conditions, doesn’t give rise to populational expansion but a slow proliferation resulting in upkeep of the population size. Intracellular pathways triggered by self-peptide–MHC complexes seem to be the same as in true activation, but in use on a lower level (Goldrath et al. 2004). Dire circumstances that result in severe lymphopenia can induce, however, a more replenishing proliferation producing even effector cells, possibly because of an excess of self-peptide–MHC and cytokine signals (Surh and Sprent 2008). Memory T cells are maintained in TCR-independent ways (Boyan et al. 2009), as discussed below.

3.2.2 IL-7 and other homeostatic cytokines

Cytokines can be perceived as hormones of the immune system, and for homeostasis, the common γ-chain receptor family cytokines, most importantly IL-7, IL-2 and IL-15, are fundamental (Rochman et al. 2009).

For naïve T cells, IL-7 is the other major homeostatic signal in addition to specific self-peptide–MHC contacts (Takada and Jameson 2009). IL-7 is produced for them to compete upon by the fibroblastic reticular cells of the T cell zones of lymph nodes, and is there mostly matrix-bound and therefore paracrine in nature (Link et al. 2007). IL-7 is also produced more systemically by other nonhaematopoietic stromal cells such as bone marrow stromal cells and endothelial cells, and is present in most organs of the body (Capitini et al. 2009). IL-7 binds to the IL-7 receptor (IL-7R) that comprises the IL-7Rα (CD127) and the common γ-chain receptor (CD132). Signalling through the IL-7R triggers STAT-5 phosphorylation that eventually results in gene expression of, for example, the Bcl-2 (anti-apoptotic B-cell lymphoma 2) gene, enhances proliferation and lowers the threshold needed for activation through the TCR (Mazzucchelli and Durum 2007). Recently, a transcription factor called forkhead box O1 (Foxo1) was described as the intracellular control centre of naïve T cell homeostasis: Foxo1 is suggested to integrate environmental signals so that when
growth factors are low it upregulates CCR7, CD62L and CD127, and when signalling through the TCR or by cytokines is abundant, Foxo1 is inactive (Kerdiles et al. 2009).

CD127 expression is downregulated in response to IL-7 or TCR-mediated signalling (Alves et al. 2008). Accordingly, effector T cells are independent of IL-7 whereas memory T cells again rely on IL-7 and the cytokine IL-15 for survival (Rochman et al. 2009). The elevated expression of CD127 has even been suggested, in line with the descendence model of memory generation, to identify those 5-10% of effector cells that will survive as memory cells (Kaech et al. 2003). IL-15, produced for the most part by DCs, helps also the effector cells to resist going into apoptosis (McGill et al. 2010).

TSLP is a homeostatic cytokine that needs to be taken into account when describing IL-7. This is because TSLP signals through a combination of CD127 and the TSLP receptor. TSLP, expressed by epithelial cells, promotes Th2 differentiation, increases the effector responses of CD8+ T cells, and might promote Tregs as well. (Ziegler and Artis 2010). The role of TSLP in T cell homeostasis is, however, seen as secondary to IL-7, because most activation of CD127 has been shown to come from IL-7 (Mazzucchelli and Durum 2007).

IL-2, in the other hand, is a major auto- and paracrine cytokine promoting activated T cell proliferation (Wells 2009). Even though IL-2 is potent in accelerating clonal expansion and effector development, the only irreplaceable function of IL-2 is in the generation and homeostasis of Tregs (Malek 2008). IL-2 signals through the IL-2 receptor (IL-2R), which consists of IL-2Ra (CD25), IL-2Rb (CD122) and the common γ chain receptor. CD25 is highly expressed on Tregs, who at the same time are low on the expression of CD127 (Seddiki et al. 2006). In the homeostasis of peripheral Tregs, IL-2 is the key cytokine facilitating proliferation (Malek 2008). By not allowing intracellular production, however, FOXP3 renders Tregs dependent on exogenous IL-2, which is then scavenged from activated non-Treg cells – this mode of action is a substantial mechanism of Treg suppressive function (Sakaguchi et al. 2008).

3.3 Local T cell residency: the skin as an example

The skin is an important immunological organ in many ways. It provides a physical barrier against the surrounding environment and its pathogens, and as this barrier is often breached, the skin is also accustomed to acting as the centre stage for inflammatory responses. The skin has long been known to be populated abundantly by actors of innate immunity, but skin resident effector memory T cells are only recently being appreciated for their significance in early local recall responses and also in autoimmune diseases (Clark 2010). Experiments with Herpes simplex virus re infection models showed that there are two groups of
antigen-specific T cells rapidly responding – those recruited from the peripherally circulating pool and those already resident in the skin (Gebhardt et al. 2009). More importantly, the peripheral tissue residency of T cells doesn’t seem to depend upon constant exposure to a pathogen (Wakim et al. 2010).

An overview on the functioning of memory in a cutaneous rechallenge with a pathogen can be given. First, the pathogen is encountered by APCs and residing effector memory T cells, which immediately start to resist the invasion, the effector T cells not requiring help from APCs. Peripherally circulating TEM cells are also locally attracted by this inflammatory process, and driven to function through the TCR-mediated stimuli. DCs convey the encounter to the lymph nodes, where they present the antigens they have processed and recruit TCM cells specific for these antigens. TCM cells rapidly proliferate and form new effector T cells that home to the site of the invasion. After the pathogen is successfully defeated, the maintenance of memory cells in the skin and in the lymph nodes is mediated by cytokines such as IL-7 and IL-15. Residual antigen kept by the DCs and presented to the memory cells might somewhat augment their upkeep as well. The signals leading to residential memory cells are unclear, but CD103, a surface molecule that binds E-cadherin produced by epithelial cells, seems to be a common finding in resident T cells, possibly mediating their homeostasis. (Sheridan and Lefrançois 2011)

Nesting of T cells in peripheral tissues is a finding not entirely auspicious in character. Grafting healthy appearing skin from psoriasis patients onto immunodeficient mice led to typical psoriatic lesions (Boyman et al. 2004), suggesting autoreactive T cells residing in non-inflamed skin as the culprit population for disease upkeep. As to the Th lineages that might be involved in residential autoreactivity, accumulation of Th1 and Th17 cells has been implicated in psoriatic skin (Lowes et al. 2008).

4 T CELL DEVELOPMENT AND CENTRAL TOLERANCE

Autoimmunity occurs when the immune system directs its pathogen-associated functions towards the structures of the body. For T cells, two levels are distinguished in its prevention: the central, i.e. intrathymic, and peripheral. As the key features of central and peripheral tolerance will be discussed, it will become apparent that the prevention of autoimmunity is built inside the normal life cycle of T cells in its every vital phase.
4.1 Early stages of T cell development

All lymphocytes derive from haematopoietic stem cells that reside in the bone marrow. T lymphocytes, however, can be considered born only after subsequent developmental phases in the thymus, where the T cell commitment occurs. (Zlotoff and Bhandoola 2011)

4.1.1 Thymus as the site of T cell development

Thymus is a mediastinal organ that is constructed mainly from the endodermal embryonic layer with possible minor ectodermal influence. Thymus is organized into lobules separated by connective tissue, but the key unifying division of structure across the whole of thymus is the distinction of a cortex and a medulla. (Rodewald 2008). Thymic epithelial cells interplay with developing T cells, inside the thymus called thymocytes, in different ways in the cortex and in the medulla, and allow for the sensitive maturation process to follow its path. Cortical thymic epithelial cells (cTECs) are hence quite different from medullary thymic epithelial cells (mTECs). Other cells with vital tasks in this process are the intrathymic DCs and B cells, and macrophages that clear apoptotic thymocytes. (Kyewski and Klein 2006)

T cell precursors enter the thymus at the corticomedullary junction, after which they immediately migrate to the farthest corner, the subcapsular region of the cortex. The cortex is where the majority of thymocytes reside, and their maturation is reflected in their transit towards the medulla (Figure 3). Three important phases are recognized in the development of thymocytes: variable-diversity-joining (V(D)J) recombination, positive selection and negative selection, of which the two first phases take place in the cortex and the last one mostly in the medulla. (Miller 2011). Overall, 95% of T cell precursors die in the thymus, showing the stringency of these selection processes (Kyewski and Klein 2006).
Figure 3. The structure of the thymus and the migratory path of T cell development within (modified from Kyewski and Klein 2006). T cell precursors enter the thymus from the blood vessels of the corticomedullary junction, and begin their migration in the thymus by transferring to the subcapsular region. After this they enter the cortex, where most thymocytes reside and undergo V(D)J recombination and positive selection. The thymocytes interact in the cortex with the cTECs, and apoptotic thymocytes failing to succeed in these developmental phases are cleared out by macrophages. From the cortex thymocytes migrate to the medulla, where the step of negative selection is thought to mostly occur, facilitated by mTECs and DCs and to a lesser degree B cells.

4.1.2 V(D)J recombination

V(D)J recombination is a unique mechanism of nearly unlimited generation of genetic variability into the developing TCRs (Schatz and Ji 2011). This requires the function of a tightly controlled enzyme complex, namely the recombination activating gene (RAG) enzymes 1 and 2 (Matthews and Oettinger 2009). For a full and functional αβ TCR to develop, the thymocyte engages in rearrangement of the β chain that occurs during the double-negative (DN) stage, when neither the CD4 nor the CD8 molecule is expressed on the thymocyte. Rearrangements, facilitated by the RAG enzyme complex, take place between the Dβ and Jβ - segments and thereafter between the Vβ and the D-Jβ. For extra diversity, nucleotides are also added to the junctional regions of the segments. After successful rearrangements and expression into a protein, the β chain associates with a pre-TCR-α chain, which the lymphoid cells developmentally express already before β chain rearrangements (Carrasco et al. 2002), and thereby a pre-TCR is formed. The pre-TCR is needed for further thymocyte survival, and selection based on a proper pre-TCR occurs somewhat parallel to differentiation
to the double-positive (DP) stage, CD4+CD8+. These two processes can however vary in order from cell to cell. At the start of the DP stage there is great proliferation of the cells, and thereafter they are ready to perform α-chain rearrangements between their V and J regions, a process that continues until their TCRs are proven functional in the phase of positive selection, discussed below. (Gascoigne and Palmer 2011, Schatz and Ji 2011)

γδ T cells, the small minority of T cells, can form when γ and δ chain rearrangements have been fruitful but the β-rearrangements unsuccessful. Full γδ T cell lineage commitment, although still rather obscure, is however likely dependent upon additional factors. (Kreslavsky et al. 2010)

### 4.1.3 IL-7 in the thymus

IL-7 is an important cytokine in the upkeep of thymocytes during their development, as can be noted from the expression of CD127 beginning almost immediately after thymic entry. Indeed, thymocytes need IL-7 in the early DN stages to survive. (Hernandez et al. 2010). However, CD127 has to be sharply downregulated when entering the DP stage for normal further development to occur (Fry and Mackall 2005). IL-7 becomes important again in the CD4/CD8 lineage commitment step, as discussed below.

### 4.1.4 Positive selection

A successfully formed DP thymocyte has to survive the positive selection phase. Here cTECs present self-peptide–MHC complexes, and only thymocytes with a responding TCR avoid apoptosis. (Klein et al. 2009). Positive selection is also connected to determination of the final fate between becoming a CD4+ or CD8+ T cell, but in unresolved ways. All DP thymocytes seem to downregulate CD8 expression during positive selection. As to what happens next, one popular model of kinetic signalling proposes that when the TCR binds here to a class II MHC, lengthy interaction is possible and CD4+ fate sealed. However, if the TCR was in a successful interaction with a class I MHC, this downregulation disturbs the intracellular signalling, leading to a different track. (Carpenter and Bosselut 2010). Next cytokines, especially IL-7, are thought to step in, as the disruption in TCR signalling permits the thymocyte to again sense IL-7, a feature found necessary for the full CD8-lineage commitment (Park et al. 2010).

### 4.2 Negative selection

Positive selection demands for a certain extent of autoreactivity of the developing T cells. To protect from autoimmunity, this phase is followed by a
term of negative selection, also known as central tolerance induction. (Carpenter and Bosselut 2010)

Negative selection involves testing the thymocytes for excess affinity to self-peptide–MHC complexes. This is mainly mediated by mTECs and intrathymic DCs, and to a lesser degree by B cells, in the medulla. (Kyewski and Klein 2006). mTECs and DCs differ in their means of access to self-peptides, as DCs can take up blood-borne antigens, and up to 50% of DCs in the thymus have recently been unveiled to be immigrants from peripheral tissues, carrying a wide range of self-peptides (Li et al. 2009). mTECs, on the other hand, present thymic self-peptides, and have the special ability of promiscuous gene expression leading to the generation of hundreds of self-antigens (Derbinski and Kyewski 2010; discussed in more detail in the following chapter 4.3). DCs have also been shown to cross-present these mTEC-derived self-antigens, made accessible by secretion or apoptosis, and this seems to be important especially for the tolerance induction of CD4+ T cells (Gallegos and Bevan 2004).

The precise requirements for negative selection remain still enigmatic. Intrinsic tuning after positive selection might be involved, where thymocytes with high affinity TCRs for self-antigens would become susceptible to apoptosis. More convincingly, migration in the thymus and mirroring alterations in the milieu and the interacting APCs are given emphasis. (Klein et al. 2009). As an example of the importance of thymic journeying, the loss of cortex-to-medulla homing receptor CCR7 has been shown to lead to impaired negative selection (Nitta et al. 2009). Sensitivity to place seems to be a key feature of negative selection, as any major disturbances in the medullary phase of thymocyte development cause, at least in mouse models, drastic systemic autoimmunity (Klein et al. 2009).

4.3 Role of AIRE in central tolerance

A medullary specialty is the promiscuous gene expression in mTECs mentioned in the previous chapter. Although already sketched in Frank Macfarlane Burnet’s clonal selection theory from 1958, the depth of tolerance induction in the thymus came to light in the 1990’s with findings of first insulins and then other peripheral tissue restricted antigens’ broad expression in mTECs (Taniguchi and Anderson 2011). The great interest in the role of peripheral tissue restricted antigen expression in the evasion of autoimmunity rose with the discovery of the Autoimmune regulator (AIRE) gene (Finnish-German APECED Consortium 1997, Nagamine et al. 1997). This was because AIRE was identified in these genetic linkage studies as the sole cause of Autoimmune polyendocrinopathy - candidiasis - ectodermal dystrophy (APECED, also known as autoimmune polyendocrine syndrome type 1), a severe autoimmune disease (discussed in
depth in chapter 6). Subsequently, studies on \( \textit{Aire}^{-/-} \) knockout mice led to the conclusion that \( \textit{Aire} \) widely controls the peripheral tissue restricted antigen expression in mTECs. (Anderson et al. 2002). A new link between autoimmunity and T cell development had emerged.

AIRE contains nuclear localization sequences and structural domains familiar to many common transcription factors (Figure 4), and was quickly denoted as a transcriptional regulator (Peterson et al. 2008). The highest expression of Aire localizes in the mTECs (Zuklys et al. 2000). The microarray based comparison of isolated mTECs from \( \textit{Aire} \) knockout mice and wild-type mice showed that many of the peripheral tissue restricted antigens detected from intact thymus were absent from \( \textit{Aire} \) knockout mice – altogether 200 to 1200 genes were estimated to be activated by \( \textit{Aire} \) and of these the vast majority were those coding peripheral tissue restricted antigens (Anderson et al. 2002). These \( \textit{Aire}^{-/-} \) knockout mice developed tissue-infiltrating lymphocytes and autoantibodies (Anderson et al. 2002). Mechanistic evidence for loss of Aire to result in prevailing autoreactive T cells is founded on transgenic mouse models where TCRs specific for neo-self-antigens, which were driven by Aire-regulated promoters, were not deleted (for example Liston et al. 2003, Su et al. 2008). This condition has however been regarded as rather manipulated compared with endogenous self-antigen regulation (Anderson and Su 2011). It is important to note that Aire's control over promiscuous gene expression doesn't include all peripheral tissue restricted antigens expressed in the mTECs (Derbinski et al. 2005), and that in Aire-deficient mice even those peripheral tissue restricted antigens whose transcription is unaffected by Aire can be targets of autoimmunity (Kuroda et al. 2005). One study even reported AIRE expression in thymic and peripheral blood B cells, and in DP cells in the thymus (Suzuki et al. 2008), however, with an unclear significance.

\[\text{Figure 4. Functional domains of AIRE and position of identified mutations} \text{ (modified from Peterson et al. 2008, Mathis and Benoist 2009). AIRE is a protein of 545 amino acids. It comprises a caspase recruitment domain (CARD)/homogenously staining region (HSR), nuclear localization sequences (NLSs), a SAND domain, four LXXL-motives (L), two plant homeodomain zinc fingers (PHD), and a proline-rich region (PRR). Functionally important mutations in AIRE have been identified to affect the positions marked with a line; the thin lines indicate recessive mutations and the thicker bar the dominant-negative mutation.}\]
Although clearly involved in negative selection, the intracellular basis of AIRE’s function is complex. Analysis of interaction patterns showed that Aire associates with four major intracellular protein classes, namely those of transcription, nuclear transport, chromatin binding and pre-messenger ribonucleic acid (mRNA) processing (Abramson et al. 2010). Such widespread functions give way to proposing that AIRE is not a traditional transcription factor but a delicate sensor of the intracellular milieu (Taniguchi and Anderson 2011). The above-mentioned protein classes link AIRE to processes such as the DNA-damage response (Abramson et al. 2010), and modification of chromatin methylation, which brings about epigenetic flexibility into negative selection (Org et al. 2008). Yet another interesting feature of Aire is its ability to promote apoptosis in mTECs (Gray et al. 2007a), and therefore even feed cross-presentation by DCs (Hubert et al. 2011).

An entirely different prospect of AIRE’s influence on central tolerance is the effect it might have on the development of mTECs, the organogenesis of the thymus and even on the whole early embryo (Matsumoto 2011). In Aire-deficient mouse thymus the morphological alterations, such as the near absence of Hassall’s corpuscles, structures consisting of terminally differentiated mTECs with a function yet to be resolved, and diminished expression of a marker for terminal epidermal differentiation together point towards a role for Aire in mTEC differentiation (Gillard et al. 2007, Yano et al. 2008). Analysis of differentiating mTECs, however, showed that Aire-positivity appears in mTECs as a late-stage post-mitotic phenomenon (Gray et al. 2007a), and therefore Aire’s role in mTEC differentiation is still quite disputed (Mathis and Benoist 2009, Matsumoto 2011). Evidence of Aire affecting the thymic machinery in its entirety is found in the decreased expression of intrathymic migration markers such as CCR7 in thymi of Aire-deficient mice, and in the control Aire has over emigration of mature CD4+ T cells from the thymus (Laan et al. 2009). A developmental anomaly behind autoimmunity caused by AIRE deficiency is an even further claim, based on the transient expression of Aire before the three germ cell layers of the embryo are formed in mice (Nishikawa et al. 2010). The significance of these findings for understanding human AIRE remain unproven (Figure 5).
Figure 5. AIRE’s role in central tolerance. The current postulate of AIRE function in negative selection acknowledges most importantly two mechanisms: the control of the expression of peripheral tissue restricted antigens (marked here as PTA) in mTECs, and feeding cross-presentation of DCs by inducing apoptosis in mTECs. Furthermore, AIRE is involved in facilitating the migration of thymocytes inside the thymus and their emigration to the periphery. AIRE has been implicated in organizing the thymic structure and in mTEC development, and even in the development of the whole early embryo.

4.4 Natural Treg development in the thymus

At the border of central and peripheral tolerance is the population of natural Tregs that is born in the thymus but asserts its suppressive functions in the periphery. The process by which Tregs are born is a disputed one, and many of the developmental phases undergone by T cells in the thymus have been suggested to be the stage of Treg commitment. The characteristic transcriptional regulator of Tregs, FOXP3, is expressed clearly the most in CD4+CD8- cells of the medulla (Fontenot et al. 2005). This is in line with a proposed two-step model, where natural Tregs are generated by deviation from the main developmental path in the step of negative selection. Here, intermediate to high affinity TCR-self-peptide-MHC interaction would serve as the instructor, which would then be followed by a cytokine-driven consolidation. (Lio and Hsieh 2008). More precisely, certain TCR and CD28 activation would cause sensitivity to cytokines such as IL-2, which then were to trigger STAT-5 signalling, leading to expression of FOXP3 (Burchill et al. 2008). TCR-based commitment has gained strong support (Hinterberger et al. 2010). Although IL-2 is viewed as the preferred cytokine in physiological conditions, other common γ-chain cytokines, such as IL-7 or TSLP produced by Hassall’s corpuscles, could possibly compensate for its
absence (Wirnsberger et al. 2011). FOXP3 expression is encountered, however, also at the DN and DP stages, and in the human thymus even before TCR expression in DN cells (Tuovinen et al. 2008). Also the hallmark of the two-step model, selection to Treg lineage based on increased reactivity to self-antigens (Figure 6), has been debated against. Although autoreactive TCRs are relatively enriched in Tregs compared to all CD4+ T cells, their frequency is still low, and there is notable overlap between the TCR repertoires of Tregs and CD4+ T cells. This suggests that enhanced endurance of negative selection could just as well explain the TCR repertoire findings. (Pacholczyk and Kern 2008). Treg commitment remains thus unresolved (Bettini and Vignali 2010).

Supporting again the importance of the step of negative selection in Treg development, Aire+ mTECs have been demonstrated to be involved (Aschenbrenner et al. 2007, Hinterberger et al. 2010). Whether Aire is essential for natural Treg generation is however controversial: several reports conclude that the production of Tregs in Aire-deficient thymus is unaltered (Anderson et al. 2002, Liston et al. 2003, Kuroda et al. 2005, Hubert et al. 2009), while a recent study reported a halved number of Tregs in the thymi of mice lacking Aire (Lei et al. 2011). According to this study, the mechanism could be, perhaps a little surprisingly, altered migration of DCs to the medulla.
Figure 6. The TCR affinity model of thymocyte selection. According to this model, maturing thymocytes in the thymus are selected according to the affinity of their TCR to self-peptide–MHC complexes. In these selections, thymocytes with low affinity TCRs are induced to undergo apoptosis in the step of positive selection, thereby favouring a certain extent of self-reactivity in the thymocytes. High affinity to self-peptide–MHC complexes results in the deletion of the thymocyte in the step of negative selection, or alternatively, this thymocyte is led to become a natural Treg. Thymocytes with intermediate affinity TCRs to self-peptide–MHC complexes are thus preferred in becoming conventional T cells of the immune system.

5 PERIPHERAL TOLERANCE

Despite an orchestra of tolerance inducers in the thymus, a normal mature immune system hosts autoreactive T cell clones in the peripheral circulation (Akirav et al. 2011). The leaky central tolerance is, however, complemented by a heterogeneous group of mechanisms termed the peripheral tolerance.

5.1 Tregs in the periphery

5.1.1 The discovery of Tregs

The existence of Tregs and their role in warding off autoimmunity was first acknowledged in mouse studies, where autoimmune manifestations occurring in thymectomized mice could be prevented by transferring T cells, especially CD4+ T cells (Sakaguchi et al. 1982, Fowell and Mason 1993). Later it was found to be more specifically the CD25+CD4+ T cells that were needed here (Sakaguchi 1995). Another trail of studies identified the dysfunction of FOXP3 to cause the severe
human autoimmune disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; Bennett et al. 2001, Wildin et al. 2001, Chatila et al. 2000), and a similar lymphoproliferative disorder in mice termed Scurfy (Brunkow et al. 2001). When Foxp3 was then shown to be preferentially expressed by CD25⁺CD4⁺ T cells in the periphery and the thymus (Khattri et al. 2003, Fontenot et al. 2003), the population of Tregs became largely recognized in maintaining peripheral tolerance.

5.1.2 FOXP3 and Treg modes of action

FOXP3 is essential for Treg function. Without its continuous expression, the phenotype is lost, and moreover the level of FOXP3 expression correlates with Treg suppressive function in humans (Yagi et al. 2004, Roncador et al. 2005). Although activated non-regulatory T cells can also express FOXP3, its expression in them, unlike in Tregs, occurs on a low level and in a transient manner (Gavin et al. 2006, Tran et al. 2007, Wang et al. 2007). The consequences of FOXP3-mediated transcriptional regulation feature the stable upregulation of CD25 and of the cytotoxic T cell -associated antigen 4 (CTLA-4), the downregulation of CD127, the halt of IL-2, IL-4 and IFN-γ production, and hindered TCR signalling, all of which are related to the Treg phenotype (Sakaguchi et al. 2008).

Tregs can suppress multiple aspects of the immune system: the proliferation and differentiation of naive T cells, and the functions of effector T cells and various members of the innate immunity, most importantly APCs. They achieve this through direct cell-to-cell contacts or through cytokine-mediated effects. (Sakaguchi et al. 2010). A close contact is needed in particular to control APCs, which is for the most part thought to occur through CTLA-4, an inhibitory molecule that binds B7-costimulatory molecules on the APC (Bour-Jordan et al. 2011). Tregs use CTLA-4 to downregulate the expression of B7 molecules and thereby to diminish the APC's potential to activate T cells (Wing et al. 2008). CTLA-4 also upregulates the production of indoleamine 2,3-dioxygenase (IDO) enzyme inside the APC, which can disrupt proliferation and even cause apoptosis of the APC (Bour-Jordan et al. 2011). Tregs might use CTLA-4 also directly on activated effector T cells, which seem to express B7 on their surface, to deliver an inhibitory signal (Paust et al. 2004). Other molecules used in cell-to-cell contact include for example LAG3 (lymphocyte activation gene 3), which uses MHC II molecules to relay inhibition, granzyme A that Tregs can use to lyse T cells, and CD95 to induce apoptosis in T cells (Sakaguchi et al. 2010).

Cytokine-mediated suppression is distinguished by IL-10 and TGF-β secretion that affects both DCs and T cells. Additionally galectin-1 and IL-35 have been recently proposed to mediate suppression, but at least IL-35 was shown not to be involved in human Treg function. (Sakaguchi et al. 2010). IL-10 produced
by Tregs is essential in preventing inflammatory diseases especially on the mucosal surfaces and in the skin (Rubtsov et al. 2008). An interesting mode of action of Tregs is also cytokine-deprivation, best described for IL-2: Because FOXP3 renders Tregs CD25high and unable to produce IL-2 by themselves, they scavenge the IL-2 available in the milieu (Pandiyan et al. 2007). This mechanism indeed restrains the differentiation of effector T cells in vivo (McNally et al. 2011).

Many common human autoimmune diseases are linked to defects in Treg function. Tregs can prevent excessive immune responses, but they unfortunately also foster cancer and promote chronic infections (Sakaguchi et al. 2008). As other positive effects, Tregs have been suggested to enhance viral defence against *Herpes simplex* (Lund et al. 2008) and recruit neutrophils by producing chemokines (Himmel et al. 2011). The full power of Tregs in human biology is clearly still unresolved.

### 5.1.3 Peripheral conversion of Treg cells and Treg homeostasis

Tregs constitute a population of approximately 5-10% of CD4+ T cells in the adult peripheral blood (Sakaguchi et al. 1995). CD8+ T cells appear to contain a minor group of cells with a regulatory phenotype very much similar to the classical Tregs (Cosmi et al. 2003, Siegmund et al. 2009), but further investigations are needed to assure their significance in vivo. Much interest has focused recently on how the population of CD4+ Tregs is maintained through life, especially with the drastic involution of the thymus after puberty being an eminent factor in human biology. The findings of peripheral conversion of naive T cells (O’Shea and Paul 2010) or memory T cells (Akbar et al. 2007) into induced Tregs have arisen as considerable mechanisms of populational replenishment.

Manipulative expression of Foxp3 by a gene transfer into effector T cells reprograms them to become Tregs in mice (Hori et al. 2003). For this conversion into a functional Treg to occur by nature, it is important that the expression of FOXP3 is accomplished in a certain way. The presence of two cytokines, TGF-β and IL-2 are regarded non-redundant for this (Campbell and Koch 2011). An exception lies in the special ecosystem of the gut, where vitamin A converted to retinoic acid together with TGF-β induces naive T cell differentiation into induced Treg – a process very much controlled by an immature subpopulation of DCs called tolerogenic DCs (Coombes et al. 2007, Sun et al. 2007). A similar effect is seen in the skin by the active metabolite of vitamin D (Jeffery et al. 2009). Cytokine-mediated induction alone gives however only a weak suppressor phenotype (Imamichi et al. 2008), and it has become clear that in humans TCR-mediated stimulation is an essential additional prerequisite for full functionality. Interestingly, the transcription factor Foxo1 that is in a key action in the
homeostasis of all naive T cells (discussed in chapter 3.2.2), is together with Foxo3 also an essential intracellular mediator of TGF-β induced Foxp3 expression (Ouyang and Li 2011). After antigen-specific stimulation through the TCR, the Treg is free to function in an antigen-nonspecific manner. (Sakaguchi et al. 2010).

The homeostasis of the formed Treg population in the periphery is strongly influenced by the availability of IL-2, produced by nonregulatory T cells, thereby forming a feedback loop between the frequency of activated effector T cells and Tregs (Sakaguchi et al. 2008). Similar balance exists between the frequency of DCs and Tregs, with a direct correlation: deleting DCs impairs the Treg population and diminishes their Foxp3 expression, and elevating the amount of DCs causes Tregs to proliferate through an MHC II -dependent mechanism (Darrasse-Jeze et al. 2009, Swee et al. 2009, Figure 7).

An interesting relation lies between the differentiation of naive T cells towards Th17 and induced Treg lineages. TGF-β is needed for differentiation to both lineages. The presence of IL-6 however tips the scales in the favour of Th17: IL-6 inhibits TGF-β from inducing Foxp3 expression. (Bettelli et al. 2006, Mangan et al. 2006, Veldhoen et al. 2006). In addition, IL-2 inhibits differentiation towards Th17 (Laurence et al. 2007). A recent investigation brought new depth into the relationship between Treg and Th17, as it proved that the consumption of IL-2 by Tregs is needed for Th17 differentiation to occur (Chen et al. 2011). Tregs use however similar mechanisms to suppress Th17 cells as other effector T cells, for example IL-10 (Chaudhry et al. 2011).

The significance of the phenomenon of peripheral conversion in humans is still an open question. The differences in the life span of mice and humans affect studies on Treg homeostasis. In mice, natural Tregs appear to suffice lifelong, as peripheral conversion accounts for merely 4-7% of the normal adult peripheral Treg population, based on a Foxp3-CD4+ T cell transfer study (Lathrop et al. 2008). The TCR repertoires of thymic and peripheral Tregs overlap to a large extent in mice, which further supports thymus-based maintenance of the population (Hsieh et al. 2006). Lymphopenic conditions however favour peripheral conversion in mice. (Lathrop et al. 2008). In humans, the situation is more complex, and more difficult to address. Overall Treg population size increases with aging (Gregg et al. 2005). However, peripheral Tregs are known to be rapidly dividing, and to lack an active telomerase, and therefore they are prone to apoptosis through proliferative exhaustion (Akbar et al. 2007). Therefore, the dilemma of populational replenishment is clear. The origin of peripheral Tregs has been approached by classifying them into the CD45RA+ naïve population and the CD45RO+ activated population, which shows that while the naïve population represents 75% of Tregs in the cord blood (Wing et al. 2002), over 90% of adult blood Tregs belong to the activated population.
CD31 appears a relevant marker of recent thymic emigrant Tregs, as TREC expression is limited to the CD31+ subpopulation in Tregs, too (Haas et al. 2007). Of all FOXP3+ cells, 7% are RTEs in subjects under 30 years of age, whereas in subjects over 45 years of age only 3% (Haas et al. 2007), and RTEs virtually disappear by the age of 80 (Booth et al. 2010). As expected from these numbers, a recent study showed that rapidly dividing, highly differentiated memory CD4+ cells convert into Tregs in humans, and based on the similarity of the TCR repertoires, this subset of memory T cells is suggested to be the major clonal precursor of peripheral Tregs in adults (Vukmanovic-Stejic et al. 2006). Another study noted that the gene expression patterns between naive and activated Tregs are in humans quite distinct, and that they locate differently so that naive Tregs nest in the bone marrow whereas activated prefer homing to the skin (Booth et al. 2010). Interestingly, the frequency of RTE Tregs correlates with suppressive capacity of the total Tregs, and in MS specifically the RTE Treg frequency is decreased (Haas et al. 2007), indicating that the different Treg subpopulations might have distinct functions.

Non-FOXP3+ Tregs are also induced in the periphery. For example, type 1 regulatory T cells require IL-10 and signals from immature, tolerogenic DCs to develop and to function in their task of IL-10 secretion mainly in the epithelial and mucosal surfaces. Immature DCs are highly appreciated as inducers of regulatory T cells, both the conventional FOXP3+ Tregs and the smaller populations with suppressive functions. (Saurer and Mueller 2009)

**Figure 7. Upkeep of the FOXP3+ Treg population.** The peripheral FOXP3+ Treg population consists of natural Tregs that have emigrated from the thymus, and induced Tregs that have undergone peripheral conversion from naive CD4+ T cells to become activated Tregs. A subset of the natural Tregs is in the periphery in a resting state, thus forming a reservoir, whereas others have been activated. The activated Treg pool is rapidly dividing and prone to apoptosis, and therefore the maintenance of the activated population is essential. Their homeostasis strongly depends on the availability of IL-2 in the milieu, which the activated effector T cells produce. Tregs also depend upon interactions with DCs, which also are primarily responsible, in the presence of the cytokines IL-2 and TGF-β, for peripheral conversion of naive T cells into induced Tregs.
5.2 Additional tolerogenic mechanisms inside the lymph nodes

5.2.1 T cell anergy

T cell activation is, as described in the previous chapters, an important event in the life cycle of a T cell. Hence, it is natural that there are several mechanisms by which TCR engagement is ensured to be purposeful. The basis of these mechanisms lies in the necessity of costimulation for T cell activation, which comes from an activated, mature DC. Immature DCs are inefficient in stimulating naive T cells and this inefficiency is a mechanism of passive tolerance in itself. The absence of costimulation from an activated DC can then lead to the active induction of T cell anergy. This means an extending unresponsiveness of the T cell following TCR stimulation without costimulatory signals. (Fathman and Lineberry 2007)

Most of the mechanisms of T cell anergy induction spark from the absence of CD28 activation. CD28-mediated costimulation is needed for the production of IL-2 (Wells 2009). TCR stimulation alone without CD28-mediated activation also promotes the transcriptional expression of distinct molecules that are designed to hinder TCR signalling; two examples of such molecules are the E3 ubiquitin ligases gene related to anergy in lymphocytes (GRAIL) and the Casitas B-cell lymphoma ubiquitin ligase (Cbl-b; Chappert and Schwartz 2010). GRAIL enhances TCR-CD3 degradation (Nurieva et al. 2010), while Cbl-b mediates the control on proliferation that CD28 signalling has (Paolini et al. 2011).

T cell anergy is not only CD28-related, though. Even with sufficient CD28-costimulation, the event of T cell activation can turn into anergy, when a key metabolic signalling pathway, the mammalian target of rapamycin (mTOR) as its centrepiece molecule, is blocked. This molecule takes stimuli from cell surface sensors of energy and nutrients, as well as from side paths of both the CD28 and CD25 signalling, and, with a sufficient sum effect, turns the metabolic switch into anabolism. (Chappert and Schwartz 2010). Therefore, insufficient energy conditions, for example, can induce anergy even with sufficient TCR, CD28 and IL-2 engagement (Zheng et al. 2009). Activation of mTOR can also promote the degradation of GRAIL, a feature hinting that mTOR could even be the master regulator of anergy (Lin et al. 2009).

Anergy of a T cell upon antigen stimulation can also be introduced at the TCR level by a cell-surface molecule called CD5. This pan-T-cell marker inhibits TCR signalling through yet unresolved mechanisms that raise the threshold of TCR-CD3 engagement. Subsequently, elevated levels of CD5 seem to protect from autoreactivity. (Dalloul 2009)
5.2.2 Clonal deletion

The actions and passive effects of DCs are at the heart of peripheral tolerance at many levels: the peripheral induction of Tregs, especially in the gut, or induction of T cell anergy by refraining from costimulatory actions. The most important active form of DC-mediated tolerance is, however, clonal deletion taking place in the secondary lymphoid organs, especially the lymph nodes. Clonal deletion is an event where naive T cell is activated, prompted to proliferate, but then in the phase of clonal expansion is signalled to undergo apoptosis. (Lukacs-Kornek and Turley 2011). What exactly causes a DC to trigger deletion is obscure. The mechanism of DC cross-presentation, referring here to the sampling of antigens from nearby tissues and processing them for presentation to T cells, has been implicated, as CD8+ T cells readily undergo clonal deletion if they recognize self-antigens in this setting (Luckashenak et al. 2008).

The reports on whether absence of DCs causes T cell -mediated autoimmunity are however conflicting. Constitutive deletion of DCs causes defects in antiviral responses, but as this murine study reported no T cell-related autoimmune phenomena (Birnberg et al. 2008), another depicted a Th1 and Th17 -based overactivation and an inflammatory bowel disease – without a Treg or CD8+ T cell disturbance (Ohnmacht et al. 2009).

5.3 The role of T cell homeostasis in peripheral tolerance

Immunodeficiency and autoimmunity connect with each other in primary immunodeficiencies such as the Omenn’s syndrome, where lymphopenia-driven proliferation is perceived to drive autoimmune manifestations (Waterfield and Anderson 2011). Such diseases encourage the search of common defective mechanisms in the upkeep of the T cell population behind autoimmunity and immunodeficiency. One signalling pathway suggested to be important for both homeostasis of T cells as well as prevention of autoimmunity is the one controlled by the protein kinase Janus kinase 3 (Jak3). It responds to signalling through the common γ chain receptor, first activated by for example IL-7 or IL-2. Mutations of Jak3 cause in humans a severe combined immunodeficiency, where T cells are absent. In mice with this deficiency, T cell numbers are only reduced, and a T cell -dependent autoimmune disease emerges. (Pesu et al. 2008). It remains to be speculated, whether less severe alterations in Jak3 signalling might thus promote autoimmunity also in humans.

At the extracellular level, T cell homing guides T cells to proper sites, at the same time limiting unwarranted contacts. If the physical boundaries, such as the blood-brain barrier, are broken, the possibility for improper T cell function arises (Correale and Villa 2007). In steady-state conditions, a naive T cell cannot enter tissues but only secondary lymphoid organs, and this controls the
activation. The knockout of Ccr7 causes systemic autoimmunity in the form of autoantibodies and multiorgan T cell infiltrates (Davalos-Misslitz et al. 2007), which could be due to defects in Treg homing and function in lymph nodes (Schneider et al. 2007), defective negative selection in the thymus (Nitta et al. 2009) and also damaged DC homing to lymph nodes (Ohl et al. 2004). CCR7 appears to have a role in the formation of organized tertiary lymphoid structures at sites of inflammation ( Förster et al. 2008), a phenomenon commonly seen, for example, in lesions of autoimmune thyroiditis or sialadenitis of Sjögren’s syndrome, and the tertiary structures are thought to maintain the chronic autoimmune responses (Aloisi and Pujol-Borrell 2006). Ccr7−/− mice also experience delayed responses of the adaptive immunity against viral infections, although the defence system seems to be able to by-and-large circumvent its absence ( Förster et al. 2008).

Another example of a molecule implicated both in autoimmunity and defective host defence is IL-22, a member of the IL-10 family of cytokines but with unique functions. At the epithelium, IL-22 is important in maintaining normal barrier homeostasis, and attracting both proper immune responses against pathogens and inducing tolerance. The effects of IL-22 appear context-dependent, as for example the presence of IL-17 is needed to elicit inflammatory responses. (Sonnenberg et al. 2011). Interestingly, TGF-β, vital for Th17 differentiation, suppresses IL-22 production. IL-22, mediating the cross-talk between leukocytes and the epithelium, has been shown important for responses against various extracellular bacteria by increasing the production of antimicrobial agents. IL-22 is however found overexpressed in many autoimmune diseases such as psoriasis or inflammatory bowel diseases. (Witte et al. 2010). These examples demonstrate the multi-faceted functions of immunological molecules, and the importance of their right regulation.

### 5.4 Role of AIRE in peripheral tolerance

It was clear from the beginning that Aire is transcribed also outside the thymus, on a lower scale (Anderson et al. 2002). Although at the protein level it was at first difficult to prove, the presence of AIRE protein in mouse and human lymph nodes has become acknowledged (Metzger and Anderson 2011). The initial studies on peripheral Aire were focused on DCs, because peripheral blood monocytes and DCs had AIRE transcripts (Kogawa et al. 2002), and were therefore regarded the major peripheral population even in lymph nodes (Taniguchi and Anderson 2011). Aire−/− DCs were reported to activate naive T cells in a more efficient manner, and the amount of DCs in the lymph nodes and spleen and of monocytes in both patient and mice blood was elevated in the absence of AIRE (Ramsey et al. 2006). Furthermore, DC responses to pathogens
appeared lowered at the transcriptional level in APECED patients (Pöntynen et al. 2008).

This was however followed by a study where a special group of extrathymic Aire-expressing cells in the secondary lymphoid organs of mice were identified (Gardner et al. 2008). Extrathymic Aire-expressing cells were void of classical DC markers, but had the ability to mediate deletional tolerance. These stromal cells differed from mTECs in that they had no costimulatory molecules, but similar to mTECs had high levels of MHC II, indicating a capability to interact with CD4+ T cells as well. Further comparison of extrathymic Aire-expressing cell -fractions from Aire−/− mice and normal mice using a microarray revealed that approximately 160 genes were dependent on Aire in these cells. The majority of the genes were peripheral tissue restricted antigens, and separate from the peripheral tissue restricted antigens that Aire controls in the thymus. (Gardner et al. 2008). These findings gave Aire a new, thymus-complementing peripheral function that might possibly adapt to infection or inflammation (Tanguchi and Anderson 2011). This is especially interesting because of new knowledge on the lymph node stromal cells and their ability to mediate deletional tolerance. It appears that many cell populations in the secondary lymphoid organs have the ability to express peripheral tissue restricted antigens (Lee et al. 2007). Another transcription factor called Deaf-1 (deformed epidermal autoregulatory factor-1) is an Aire analog in many of these cell populations (Yip et al. 2009). Whether extrathymic Aire-expressing cells have a role in the peripheral control of Tregs has not been properly explored, but mechanistic qualifications for this exist (Roozendaal and Mebius 2011).

The peripheral expression of AIRE in humans appears however a little different from mice (Poliani et al. 2010). Secondary lymphoid tissues, except the spleen, contain peripheral tissue restricted antigen -expressing AIRE+ cells that have a DC phenotype, and interestingly produce IL-10 and IDO, suggesting tolerogenicity. They appear only after birth, and are more frequent in abdominal than in superficial lymph nodes. The extrathymic human AIRE+ population increases in size with aging, but not to a statistically significant extent. (Poliani et al. 2010). This temporal regulation however has sparked others to suggest that peripheral AIRE might represent the means of meeting the challenges of age-related thymic involution (Metzger and Anderson 2011).

Tregs in Aire−/− mice form a normal population in appearance and function (Liston et al. 2003, Kuroda et al. 2005, Anderson et al. 2005), although one recent study did find the intrathymic frequency of Tregs lowered (Aricha et al. 2011). Human AIRE deficiency is however associated with severe Treg defects (Kekäläinen et al. 2007). The expression of FOXP3 mRNA and protein was twofold higher in total peripheral blood mononuclear cells (PBMC) of control subjects and four times higher in the subset of CD25high cells. In vitro functional
assays using polyclonal stimulation of nonregulatory T cells by anti-CD3 or phytohemagglutinin, and thereafter measuring the decrease in proliferation when co-cultured with Tregs, showed an impaired suppressive ability. The TCR repertoires of Tregs were significantly less skewed from the naive repertoire of cord blood samples than those of the controls, indicating impaired TCR activation. (Kekäläinen et al. 2007). Another study had already reported decreased frequency of CD4+CD25+ Tregs (Ryan et al. 2005); however this finding did not repeat itself in the latter study (Kekäläinen et al. 2007). It has been shown in mice that Aire and Foxp3 mutations have a synergistic effect, a combination of the two leading to an accelerated disease progression compared to only either one (Chen et al. 2005). Tregs are active in the lymphocyte infiltrates in Aire deficient mice (Kekäläinen et al. 2007), probably involved in keeping the disease phenotype mild. Treg function has therefore clearly an impact on the manifestation of Aire deficiency (Figure 8).

An interesting study sought to determine which class of peripheral tolerance would need to fail in order for Aire deficiency to lead to rapid autoimmune manifestations (Teh et al. 2010). Crossing mice into different backgrounds showed that when, for example, deleting the Fas ligand or decreasing the number of Tregs gave no serious sequelae, defects in anergy and requirement for CD28 costimulation (Cbl-b−/−) in combination with Aire deficiency resulted in dire autoimmune tissue destruction. Furthermore, T cells from these mice were sufficient to transfer the outcome onto another mouse. (Teh et al. 2010). This would suggest that lack of costimulatory signals is needed to keep the autoreactive T cell clones in check, although it is difficult to draw conclusions of how physiological these findings are.
Figure 8. Current understanding on AIRE’s influence on peripheral tolerance. AIRE is expressed in the extrathyMIC Aire-expressing cells (here marked eTAC) of the lymph nodes, where AIRE is suggested to be important for the clonal deletion of T cells. DCs express AIRE, and thereby AIRE can have a role also in DC-mediated deletional tolerance. DCs are affected by AIRE deficiency, namely their frequency and functional capacity in anti-pathogen responses become diminished, and this can indicate a larger effect of AIRE on DC-mediated peripheral tolerance. AIRE is involved in the development of Tregs in the thymus, and the peripheral Treg activation and functional capacity have been suggested to be influenced by AIRE, possibly through AIRE expression in the extrathyMIC Aire-expressing cells and DCs.

6 APECED

APECED is the disease that brings AIRE to medicine. First connected to an autoimmune background by the detection of autoantibodies in the patients in 1963 (Blizzard and Kyle 1963), APECED has since then kept revealing new insights into central and peripheral tolerance.

6.1 Genetic background: mutations of AIRE

Homozygous or compound heterozygous loss-of-function mutations of AIRE generally cause APECED (Akirav et al. 2011). What is striking about this rare autosomal recessive disease is the variety of possible phenotypes, all arising from mutations in one gene.

AIRE is located in the human chromosome 21q22.3. More than 60 mutations have been identified, of which the mutations R257X in exon 6 (also known as c.769C > T) and a 13-base pair deletion in exon 8 (c.967-979del) are the most common ones. (Björses et al. 2000, Wolff et al. 2007). The mutations in APECED occur throughout the coding region of AIRE and include nonsense mutations causing premature stop codons, frameshifts caused by deletions, and missense mutations (Akirav et al. 2011).
APECED shows populational enrichment: the Iranian Jews (lifetime prevalence 1: 9 000) Sardinians (1: 14 500) and the Finns (1: 25 000) are especially affected. Patients are however diagnosed all over the world, and Norway and Northern Italy are amongst the more frequent locations. (Betterle et al. 1998). A founder effect in the Finns and the Iranian Jews is suspected, with just one or two individuals thought to have carried the original mutations in these isolated populations (Akirav et al. 2011).

APECED belongs to the Finnish disease heritage, and has been intensively investigated in the Finns. Approximately 90 Finnish patients have the diagnosis, and 77% of the patients tested are homozygous and 17% compound heterozygous for the R257X (Perheentupa 2006). In fact, this mutation accounts for the majority of cases in Italy, Norway, Great Britain and the USA (Husebye et al. 2009). Heterozygous carriers of this mutation are present at the frequency of 1: 250 in the general Finnish population (Finnish-German APECED Consortium 1997). Heterozygosity does not cause APECED, although one Italian family forms an exception to this: a G228W mutation acts in a dominant negative way to cause a phenotype of APECED that fulfils the diagnostic criteria (Getani et al. 2001). Also in a mouse model this mutation caused spontaneous autoimmunity distinct from the Aire knockout mice (Su et al. 2008).

All the Iranian Jews with APECED carry their own unique mutation, Y85C (c.374A>G), which is thought to influence the phenotype as Iranian Jews do not develop candidiasis or keratopathy (Zlotogora and Shapiro 1992). Also, the mutation R257X correlates with a higher prevalence of candidiasis than with other mutations (Halonen et al. 2002). Apart from these findings, the mutations of AIRE do not seem to have a strong correlation with the disease phenotype (Halonen et al. 2002). Other factors, such as disease-modifying genes or environmental factors together with possible stochastic elements such as the random generation of the TCR repertoire, could determine the phenotype of an individual APECED patient (Mathis and Benoist 2009). Indeed, the phenotype varies greatly from individual to individual, even between siblings (Perheentupa 2006). Certain HLA class II alleles predispose to individual disease components (alopecia, Addison’s disease; protective allele for type 1 diabetes) in the same manner as they do in non-APECED patients (Halonen et al. 2002). Polymorphisms of the HLA are a common predilect to autoimmunity in humans (Wiebolt et al. 2010), but in APECED these are insufficient to explain the phenotypic variation as a whole (Perheentupa 2006). The occurrence of APECED shows also no gender-bias, although the disease component hypoparathyroidism is more common in women (Gylling et al. 2003).
6.2 Clinical features of APECED

The classical triad of disease components in APECED is chronic mucocutaneous candidiasis (CMC), hypoparathyroidism and Addison's disease, of which two are required for the diagnosis (Betterle et al. 1998). The first patient was depicted in the literature with APECED-like symptoms already in 1929 (Puel et al. 2010), and a patient with the triad of symptoms in 1946 (Leonard 1946). APECED is classified as a primary immunodeficiency, in the subclass of diseases of immune dysregulation (Mathis and Benoist 2009). The autoimmunity in APECED is organ-specific, but with many components (Table 2) – five is the median in one patient but even ten have been reported. First symptoms occur at the age of 0.2-18 years (median 3.3 years). New tissue-specific disease components appear through life, and their prevalence increases at least until the age of 60. (Perheentupa 2006).

6.2.1 Disease components and autoantibodies

A relevant feature of APECED is the existence of high titre IgG autoantibodies, most of them against various targets of the disease process and preceding the onset of target organ failure. Autoantibodies can however be present in the patients even without the target organ failure. (Betterle et al. 1998)

In addition to the most common endocrinopathies, hypoparathyroidism (usually at the age of 3-5 years) and Addison's disease (at 11-15 years), the patients develop often later on also gonadal failure, autoimmune hepatitis, and some also hypothyroidism or type 1 diabetes (Table 2). Proteins of these organs, many of which are intracellular key enzymes, are targeted by autoantibodies: for example NALP5 (NLR family, pyrin domain containing 5) in hypoparathyroidism, 21-hydroxylase in Addison's disease, insulin in type 1 diabetes or side-chain cleavage enzyme in gonadal failure. The presence of autoantibodies correlates with the disease component. Some of these autoantibodies are specific for APECED, and others are present also in non-APECED patients. (Husebye et al. 2009). AIRE is not expressed in these typically affected organs of APECED autoimmunity (Fierabracci 2011), with one exception: the testes and ovaries. Even there mutated AIRE has not been implicated as a target of autoimmunity, but has been suggested to function for the germ-line stability especially in spermatogonia and spermocytes (Schaller et al. 2008). Ovarian insufficiency is the most common additional endocrinopathy besides the diagnostic triad in APECED, reaching approximately a prevalence of 65% in women and starting at early adulthood, whereas testicular failure has a maximum prevalence of 25% in the men and starts usually at an older age. Occurrence of gonadal failure is connected to Addison's disease. (Husebye et al. 2009)
CMC is usually the first sign of APECED, and reaches a prevalence of 100% in the Finnish patients. Chronic *Candida albicans* infection can occur in the oral cavity, the oesophagus, the vagina or the nails. (Perheentupa 2006). CMC has gained much attention recently, because of a correlation between autoantibodies against Th17 class cytokines and CMC, both in patients with APECED and in those without it (Puel et al. 2010, Kisand et al. 2010). CMC is an interesting disease component also because inherited CMC syndromes associate with autoimmune endocrinopathies, and such patients rarely develop systemic *Candida* infections, as opposed to patients with immunosuppressive states. This suggests a special connection between autoimmune endocrinopathies and the environment in the skin and mucosal surfaces. (Eyerich et al. 2010). T cells are crucial in the defence against CMC, and Th17 cytokines especially important, since inborn errors in them cause CMC (Puel et al. 2011). The autoantibodies found were against IL-22 (in 91% of patients), IL-17F (75%) and IL-17A (41%), their amount correlated with the presence of CMC, and they were shown to neutralize the cytokines in question (Puel et al. 2010, Kisand et al. 2010). Two recent studies also found that STAT-1 mutations are a cause of autosomal dominant chronic mucocutaneous candidiasis, further emphasizing the connection to IL-17 signalling (van de Veerdonk et al. 2011, Liu et al. 2011).

Ectodermal manifestations include enamel hypoplasia of the teeth, alopecia, nail dystrophies, vitiligo, and numerous eye diseases. Of the eye diseases, the most common is ocular keratopathy that occurs in 25% of the patients and creates a risk for vision loss. Vitiligo, alopecia and keratopathy have been regarded as autoimmune manifestations. (Husebye et al. 2009). A recent study identified AIRE in epidermal and follicular keratinocytes, where it associated with the cytoplasmic filament network, suggesting that structural defects here might be behind the ectodermal abnormalities (Kumar et al. 2011). However, even enamel hypoplasia in the patients has been argued to reflect chronic infections, and thereby the existence of ectodermal dystrophy in APECED has been altogether questioned (Collins et al. 2006).

One group of autoantibodies stands out however from the rest: the autoantibodies against type 1 IFNs. Their combined prevalence is a fascinating 100% in the patients, and so far they are unconnected to any of the disease components of APECED in non-APECED patients. Even more importantly, their appearance precedes all the disease manifestations of APECED, thus implying a causative role in organ-specific autoimmunity. (Meager et al. 2006). Together with the antibodies against Th17-cytokines, the anti-IFN antibodies form an interesting chapter on APECED autoimmunity, as they evidently indicate autoimmune reactivity against the immune system, in addition to the endocrine target organs that are more familiar from other autoimmune diseases (Meager et al. 2008). Type 1 IFNs are associated with human autoimmune diseases as such,
for example systemic lupus erythematosus, where their serum levels are increased (Pascual et al. 2006). Of some interest is that mTECs are a major source of IFN-β in noninflammatory conditions, and its expression in them is Aire-dependent (Lienenklaus et al. 2009). However, APECED patients are not susceptible to viral infections, which would be expected from alterations in the function of type 1 IFNs (Akirav et al. 2011).

In accordance with the interest in understanding the reason for the autoantibodies, the research focus on T cells in APECED has been on the side of the CD4+ cells. This stems also from the report that the autoreactivity would lie in the CD4+ population instead of the CD8+ T cells: their depletion from an Aire−/− mouse significantly reduced the autoimmune findings, and especially Th1 cells were identified as the ones infiltrating tissues (Devoss et al. 2008). In humans, however, the Th1 population in the peripheral blood appears normal (Ahlgren et al. 2011). Few and conflicting studies have been published on the T cell profile of APECED patients, with the most recent and largest stating reduced numbers of the memory CD4+ cells exhibiting homing markers for entry into epithelial surfaces and sites of inflammation (Wolff et al. 2010). Much is therefore yet to be learned about T cells in APECED.
Table 2. Most common disease components of APECED and autoantibodies connected to them. Disease components and their prevalence are based on a series of 91 Finnish patients between 30 and 40 years of age (Perheentupa 2006), except enamel hypoplasia, which had been studied separately in 43 Finnish patients and reported to have an onset before the age 7 (Ahonen et al. 1990). Autoantigens associated with the disease components are based on studies on autoantibodies in the patients, and are listed here as reviewed in Akirav et al. 2011 and Husebye et al. 2009.

<table>
<thead>
<tr>
<th>Disease component</th>
<th>Prevalence (% of patients)</th>
<th>Autoantigen associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>98</td>
<td>IL-17A, IL-17F, IL-22</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>85</td>
<td>NALP5</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>78</td>
<td>Steroid 17α- and 21-hydroxylase, side-chain cleavage enzyme</td>
</tr>
<tr>
<td>Enamel hypoplasia</td>
<td>77</td>
<td>-</td>
</tr>
<tr>
<td>Gonadal failure</td>
<td>Ovarian 60, testicular 12</td>
<td>Side-chain cleavage enzyme</td>
</tr>
<tr>
<td>Alopecia</td>
<td>39</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Vitiligo</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Intestinal malabsorption</td>
<td>Chronic diarrhea 22, obstipation 21</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>Keratoconjunctivitis/ keratopathy</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Autoimmune gastritis</td>
<td>20</td>
<td>Intrinsic factor, parietal cells</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>18</td>
<td>Cytochrome P450 1A2, aromatic L-amino-acid decarboxylase</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>14</td>
<td>Thyroglobulin, thyroperoxidase</td>
</tr>
<tr>
<td>Rash with fever</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>13</td>
<td>Insulin, islet antigen 2, glutamate decarboxylase</td>
</tr>
</tbody>
</table>

6.2.2 Treatment

No curative treatment for APECED exists. The symptoms of endocrinopathies are managed with hormone and electrolyte replacements, for example calcium and vitamin D in hypoparathyroidism, hydrocortisone and fludrocortisone acetate in Addison’s disease and insulin in type 1 diabetes mellitus. To prevent CMC from
provoking squamous cell carcinoma, the need for aggressive treatment with antifungal agents early on is emphasized. (Husebye et al. 2009). The use of immunosuppressants, for example cyclosporine, methotrexate or cyclophosphamide, in treating especially severe manifestations has been reported successful, and is to be considered in manifestations that do not respond to conventional strategies (Akirav et al. 2011).

6.3 Features as a model for human autoimmunity

Autoimmune diseases have a combined prevalence of approximately 5% in a Western population (Jacobson et al. 1997). Despite intensive research, the understanding of them remains insufficient (Davidson and Diamond 2001). The value of APECED in studying human autoimmunity will be assessed next.

6.3.1 Common autoimmune diseases

All autoimmune diseases are considered antigen-specific. The disease manifestations however allow for a classification into organ-specific or systemic autoimmune diseases. Typical examples of common organ-specific diseases are MS (prevalence 0.1% in the USA) or type 1 diabetes (incidence in Finland 64 per 10^5 in the 0-14 years age group in 2005, the highest in the world; Borchers et al. 2010), where mainly one organ serves as the target. (Marrack et al. 2001). Endocrine organs are for some reason a favoured target of autoimmunity (Wiebolt et al. 2010). Some organ-specific autoimmune diseases are noted as non-destructive, for example myasthenia gravis, where autoimmune attack against a cellular target, here the acetylcholine receptor, causes no tissue destruction but has harmful effects on the function of the body. In systemic autoimmune diseases, such as systemic lupus erythematosus, the attack is targeted against antigens expressed broadly in the body. (Marrack et al. 2001)

The genetics of common autoimmune diseases is complex. The overall effect of genetics can be estimated by the concordance rate for monozygotic twins, which is for example in type 1 diabetes maximally 50% (Borchers et al. 2010). The cause of this susceptibility is however multifaceted. For individual common autoimmune diseases, the highest genetic susceptibility comes from HLA allele polymorphisms (Marrack et al. 2001), and for type 1 diabetes there are several risk and protective alleles identified. Polymorphisms in other genes, the CTLA-4 for example, are associated with a risk for autoimmunity, but for individual polymorphisms the risk is low. Disease clustering is, however, an eminent factor in autoimmunity, so that family members are affected by separate autoimmune diseases but their overall occurrence in the family is higher than in
the general population. Environmental factors are given considerable emphasis as the triggers of autoimmunity. (Davidson and Diamond 2001)

6.3.2 Challenges with autoimmune mouse models

The disease burden from autoimmunity has prompted much investigation, a great deal of it with mouse models. Mouse models have provided plenty of new knowledge, but for example in type 1 diabetes or MS, the full human disease has remained irreproducible and models inefficient in finding effective treatments for the patients (Driver et al. 2011, Pachner 2011). The human and murine immune systems have many differences, and the limitations to extrapolating findings from mice to humans have to be taken into account (Mestas and Hughes 2004).

In APECED, the Aire−/− mouse model develops tissue infiltrates of lymphocytes and autoantibodies, T cells are overactivated and the TCR repertoire is altered (Anderson et al. 2002, Ramsey et al. 2002, Kuroda et al. 2005). However, without crossing onto another severe genetic background, such as the autoimmune-prone nonobese diabetic mouse, the clinical findings in Aire−/− mice remain mild, with the exception of infertility, and the targets of autoreactivity show differences (Jiang et al. 2005). In light of the recent findings of CMC and Th17 alterations, the Aire−/− mouse model is a poor subject for studies on the skin and the mucosal surfaces, as the mice generally seem not to have a susceptibility for Candida (Hubert et al. 2009), although one recent study debates this (Ahlgren et al. 2011). Research on human patients with APECED is therefore valued (Peterson et al. 2008).

6.3.3 Other monogenic autoimmune diseases

Besides APECED, only a few monogenic autoimmune diseases are known. Of these the most notable ones are IPEX (see chapter 5.1.1) and autoimmune lymphoproliferative syndrome, which have helped in understanding the fundamentals of the immune system (Bussone and Mouthon 2009). In IPEX, the FOXP3 gene is mutated and inherited in an X-chromosome linked, recessive way. The major disease components include diarrhoea, type 1 diabetes, thyroiditis and eczema. Autoimmune lymphoproliferative syndrome, on the other hand, is caused by mutations in the genes of the Fas-mediated pathway of apoptosis, most notably in the Fas or Fas ligand, and the disease is inherited in an autosomal dominant fashion. Autoimmune lymphoproliferative syndrome patients experience lymphadenopathy, splenomegaly and autoantibody-mediated cytopenias, and have a 10-20% risk for a malignancy, usually lymphoma. The autoimmunity in autoimmune lymphoproliferative syndrome is
postulated to rise from defective homeostasis of the lymphocyte populations, as Fas-mediated apoptosis is an essential mechanism for this. (Waterfield and Anderson 2010)

### 6.3.4 AIRE in more common autoimmune diseases

AIRE’s role in more common autoimmune diseases has been studied to some extent. Heterozygous carriers of loss-of-function mutations in AIRE, mostly relatives of APECED patients, have been studied in rather small cohorts. One study reported high levels of IgA antibodies, active T cells and some autoantibodies in the parents of four APECED patients, suggesting subclinical autoimmunity in the heterozygotes (Sediva et al. 2002). None of the studies found diseases of the diagnostic triad of APECED from the heterozygotes (Sediva et al. 2002, Cervato et al. 2009, Capalbo et al. 2011), except for the autosomal dominant mutation described before that had also the special feature of high prevalence autoimmune thyroiditis accompanying the disease (Cetani et al. 2001). Two patients with endocrine autoimmune diseases and heterozygous mutations of AIRE were recently reported, but also healthy controls carried the mutations and therefore the role of the mutations in the pathogenesis was disputable (Cervato et al. 2010, Toth et al. 2010). The possibility of heterozygous effects in common autoimmune diseases is still however attractive (Fierabracci 2011). Polymorphisms of functional AIRE have not so far been connected in association studies to susceptibility to individual disease components such as Addison’s disease, type 1 diabetes or autoimmune thyroiditis; however the results on vitiligo and alopecia were conflicting (Mathis and Benoist 2009).

Of note is that many common autoimmune diseases, such as the organ-specific MS, are missing from the wide spectrum of disease components depicted in APECED. Alterations in thymic function have however been connected to MS (Haas et al. 2007) and many other common autoimmune diseases such as type 1 diabetes. Populational studies have shown that alleles leading to high expression of insulin in the thymus associate with protection from type 1 diabetes. (Vafadiis et al. 1997, Pugliese et al. 1997). AIRE and the IFN-signalling pathway together have been shown to control the expression of a myasthenia gravis autoantigen, the CHRNA1, in the thymus (Giraud et al. 2007). Furthermore, even Down syndrome seems to associate with a defect of the thymus and a decreased expression of AIRE within (Lima et al. 2011).

It can be thus stated that with AIRE polymorphisms and heterozygosity for loss-of-function mutations conferring possibly some risk for common autoimmune diseases, the significance of APECED as a model for human autoimmunity rises most importantly from the knowledge it can bring on the mechanisms of autoimmunity.
AIMS OF THE STUDY

The main aim of this study was to discover mechanisms of autoimmunity in APECED, a human model disease. Specific aims were to understand

I  populational factors affecting the defect of regulatory T cells in APECED

II  CD8+ T cell function in APECED

III  the *in vivo* memory response of CD4+ T cells in the skin of APECED patients
SUMMARY OF MATERIALS AND METHODS

Detailed description of the methods used in the study is provided in the original publications I-III.

1 SAMPLES

1.1 APECED patients (I-III)

The study included 13 APECED patients (eight were women), with a mean age of 40.9 years (range 22 to 64). Their diagnosis had been verified by sequencing the AIRE gene as described (Halonen et al. 2002). Twelve of the patients were homozygotes for the Finn-major mutation R257X, and one patient had R257X mutation accompanied by 1085/1097 deletion. At the time of the sampling, the patients had no ongoing treatment with systemic immunosuppressive medications, had no infections, and none were pregnant. The most common disease components of the patients studied are given in Table 3.

Table 3. Most common disease components of the APECED patients studied.

<table>
<thead>
<tr>
<th>Disease component</th>
<th>Prevalence (number of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucocutaneous candidiasis</td>
<td>13/13</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>11/13</td>
</tr>
<tr>
<td>Hypoparathyroidism</td>
<td>10/13</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>4/13</td>
</tr>
<tr>
<td>Ovarian atrophy</td>
<td>4/13</td>
</tr>
<tr>
<td>Severe chronic constipation</td>
<td>4/13</td>
</tr>
</tbody>
</table>

Venous blood samples were drawn into EDTA Vacutainer tubes (BD Biosciences, San Jose, CA) and plasma separated by centrifugation. For the isolation of PBMC, Ficoll-Paque (GE Lifesciences, Uppsala, Sweden) gradient centrifugation was applied. Cryopreservation and thawing of PBMC, when not analyzed fresh, was performed using the CTL CryoABC-Kit (Cellular Technology, Cleveland, OH).
1.2 Healthy subjects (I-III)

Healthy adult volunteers were recruited to match the patients by age and sex. Their mean age was 34.5 years (range 23 to 56), and 8 were women. Venous blood samples were drawn and PBMC isolated as described above. For studies on thymic tissue and pediatric blood, samples were obtained from 10 otherwise healthy children undergoing corrective cardiac surgery. Their mean age was 3.5 years (range 1 day to 10.4 years, 4 were female). Thymic tissue was mechanically homogenized to release the thymocytes, and analyzed the same day.

1.3 Mantoux testing and blister induction (III)

To elicit a local memory response of the skin, Mantoux testing, i.e. intradermally injecting clinical grade tuberculin purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark), was selected as the approach. This setting was used recently to describe the kinetics of Treg accumulation (Vukmanovic-Stejic et al. 2008). Injections were given on the skin of the lower abdomen, and clinical responses in the form of induration and erythema measured 48 hours later. To gain access to the cells infiltrating the skin, suction blisters were raised over the reactions, as well as on unexposed skin, by a method that separates the epidermis from the dermis (Kiistala and Mustakallio 1964). A clinical suction pump (Itkavec WS-85, Instrumentarium Itka, Lahti, Finland) and Dermovac suction cups (Ventipress, Lappeenranta, Finland) produced a negative pressure that was applied on the skin until the blisters were visible (30-90 min).

Blister fluid was harvested 23 hours later. This period of maturation was necessary due to our preliminary testing showing low cell count if the sampling was done straight after the blister induction. An earlier report had described the peak of T cell retrieval on day 7 after the injections, whereas the clinical reaction reached its height at day 3 (Reed et al. 2004). Our testing however indicated decreasing cell count after 3 days from the injections, and we decided to retrieve the cells on day 4 of the experiment. The blister fluid was microcentrifuged at 600 g for 5 minutes to pellet the cellular contents. Cell numbers were estimated by microscopic Bürker chamber count, and viability assessed by trypan blue exclusion. With every sample, one-third of the cells were lysed with TRI Reagent (Ambion, Austin, TX) and stored at -70°C, with two-thirds analyzed immediately by flow cytometry.
2 FLOW CYTOMETRIC ANALYSIS (I-III)

Flow cytometric analyses were designed to allow for simultaneous staining of up to ten different cell surface and intracellular markers. Directly conjugated fluorescent monoclonal antibodies (mAbs) used in the different experiments of the study were: CD4-APC-Cy5, CD5-PerCP-Cy5.5, CD45RO-APC, CD45RA-APC, CCR7-PerCy7, perforin-FITC (all from BD Biosciences), CD4-Alexa Fluor 700, CD8-PacificBlue, CD25-PE-Cy7, CD62L-APC-Cy7, and CD127-APC-Cy7 (eBioscience, San Diego,CA). CD45RO-biotin (BioLegend, San Diego, CA), CD31-biotin (BD Biosciences) or CD8-biotin (ImmunoTools, Freisoythe, Germany) was used with the enzymatically conjugating antibody PE-TexasRed-Streptavidin (BD Biosciences). Anti-CD28 (ImmunoTools) together with PE-labeled goat-antimouse Ig (BD Biosciences) was another primary-secondary antibody complex that, when used, was added before any other mAbs.

The cells were permeabilized with the FOXP3 permeabilization and fixation kit (eBioscience), after which staining with FOXP3-PE (eBioscience), Ki-67-FITC (BD Biosciences) or Ki67-PE (Santa Cruz Biotechnology, Santa Cruz, CA) was possible.

The samples were run in most of the experiments on FacsAria instrument and analyzed on FacsDivia software (BD Biosciences); alternatively, Cyan instrument and Summit software (Beckman Coulter, Brea, CA) were used. Spectral overlap was compensated in the setup of the analyses using single-stained cells as well as BD Comp beads (BD Biosciences).

3 DETECTION OF APOPTOTIC CELLS (I)

Apoptotic Tregs were detected using the Annexin V analysis staining kit (BD Biosciences). Briefly, PBMC were stained with directly conjugated mAbs CD4-PE (BD Biosciences), CD25-PE-Cy7, CD127-AlexaFluor 750 (both from eBiosciences) and CD31-APC (ImmunoTools), and CD45RO-biotin (BioLegend) was used together with PeTexasRed-Streptavidin (BD Biosciences). After washes with phosphate-buffered saline (PBS), Annexin V binding buffer was added and the Annexin-FITC staining carried out. The samples were run on Cyan instrument within 1 hour of the staining.
4 CELL SEPARATION

4.1 Sorting (II)
Sorting for mRNA extraction was prepared by staining PBMC cells with mAbs against the surface markers CD8-FITC, CD31-APC (both from ImmunoTools) and CD45RA-PE (BD Biosciences). Thereafter, flow cytometric cell sorting was set-up and performed on the FacsAria instrument, and the purity of the sorting verified by separate runs of the sorted populations. The sorted cells were lysed the same day.

4.2 Immunomagnetic cell separation (II)
Enrichment of selected subpopulations of T cells involved staining of anti-mouse IgG-coated M450 Dynabeads (Dynal, Oslo, Norway) with an mAb with desired selectivity, washes to remove excess mAb, and incubation with the sample leading to cell surface marker mediated binding to the beads. After a repeat with another mAb coated on magnetic beads, and washes to discard loose attachments, the bound cells were lysed directly.

5 CELL CULTURE (III)

PBMC were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated human AB serum (Finnish Red Cross Blood Service, Helsinki, Finland), 20 mM HEPES, 2 mM L-glutamine and 50 μM 2-mercaptoethanol (all from Sigma, St. Louis, MO). To polyclonally stimulate the cells, mitogenic anti-CD3 mAb (ImmunoTools, Friesoythe, Germany) was immobilized to the bottom of the wells by a 1h incubation followed by a gentle wash to remove unbound mAb. The culture was performed in duplicate for 3 days at +37°C in 5% CO₂, and the cells then collected for RNA isolation.

6 PCR

6.1 RNA/DNA isolation and cDNA synthesis (II-III)
For lysis of the cells, TRI Reagent was used, followed by isolation of total RNA according to the protocol of RNeasy Mini Kit (Qiagen, Crawley, UK). First-strand
cDNA synthesis was done with oligo-dT primer (Sigma) and an AMV reverse transcriptase (Finnzymes, Espoo, Finland). Genomic DNA isolation was performed using the QIAamp Blood Kit (Qiagen).

6.2 Quantitative PCR (II-III)

Quantitative polymerase chain reaction (PCR) was performed with commercially available human intron-spanning primer-probe sets and the TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA). An exception was TCR Ca, which was an assay-by-design product; the sequence of this assay was: GCCTTCAACAACAGCATATTCCA, CTCGACCAGCTTGACATCACA, and FAM-CAGCCCAGAAAGTTTC-5 quencher. The iCycler-IQ instrument (Biorad, Hercules, CA) ran the reactions in duplicate for 45 cycles. The data were normalised against β-actin or TCR Ca, as indicated.

6.3 TREC analysis (II)

TRECs were quantified from isolated genomic DNA with a PCR-assay using TREC-specific primers (Sigma) 5’-CATCCCTTCAACCAGCT and 5’-GCCAGC-TGCAGGTTTGG, and probe 5’-6FAM-GACACCTCTGGTTTGTAAAGGTG-CCC-3’ for normalization of the data, signal from exon 2 of the SOCS1 gene (TaqMan assay, Applied Biosystems), was measured as a quantifier of genomic DNA. The iCycler-IQ instrument (Biorad) was again used to run the reactions.

6.4 TCR repertoire analysis (II)

The TCR repertoire was analyzed following an established approach (Pannetier et al. 1995). First, the samples were quantitatively adjusted by the level of β-actin expression. Then, a set of Vβ-specific primers were randomly selected to react together with a Cβ-specific primer and thereby to amplify a partial Vβ repertoire. This was followed by a run-off reaction, for which an internal FAM-labeled Cβ primer was used. All primers were purchased from Sigma, and the Amplitaq Gold enzyme was used for the PCR (Applied Biosystems). The fluorescent amplification products were separated and detected by the ABI3730x1 DNA Analyzer (Applied Biosystems) and analysis was performed on the GeneMapper 4.0 Software (Applied Biosystems).

To evaluate the extent of life span and antigen exposure-related alterations in the repertoire, an average cord blood repertoire served as the comparison (Talvensaari et al. 2002). Calculation of similarities was made feasible by a method that relates the areas of all the individual peaks in a Vβ-Cβ
profile to the combined area of the profile (Gorochov et al. 1998). This is described in more detail in the original article II.

7 ELISA (II)

IL-7 plasma levels were measured with the Quantikine HS IL-7 kit (R&D Systems, Minneapolis, MN) as instructed by the manufacturer. The existence of autoantibodies against IL-7 or IFN-α was evaluated and quantified with separate enzyme-linked immunosorbent assays (ELISA), where Maxisorp microtiter plates (Nalgene Nunc, Thermo Scientific, Rochester, NY) were coated overnight with recombinant human IL-7 (2 μg/ml; ImmunoTools) or recombinant human IFN-α (2 mg/ml; Roche, Basel, Switzerland). After the plates were washed and unspecific binding sites blocked, diluted plasma samples were incubated on the plates. A following incubation with HRP-conjugated rabbit-anti-human IgG (2 μg/ml; Dako, Glostrup, Denmark) made the bound antibodies detectable and, after washing, adding of the substrate produced a color reaction for the iEMS Reader MF instrument (Labsystems, Vantaa, Finland). All assays were performed in duplicate.

8 STATISTICAL ANALYSIS (I-III)

Statistical analysis was performed on the SPSS software (SPSS Inc., Chicago, IL). For testing the normality of the data, the Shapiro-Wilk test or the Kolmogorov-Smirnov test was used. Given that the distribution was shown to be normal, the statistical significance of the differences was determined by Student’s two-tailed t-test; otherwise the nonparametric test of Mann-Whitney U was used. Correlations were calculated using the Pearson’s correlation coefficient or, for data sets not conforming to normality, the nonparametric Spearman’s rank correlation coefficient was applied. Linear regression analysis was used to fit a slope between two correlating phenomena, and to calculate its confidence intervals. The limit of P < 0.05 was assigned significant. Unless otherwise indicated, the results are given as mean ± SD.
9 ETHICAL CONSIDERATIONS (I-III)

Helsinki University Central Hospital Ethics Committee approved the study and the principles of the Helsinki Declaration were followed in collecting the samples. Written informed consent was obtained from the patients and the healthy controls or, in the case of pediatric samples, from the parents of the subjects.
RESULTS

1 DEFINITION OF T CELL SUBPOPULATIONS (I-III)

The markers used in the studies for different T cell subpopulations are listed below, and referred to in the text by the names given in the parentheses (Table 4).

Table 4. The markers used in the studies for different T cell subpopulations.

<table>
<thead>
<tr>
<th>T cell subpopulation</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulatory T cell (Treg)</td>
<td>CD4^+FOXP3^+CD127^- (unless otherwise specified in the text)</td>
</tr>
<tr>
<td>Naive T cell (naive)</td>
<td>CCR7^+CD45RA^- or CCR7^+CD45RO^- or CCR7^+CD62L^-CD45RA^+ or CD28^+CD45RA^+</td>
</tr>
<tr>
<td>Recent thymic emigrant T cell (RTE)</td>
<td>CD31^+ CD45RO^-</td>
</tr>
<tr>
<td>Effector memory CD45RA^+ (T_{EMRA})</td>
<td>CCR7^-CD62L^-CD45RA^-</td>
</tr>
<tr>
<td>Resting / T_{EMRA} (resting Treg, CD4_{RA} or CD8_{RA})</td>
<td>CD45RA^- or CD45RO^-</td>
</tr>
<tr>
<td>Activated/memory (activated Treg, CD4_{RO} or CD8_{RO})</td>
<td>CD45RA^- or CD45RO^+</td>
</tr>
</tbody>
</table>

These markers are described in more depth in chapters 3.1 and 5.1.2 of the review of the literature. The naive population of T cells is usually defined by its expression of lymph node homing markers CCR7 or CD62L, in addition to CD45RA^+CD45RO^-. We checked the expression pattern of CD45RA and CD45RO by flow cytometry, and it was mutually exclusive in the patients (data not shown). Therefore the denotation CD4_{RA}/CD8_{RA} for CD45RO^- cells and CD4_{RO}/CD8_{RO} for CD45RO^+ cells is used hereafter. In Tregs, this forms the division into resting and activated Tregs, respectively, and the resting subpopulation contains also the RTE Tregs (Miyara et al. 2009, Booth et al. 2010). In non-Treg T cells, all CD45RA^-CD45RO^- T cells are not resting as they contain the T_{EMRA} cells (Sallusto et al. 2004, Libri et al. 2011).

The marker CD31 has been used as an RTE marker in CD8^+ T cells (Gurkan et al. 2010) and it displays similar age-related kinetics as in CD4^+ T cells (Tanaskovic et al. 2010), but still it is established mostly in the CD4^+ T cells (Kohler and Thiel 2009). Therefore, we did additional experiments in the study II
to confirm its role in CD8⁺ T cells. Five pediatric thymus samples were analyzed, and the expression of CD31 in CD8⁺CD4⁻ thymocytes (92.6 ± 3.7%) was even higher than in CD4⁺CD8⁻ (83.3 ± 5.2%) thymocytes. This indicated that CD31 is similarly upregulated in CD8⁺CD4⁻ thymocytes to be emigrated from the thymus. We then proceeded to sort CD31⁺ and CD31⁻ CD8 RA T cells from four APECED patients and healthy controls, and to perform a TREC analysis on them. A consistent amplification was detected only in the CD31⁺ cells, and there was no difference between patients and controls in the TREC levels (Δ cycle 3.2 ± 1.4 and 3.0 ± 2.3, respectively; Δ refers to the normalization of the data against the expression of a quantifier gene for genomic DNA, in this study SOCS1). It was therefore possible to use CD31 as an at least putative RTE marker also in CD8⁺ T cells in APECED patients.

2 DEFECT OF THE ACTIVATED TREG POPULATION AND DISRUPTED RTE TREG HOMEOSTASIS (1)

Because Tregs were found defective in their ability to suppress in vitro responses in APECED patients (Kekäläinen et al. 2007), we wanted to explore further the origins of this defect. We approached the question by a multicolour FACS analysis to make a distinction between the different subpopulations of Tregs.

2.1 Activated Tregs express FOXP3 at a lowered intensity

FOXP3 mean fluorescence intensity (MFI) correlates with the suppressive function of Tregs (Roncador et al. 2005). As previously reported in Tregs from APECED patients (Kekäläinen et al. 2007), the decrease of FOXP3 MFI was apparent also in our data. Moreover, tracing this defect into the two major subsets of Tregs, the activated and the RTE, revealed that although the FOXP3 expression was lowered already in the RTE Tregs of the patients, this was not sufficient to explain the overall decrease in the FOXP3 expression of the Tregs (original publication 1, Figure 1). This suggested that the failure of Tregs in APECED was not solely of thymic origin.

2.2 RTE Tregs are decreased in frequency and undergo accelerated proliferation

We therefore analyzed further the peripheral maintenance of the Treg population. Previous studies had found the frequency of Tregs in APECED normal (Kekäläinen et al. 2007) or reduced (Ryan et al. 2005). In this study, there was no significant difference in the frequency of CD4⁺CD25⁺ cells, the
definition used in the previous studies to denote Tregs, between the patients and controls (3.0 ± 0.6% and 3.0 ± 0.6%, respectively). Also the FOXP3+CD127– fraction of CD4+ T cells was unaltered in frequency (7.5 ± 2.3% in the patients vs. 6.3 ± 1.4% in the controls, NS).

We then analyzed the distribution of Treg subsets according to their expression of CD45RO and CD31, allowing most importantly for the analysis of the frequency of activated Tregs and RTE Tregs. Here, the only difference appeared in the RTE Tregs, the fraction of which amongst the Tregs was almost halved in the patients: 7.1 ± 4.9 % vs. 13.6 ± 7.1 % in the controls (P < 0.02; original publication I, Figure 2A). To investigate whether this was due to increased apoptosis or transition to the activated pool of Tregs, Annexin V staining was deployed. This gave no evidence for an apoptotic process, as the staining was similar in patients and controls (7.1 ± 3.7% vs. 7.8 ± 3.2%, respectively). Therefore it appeared that the recruitment of RTE Tregs to the activated subpopulation was increased.

Diminished frequency of a T cell subset might result from decreased proliferation. To study this, we then analyzed the expression of Ki-67, a marker for entry into the cell cycle and thereby indirectly for proliferation, in the Tregs. No alterations in Ki-67 expression were evident in overall Tregs between the patients and controls (11.8 ± 7.0% and 11.4 ± 5.0%, respectively, NS), or the activated Tregs (14.9 ± 9.5% and 14.6 ± 7.6%, respectively, NS). In the RTE Tregs, however, we found a significant difference, with them expressing not decreased but elevated amounts of Ki-67 in APECED compared to healthy controls (original publication I, Figure 2C). As further confirmation for the naive RTE subpopulation to be specially affected, a significant inverse correlation existed between their frequency and expression of Ki-67 (Figure 9). A picture of populational exhaustion of RTE Tregs in APECED therefore emerged, because of their increased proliferation accompanied by a decrease in frequency.
Figure 9. Frequency of RTE Tregs correlates inversely to the frequency of RTE Tregs expressing Ki-67. Open circles indicate the controls and the closed circles the patients. The best-fit curve is shown. Calculated for the subjects shown in the graph, Pearson’s correlation coefficient was −0.442, P < 0.05. One control and two patients outlying from the others by their Ki-67 expression were excluded from this analysis; including them strengthened the correlation (Spearman’s correlation coefficient −0.598).

The current understanding of RTE Tregs in the adults states them as a slowly dividing population that is very resistant to apoptosis (Miyara et al. 2009), thus forming a reservoir of resting Tregs. Activated Tregs on the other hand are rapidly dividing and prone to apoptosis (Vukmanovic-Stajic et al. 2006). To evaluate the importance of the increased turnover of APECED RTE Tregs, we studied the RTE fraction of Tregs in healthy children and adults. Compared to RTE cells amongst all CD4+ T cells, the RTE Tregs behaved similarly as to their population kinetics, and the decreases in their frequency were in correlation to each other (original publication I, Figure 3). This had been shown previously with adults (Booth et al. 2010), and our analysis confirmed and extended the findings of strong populational RTE Treg maintenance in healthy subjects.

2.3 Defect of Tregs is more severe in the peripherally activated population

Peripheral activation of naive Tregs should lead into elevated expression of FOXP3 (Miyara et al. 2009, Yagi et al. 2004). The increase in FOXP3 expression
from the RTE to the activated subset in the same individual subject was clearly smaller in the patients (Figure 10). This suggested that the Tregs in APECED were peripherally unable to properly activate, which could be contributed to by the disrupted peripheral homeostasis and increased attrition of the RTE Treg population. Also, the controversial population of CD8⁺ FOXP3⁺ Tregs displayed similar alterations in FOXP3 expression, to a greater extent in the activated population (original publication I, Figure 4C).

![FOXP3 mean fluorescence intensity](image)

**Figure 10.** Increase in FOXP3 expression in each subject individually from the RTE Tregs subset to the activated Tregs. Open circles indicate the controls and the closed circles the patients; the lines connect the same individual subjects. Calculated for the difference between patients and controls in the change in FOXP3 MFI from the RTE to the activated subset, P < 0.005.

CD5 is a negative regulator of TCR signalling (Dalloul 2009), and high intensity TCR-mediated signalling is known to upregulate CD5 on the T cell surface (Azzam et al. 1998). CD5 staining showed that its expression was upregulated in all but three controls and one patient from the RTE subset to the activated Tregs, with no significant difference in the patients compared to the controls (change in CD5 MFI from the RTE to the activated subset 559 ± 331 in
the patients vs. 472 ± 526 in the controls, NS). Alterations in antigen stimulation were not therefore indicated here. A previous study had investigated the TCR repertoire of all Tregs in APECED patients, and found it to be more naive than that of healthy controls, suggesting a lack of activation in the periphery (Kekäläinen et al. 2007).

The overall activation profile of CD4+ T cells was also analyzed, in order to exclude other sources of elevated FOXP3 expression. The frequency of CD4_{RO} cells and their expression of Ki-67 were normal, which argued against a confounding increased activation status in the patients’ CD4+ T cells (original publication I, Table 1). FOXP3 expression in activated non-Treg cells is low level and transient, whereas the cells expressing FOXP3 the highest are regarded Tregs without question (Gavin et al. 2006). To further rule out a bias in the results deriving from activated non-Treg cells, we thus analyzed the highest 10% of FOXP3 intensity on the activated CD4{sup}+FOXP3{sup}+ cells, and then even the top 1% of FOXP3 intensity on all CD4_{RO} T cells, and the defect in the patients was even more evident in this analysis (original publication I, Figure 5). General activation of the immune system in the patients was therefore an unlikely reason for the findings.

3 CD8+ T CELLS ARE PERTURBED BY IL-7 DYSREGULATION (II)

Encouraged by the homeostatic defect detected in Tregs in the first study, we then turned our interest towards the overall CD4+ and CD8+ T cell populations in APECED. Recent studies had delineated lymphocytosis in the peripheral blood of APECED patients from childhood to adults (Perniola et al. 2005, Tuovinen et al. 2009), which also gave initiative for further exploration.

3.1 Increased proliferation of CD8+ T cells arising from the CD8_{RA} subset

Analysis of Ki-67 expression showed that the CD8+ T cells had an increased proliferation rate in the patients, while the CD4+ T cells displayed no such features; this finding arose from the CD8_{RA} subpopulation (original publication II, Figure 1).

The overall T cell population frequencies were unaltered in APECED: frequency of CD4+ T cells was 38.9 ± 13.7% in the patients vs. 41.3 ± 7.1% in the controls (NS), and of CD8+ T cells 28.3 ± 7.3% vs. 26.0 ± 4.6% (NS), respectively. Also, the CD45RO- and CD45RO+ subsets in both CD4+ and CD8+ T cells were maintained in APECED, as the frequency of CD4_{RA} was 54.6 ± 18.3% in patients
vs. 59.5 ± 12.3% in controls (NS), and of CD8<sub>RA</sub> 75.1 ± 12.1% vs. 69.1 ± 11.8% (NS), respectively.

3.2 CD127 expression is drastically reduced and is related to elevated IL-7 levels in CD8<sup>+</sup> T cells and the CD8<sub>RA</sub> subset

T cell homeostasis is mainly affected by TCR signalling and cytokines (Takada and Jameson 2009, Rochman et al. 2009). We then set out to identify possible causes of the increased Ki-67 expression.

CD127 expression in CD8<sup>+</sup> T cells immediately gained our attention, as the differences between the patients and controls were four-fold on the average: the patients had downregulated CD127 to MFI 204 ± 98 vs. 819 ± 297 in the controls (P < 0.001, Figure 11). CD127 expression was lowered also in the CD4<sup>+</sup> T cells, although less than in CD8<sup>+</sup> T cells (MFI 903 ± 351 in the patients vs. 1475 ± 489 in the controls, P < 0.005).

![Figure 11. Histogram of CD127 expression in CD8<sup>+</sup> cells from the control and patient representing median values. Numbers in the histograms indicate the MFI of CD127 expression.](image)

ELISA analysis of plasma IL-7 levels showed a significant increase in the patients (original publication II, Figure 2A), to which the CD127 expression is thought to respond by downregulation (Mazzuchelli and Durum 2007). Indeed, a significant negative correlation existed between the plasma IL-7 levels and CD127 expression on the CD8<sup>+</sup> cells in the patients, and in their subset of CD8<sub>RA</sub> cells (original publication II, Figure 2E), but not the CD8<sub>RO</sub> cells in the patients. In healthy controls no significant correlation could be found, which implied active regulation in the patients through this axis in CD8<sup>+</sup> T cells. It has also been suggested that decreased binding of IL-7 by T cells causes the stromal cells to secrete more IL-7 (Capitini et al. 2009), and therefore the alterations detected here could also be driven by perturbed CD8<sup>+</sup> T cell function.
Since cytokine autoantibodies are an eminent factor in APEXED, some of them even affecting the functions of the cytokines (Puel et al. 2010, Kisand et al. 2010), we measured the IL-7 autoantibody levels in the plasma, but found no significant differences between the patients and the controls (original publication II, Figure 3). Antibodies against IFN-α were readily detectable in the patients, but no connection could be drawn to CD127 expression of CD4+ or CD8+ T cells, Ki-67 expression of CD8+ T cells, or the frequency of naive CD8+ T cells.

3.3 CD5 expression is decreased and an oligoclonal repertoire is revealed in CD8+ T cells and the CD8RA subset

IL-7 is needed to sustain the naive T cell population, but it also primes naive T cells for responding to self-antigen stimulation (Takada and Jameson 2009). This effect is reflected in the downmodulation of CD5 by IL-7 (Gagnon et al. 2010).

In the CD8+ T cells, CD5 expression was significantly lowered in the patients (MFI 839 ± 477 in the patients vs. 1466 ± 413 in the controls, P < 0.003) and this change derived from the CD8RA cells (MFI 716 ± 486 vs. 1595 ± 519, respectively, P < 0.001), whereas the CD8R0 were unaffected (original publication II, Figure 4). This suggested the influence of IL-7 on the CD8+ T cells, and especially the CD8RA T cells.

We wanted then to further dissect between a directly IL-7 driven proliferation and proliferation resulting from IL-7 induced enhanced TCR stimulation. To accomplish this, TCR repertoire analyses were performed. If the proliferation were to be directly IL-7 driven, polyclonality of the TCR repertoire should be conserved (Capitini et al. 2009), whereas in TCR-mediated proliferation an oligoclonal repertoire is expected to evolve (Pannetier et al. 1995). Five randomly chosen patients and five age-and sex-matched controls were included. Following immunomagnetic separation of first CD8+ T cells and then their CD45RO+ and CD45RA+ subsets, the TCR repertoires were analyzed by spectratyping, and evaluated by comparing to a baseline repertoire obtained by averaging 11 cord blood sample repertoires (Kekäläinen et al. 2007). The TCR repertoires of CD8+ T cells from the patients were much more oligoclonal than those of the controls (original publication II, Figure 5B). Again, further analysis of the subpopulations showed that the CD8RA cells were the cause of this alteration (Figure 12), to the extent that this subpopulation had become as oligoclonal as the CD8R0 population in the patients (original publication II, Figure 5C). In the controls, the CD8R0 T cells were more oligoclonal than the CD8RA cells, as was expected. These findings supported antigen-driven expansion of the CD8RA T cells in the patients.
3.4 CD8_{\text{RA}} and naive CD8^{+} T cells produce increased amounts of perforin, and the IL-7 dysregulation is implicated to begin already in the thymus

Increased antigen-driven proliferation of CD8^{+} T cells in the context of an autoimmune disease was something to be analyzed further. We measured therefore the mRNA content of perforin, a cytotoxic effector molecule, in the CD8_{\text{RA}} T cells, and found it to be significantly elevated in the patients (51.8 ± 35.3\% in the patients vs. 18.2 ± 24.9\% in the controls, P < 0.05). The CD8_{\text{RA}} cells contain the T_{EMRA}, and to separate their influence, we analyzed further the subpopulation expressing naive markers, thus expected to be consistently resting. This revealed an even more severe alteration, as the naive CCR7^{+} CD8_{\text{RA}} T cells expressed perforin in a similarly increased way (47.0 ± 36.3\% in the patients vs. 13.2 ± 25.4\% in the controls, P < 0.05; Figure 13A), and the same was found using the marker CD28^{+} (19.9 ± 8.9\% vs. 1.8 ± 1.9\% in the controls, P < 0.01). The naive CD8^{+} T cells displayed therefore properties of activated effector cells in the patients. Also, the frequency of naive CD8^{+} T cells was found to be severely decreased in the patients (Figure 13B).
Figure 13. A, Perforin expression of naive CD8⁺ T cells in the control and patient representing median values. The numbers indicate percentages staining positive for perforin. B, Decreased fraction of naive CD8⁺ T cells in the patients shown here by the median control and patient. The numbers refer to the frequency of cells in the quadrant. The patients and controls shown in A are different from those in B.

To investigate whether the alterations in naive CD8⁺ T cells were influenced by IL-7, CD127 MFI of CCR7⁺ CD8⁺ T cells was measured, and a similar decrease in expression in the patients was detected (MFI 18 ± 13 in the patients vs. 26 ± 8 in the controls, P < 0.02). CD5 expression was also decreased in the naive CD8⁺ T cells (MFI 67 ± 26 in the patients vs. 90 ± 22 in the controls, P < 0.03). We performed a further experiment on the effects of IL-7 on CD127 expression on these naive CD8⁺ T cells, and incubated them with or without IL-7 in +4°C or in +37°C. Incubation at +37°C without IL-7 should produce shift upregulation of CD127 in in vitro conditions (Mazzucchelli and Durum 2007). This upregulation was significant in our experiment only in the naive cells of the healthy controls, suggesting that the patients' cells had undergone permanent downregulation of CD127 (original publication II, Figure 8B), in line with enhanced TCR-mediated stimulation and subsequent severe phenotypic alterations.
To further evaluate the effect of thymic imprinting, the putative RTE CD8$^+$ cells were also analyzed, and they had similarly downregulated CD127 expression, and displayed an even higher increase in the frequency of cells expressing Ki-67 than all the CD8_{RA} T cells (original publication II, Figure 8C).

4 CUTANEOUS IN VIVO EXPERIMENT REVEALS A STABLE DEFECT OF IL-22 PRODUCTION (III)

Our third study took a different approach to understanding APECED. We wanted to adopt a method to study tissue-infiltrating T cells in vivo in human patients. With the CMC-related findings of Th17 autoantibodies in APECED (Kisand et al. 2010, Puel et al. 2010), and the skin being a tissue within reach, studies on skin-infiltrating T cells seemed especially appropriate. The consequences of these autoantibodies on the function of Th17 cells in APECED have been described with conflicting results (Ng et al. 2010, Ahlgren et al. 2011), and this together with the scarcity of overall data encouraged us to study in vivo Th differentiation.

4.1 T cell accumulation and phenotype in the skin are unaltered, but pre-existing eye disease is activated in the patients

We modified a method used previously to describe Treg kinetics in healthy subjects (Vukmanovic-Stejic et al. 2008). We studied three patients and six age- and sex-matched controls, all vaccinated against Bacillus-Calmette-Guérin in childhood. Intradermal injections of PPD, i.e Mantoux testing, created therefore a memory-based delayed-type hypersensitivity response, and after a maturation period, the infiltrating cells were made accessible by inducing suction blisters on top, and harvesting them the following day from the tissue fluid. We also induced blisters on unexposed, normal skin, and took peripheral blood samples for comparison. We recovered cells from the PPD exposed skin to a similar extent from the patients and the controls: $10^6$ from the patients (range 0.1-1.6 x10$^6$) and 1.4 x10$^6$ from the controls (range 0.1-5.0 x10$^6$). Normal skin blisters gave approximately 20% of these yields in both the patients and the controls. Further analysis of the T cell subsets showed that the patients and controls both had a CD4$^+$ T cell stressed response as to be expected to result from Mantoux testing. The accumulating T cells were mostly CD4_{RO}, the expression of Ki-67 was higher in them compared to the peripheral blood indicating intracellular activation, and the expression of CCR7 decreased clearly compared to the blood cells, suggesting appropriate homing (Table 5). The T cell response was therefore evident and of expected nature in both the patients and the controls.
Table 5. Accumulation of CD4<sub>ro</sub> T cells to the exposed skin in all the subjects studied. There were no significant differences between patients and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PBMC</th>
<th>PPD-exposed skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4/CD8 ratio</td>
<td>1.9 ± 1.1</td>
<td>3.6 ± 1.6 (P &lt; 0.03)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD45RO&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>39.7 ± 10.0</td>
<td>90.4 ± 5.2 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;Ki67&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>2.4 ± 0.5</td>
<td>7.7 ± 3.4 (P &lt; 0.004)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CCR7&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>62.6 ± 10.3</td>
<td>37.0 ± 10.9 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

Clinically, the PPD injections produced similar responses of erythema on the skin of the subjects: on average 8mm (range 0-15mm) of diameter in the controls and 10mm (range 0-15mm) in the patients. Induration around the injection sites was not significantly different: on average 7mm (range 0-15mm) in the patients compared to 3mm (range 0-6mm) in the controls. To our great surprise, two of the patients had an episode of keratoconjunctivitis with onset on the day following the PPD injections. Both of them had been diagnosed with the disease component of ocular keratopathy years before the experiments, and the third patient and the healthy controls had no such pre-existing condition. The other patient was attended to by an opthalmologist to confirm the diagnosis, and the keratoconjunctivitis was transient in both patients, with local and systemic antibiotics administered to avoid further complications. This relapse of the eye disease presumably was related to the intradermal tuberculosis injections, and suggested that keratopathy in APECED might also be influenced by the immune system, instead of being a mere constant form of ectodermal dystrophy.

4.2 Mantoux testing induces normal Th1 responses

We then set out to investigate the Th profile of the response, to determine its qualitative competence after it had quantitatively measured normal. Mantoux testing produces typically a Th1-stressed response. Th differentiation was first evaluated by quantitative PCR of the mRNA production of transcription factors, and in the PCR assays the data were normalized using TCR Cα to take into account the differences in the amount of T cells in the samples. Th1-related Tbet, and Th2-associated GATA3 were expressed in the PPD responding cells in both the patients and the controls with no significant differences (original publication III, Figure 2A). The Th1-cytokine IFN-γ and Th2-lineage cytokine IL-4 were also produced at unaltered levels in the patients (original publication III, Figure 2B). The Th1-and Th2-lineages seemed therefore functional in APECED.
4.3 IL-22 expression is severely repressed with and without a challenge

To assess the Th17-lineage, we first measured the expression of the transcription factor RORC. Although the mRNA levels were slightly decreased in the patients, the difference was not significant (original publication III, Figure 2A). A clear difference however was detected in the production of IL-22 mRNA in the PPD exposed skin: while the cells of all but one control readily produced this cytokine, none of the patient cells gave a detectable signal of IL-22 mRNA (Figure 14; original publication III, Figure 2D). IL-17 levels were also lower in the patients, but not to a significant extent (original publication III, Figure 2B). These findings suggested a functional defect of IL-22 production.

![Graph](image)

**Figure 14.** TCR Cα and IL-22 mRNA expression in the PPD exposed skin of a representative age-and sex-matched patient and a control. Real time quantitative PCR was run for 45 cycles.

To assess whether this IL-22 defect was related to an antigen challenge or a constant feature on the skin of APECED patients, we analyzed the production of IL-22 mRNA from the cells of the normal, unexposed skin. The results were again clear: the patients had no detectable production of IL-22 in their skin, and the difference to controls was again significant, although also the controls produced a lower signal (original publication III, Figure 2D). The defect was therefore present also in steady-state conditions in the skin.

Despite differences in findings on the overall functionality of Th17 cells in APECED, previous studies have reported that the PBMC fail to produce IL-22 in response to stimulation with *Candida albicans* (Ng et al. 2010, Ahlgren et al. 2011). We also measured the IL-22 mRNA levels from nonstimulated PBMC and after stimulation with anti-CD3 mAb. While no difference in expression existed in nonstimulated PBMC between patients and controls, the stimulation again revealed a significant impairment of IL-22 mRNA production in the patients. The healthy controls, on the contrary, showed a marked elevation of IL-22 production with the stimulation (original publication III, Figure 2D). All the
other cytokines measured, IFN-γ, IL-4, and IL-17, showed no differences between patients and controls in the normal skin or in the PBMC, even with stimulation (data not shown). The defect of IL-22 production appeared therefore after stimulation also in the PBMC.
DISCUSSION

Put together, the studies showed in APECED a marked reduction in the frequency of RTE Tregs and naive CD8+ T cells, increased entry into the cell cycle in RTE Tregs and CD8RA and putative RTE CD8+ T cells, and severe alterations in the functions of activated Tregs and CD8RA T cells and their subset of naive CD8+ T cells. The IL-7/CD127 axis was severely disturbed in the CD8+ and CD4+ T cells, and suggested to be involved in the altered phenotype of CD8+ T cells. Influence of TCR signalling was not implied in the RTE Tregs, as CD5 expression was unaltered, but in CD8RA T cells CD5 was clearly decreased and oligoclonal expansions dominated the TCR repertoire, indicating antigen-driven proliferation, to which IL-7 could make the cells more prone. In the skin, the production of IL-22 and to a lesser degree of IL-17 was constantly defective, while other Th lineages appeared unaltered. We explored also further, in the initial analyses unrevealed, connections between the above-described alterations in Tregs, CD8+ T cells and IL-22, but no significant correlations emerged.

1 FAILURE OF PERIPHERAL TOLERANCE IN APECED

AIRE expression is highest in the thymus, and the foundation of APECED pathology has been thought to lie in defective negative selection, based mainly on studies on Aire-/- mice (Peterson et al. 2008, Mathis and Benoist 2009). However, the difference between the clinically healthy remaining Aire-/- mice and the severely affected APECED patients, in whom the AIRE mutations cause a life-threatening disease, calls for additional pathogenetic factors in humans. This conclusion has been disputed by the claim that a confounding factor, the lack of pathogen-exposure in animal model housing systems, would suffice to explain the difference (Akirav et al 2011). However, even transfer of lymphocytes from Aire-knockout mice into mice with severe lymphopenia is insufficient in triggering a disease resembling human APECED (Kekäläinen et al. 2011), nor does a wide range of different innate immune stimuli alter the mouse phenotype (Gray et al. 2007b). This allows one to infer that the inter-species differences of peripheral tolerance weigh more than severe challenges, such as infection, wearing out the T cell population. Also, there is no increase in the expression of pattern recognition receptors in the patients, receptors on cells of the innate immunity that sense microbial structures, which implies that the patients are not under increased pathogen load (Hong et al. 2009).
1.1 Treg dysfunction in APECED

Differences in Treg biology in mice and humans offer one explanation for the afore-mentioned discrepancy. We have described here a failure in the activated Tregs of peripheral blood in APECED patients, together with defective maintenance of the RTE naive Treg population. With the much longer life span of humans, such a dysregulation inevitably has more clinical relevance in humans than in mice. Although our study was overall descriptive, with no functional assays performed, the pre-existing data on defective in vitro suppression by the Treg population provides a solid background for our study. Furthermore, the TCR repertoire of Tregs in APECED patients had also been previously found to be more naive than that of healthy controls, suggesting lack of activation in the periphery. (Kekäläinen et al. 2007). The role of Treg failure in the pathogenesis of APECED has been questioned, with some researchers regarding it as a side effect of the patients suffering from a chronic illness for decades, or of the treatments the patients have received (Mathis and Benoist 2009). These arguments become less relevant in the light of our findings, where the RTE Tregs are affected. Furthermore, regarding our results on activated Tregs, the analysis of the cells with the brightest FOXP3+ expression excluded the possible low effect of activated non-Treg cells (Gavin et al. 2006, Tran et al. 2007, Wang et al. 2007), and showed the defect to be even more severe in the activated subset than in the whole population of Tregs. We confirm therefore this mechanism of peripheral tolerance to be defective in APECED, and connect it to altered peripheral homeostasis. Our findings offer not only an explanation for the differences in mice and humans, but also a cause for the emergence of new disease components with time: Increased turnover of the polyclonal Treg reservoir impairs the recruitment of functional Tregs, which due to the differences in life span can be expected to have more severe consequences in humans than in mice, and with time this disrupted maintenance gives momentum to the autoreactive T cells to cause clinical damage to an increasing number of targets. This is in agreement with the previous suggestions on peripheral AIRE’s importance in handling age-related thymic involution (Metzger and Anderson 2011).

1.2 Autoreactive CD8 RA T cells escape the control of the lymph nodes

Autoreactive T cells in autoimmune diseases are expected to be peripherally activated effector cells. We found that the APECED patients investigated hosted a population of CD8 RA T cells in their peripheral blood with very unusual features, and we presume them to thus contain the population of T cells in APECED that cause the autoimmune attacks. Several findings support our assumption. The CD8 RA T cells were highly active in producing the effector molecule perforin. Their TCR repertoire was expanded oligoclonoically implicating activation. The
expression of CD5 was decreased on the CD8_{RA} T cells, indicating increased responsiveness to antigens. Because the autoantigens T cells directly react against in APECED remain to be identified, we saw it unfeasible to prove the autoreactive nature of these cells in *in vitro* assays, although such an experiment would be needed for full certainty of our conclusion. The CD8_{RA} T cells also displayed a marked decrease in their expression of lymph node homing receptors, translating into a rise in the frequency of the T_{EMRA} population. This population is known to enlarge in chronic viral infections (Kaech and Wherry 2007), and in APECED we suggest it to reflect the constant immunoactivation of autoimmunity, thus representing the pathogenic cells in APECED.

However, the alterations cannot be explained solely by accumulation of pathogenic T_{EMRA} type cells, because the findings in CD8\(^{+}\) T cells with markers for only naive cells were equally altered. Especially, the upregulated production of the cytolytic molecule perforin was evident in the naive CD8\(^{+}\) T cells, and the result was the same with any of the markers of a naive cell used in the study. CD5 expression was decreased in the naive cells similarly suggesting antigenic stimulation, although we didn’t analyze the TCR repertoire in the naive subset separately. These findings of altered phenotype together with the CD127 downregulation extend the perturbed IL-7/CD127 regulation already to the naive CD8\(^{+}\) T cells, and suggest that the autoimmune function is imprinted already in them.

In APECED, CD4\(^{+}\) T cells have been regarded more important than CD8\(^{+}\) T cells, based on findings in mice (Devoss et al. 2008). The data on CD8\(^{+}\) T cells have been scarce, which is probably due to the CD8\(^{+}\) population appearing normal as a whole, and the alterations locating to an unusual part of the population, the CD8_{RA} cells. In healthy humans, the naive T cell population forms a stable reservoir (Lynch et al. 2009). In our study, the frequency of naive CD8\(^{+}\) T cells was severely decreased in APECED, as was the frequency of RTE Tregs, raising the interesting possibility that these populations are affected by the same homeostatic defect.

Whether loss of functional AIRE inside DCs and the defects reported in them (Pöntynen et al. 2008) have a role in the perturbed homeostasis, is a question outside the scope of this study. DCs can for example induce CD5 on T cells as one mechanism of their capability to induce tolerance (Hawiger et al. 2004). Defects in this might explain decreased expression of CD5 in our data, but would not explain why such a defect would limit to CD8\(^{+}\) T cells instead of all effector cells. Although we did not study the function of APCs in the lymph nodes, the report on increased activation of naive T cells by Aire\(^{-/-}\) DCs (Ramsey et al. 2006) is intriguing in the context of our results – irrespective of whether the defects of DCs take their effect already in the thymus or in the periphery.
A study on the expression of homing markers inside the thymus found the expression of CCR7 defective in Aire deficient mice (Laan et al. 2009), which would however imply that the CD4+ and CD8+ T cells exiting the thymus and entering the periphery lack the ability to enter the lymph nodes in the first place. Further research on the lymph nodes of APECED patients, if possible, would bring understanding on the alternatives left open here. Decreased entry into the lymph nodes was however somewhat supported in our data, where an additional analysis of the homing marker expression in the RTE CD8+ T cells showed a clear decrease in the patients (CCR7 MFI 733 ± 333 in the patients vs. 1986 ± 426 in the controls, P < 0.0005, and CD62L MFI 1712 ± 923 vs. 3287 ± 1363, respectively, P < 0.05).

1.3 Role of T cell homeostasis in APECED

1.3.1 IL-7 in APECED
Defects in IL-7 or CD127 have not been previously associated with APECED, and AIRE is not known to regulate their transcription. Our study reported however a drastic perturbation of the IL-7/CD127 axis, thereby revealing a novel aspect of APECED pathology. Furthermore, the alterations in CD8+ T cells that were related to this homeostatic dysregulation are far more widespread than could be expected from mere escape of autoreactive T cell clones, emphasizing AIRE in functions additional to negative selection.

Based on our data, the primary agent of the changes in receptor expression and cytokine production remains undetermined. Elevated IL-7 levels are known to cause downregulation of CD127 expression on T cells (Mazzucchelli and Durum 2007), but stromal cells can also upregulate their IL-7 production when the utilization of IL-7 is decreased (Capitini et al. 2009). In any case, the correlation between IL-7 levels and CD127 on CD8RA T cells supported the involvement of the IL-7/CD127 axis in the phenotypic alterations detected. However, when incubated without IL-7, the irrevocable nature of CD127 downregulation in the naive CD8+ T cells became apparent, which suggested that the effector-like features of the naive CD8+ T cells were causing the decrease in CD127 expression. Thus a combined effect of escaping autoreactivity in the absence of AIRE, and IL-7 mediated lowering of the TCR threshold and incentive to proliferate, would seem a favourable explanation for the findings.

Why did the IL-7 dysregulation have a visible effect only on CD8+ T cells, and not on CD4+ T cells? Our study did show a significant downregulation of CD127 also in CD4+ T cells. The CD4+ T cells were, however, free of the alterations in Ki-67 expression, CD5 expression, or the frequency of the
subpopulations, thereby implying that the CD127 downregulation would have a smaller effect on them. IL-7 therapy trials have also reported that CD8+ naive cells are especially sensitive to IL-7 in responding by increased proliferation (Capitini et al. 2009), and that this sensitivity might derive from differences in CD127 signal mediation between the CD8+ T cells and CD4+ T cells (Mazzuchelli and Durum 2007). It is also possible that with a larger number of subjects we would have found significant alterations in the CD4+ T cells as well, but in the current data there was no implication of this.

Could IL-7 be also behind the defects in Tregs in APECED? In the periphery, Tregs are CD127 negative or low, and in this study we also used the exclusion of highly CD127+ cells to outline Tregs. IL-7 is suggested however to be important for Tregs during their development in the thymus (Mazzuchelli et al. 2008). With the alterations in IL-7 detected, we therefore calculated correlations between IL-7 levels and RTE Treg frequencies in the patients, but no significant correlations were found. Cytokines were implicated to be affecting the Tregs, as CD5 expression was normal and therefore TCR signalling was an unlikely cause. Although we were unable to see significant correlations between the plasma IL-7 levels and alterations in the Treg populations, an intrathymic effect, invisible in the periphery, cannot be excluded. A recent study on mice with experimental autoimmune encephalomyelitis described that the thymic output of Treg cells increased with the duration of the illness, and that it involved changes in IL-7 production of the thymic stroma and in CD127 expression of Tregs (Chen et al. 2009), suggesting a role for IL-7 in Treg differentiation especially under autoimmune conditions. Since we don’t have access to thymic tissue from APECED patients, this question might instead be addressed by studies on thymomas, where functional AIRE is also often absent.

1.3.2 Special features of human Treg homeostasis reflected in APECED

The maintenance of a functional Treg population in the periphery with aging in healthy humans is still an unresolved issue, with thymic involution setting the challenge. The relevance of peripheral conversion and, on the other hand, the ability of a reservoir of naive Tregs to last long term have been debated (Walker et al. 2005, Vukmanovic-Steljic et al. 2006, Miyara et al. 2009). In our study, RTE Tregs from healthy humans formed a slowly dividing population, with kinetics of attrition similar to RTEs from non-Treg cells. The frequency of RTE Tregs was in strong correlation to the frequency of non-Treg RTEs. Together these findings support RTE Tregs as a population whose maintenance is comparable to that of naive non-Tregs, and therefore favours their significance in sustaining suppressive capacity also with aging.
In APECED, the increased attrition of RTE Tregs appearing to give rise to the defects in the activated Tregs supports the importance of the peripheral naive Treg reservoir, but we did not address the question of whether peripheral conversion of Tregs is functional in APECED. We can just note that also in the disease of MS, RTE Tregs seem to have special importance (Haas et al. 2007, Venken et al. 2010). Further experiments are however needed to make conclusions on the unutilization of conversion in the setting of human autoimmune diseases.

Activated Tregs have been described to suppress the proliferation of resting Tregs (Miyara et al. 2009), which could also be affecting the RTE Tregs in APECED, as a defective activated population could be expected to be less active also in such a function. Overall, the similarity between dysregulation of RTE Tregs and naive CD8+ T cells in APECED suggests the influence of a widely disturbing, pan-T cell factor.

2 IS THE PERIPHERAL FAILURE ACTUALLY PERIPHERAL?

A factor affecting all the T cells in APECED is naturally the lack of AIRE, which, based on the expression of AIRE, affects the thymus the strongest. Our studies had many findings suggestive of the alterations being imprinted already in the thymus. In the Tregs, although the defect was clearly influenced by peripheral activation, the RTE population was severely affected. AIRE has been reported to induce Treg development in the thymus (Aschenbrenner et al. 2007), and therefore our findings might arise from imprinted abnormality of the responses of Tregs in APECED.

When the RTE marker CD31 was included in the analysis of CD8+ T cells, a valid approach based on our results and the studies of others (Gurkan et al. 2010, Tanaskovic et al. 2010), the alterations persisted, and therefore the role of the thymus was again relevant. Increased autoreactivity rising from thymic development, together with peripheral IL-7 dysregulation, could lead to a severe disturbance as found in our study. Alternatively, the IL-7 dysregulation may begin already in the thymus.

Deductions of a general disturbance in the thymic milieu in the absence of AIRE come from studies on thymomas. Patients with thymic epithelial tumours share with APECED its feature unmimickable in Aire-/- mice, namely the presence of autoantibodies against type 1 IFNs (Meager et al. 2008). Their early prevalence of 100% in the APECED patients, and the restriction to APECED instead of any of the disease components in non-APECED patients, suggest a genesis related to AIRE deficiency (Meager et al. 2006). Thymoma patients
especially with thymoma-associated myasthenia gravis frequently have autoantibodies against type 1 IFNs, especially IFN-α, with a prevalence of approximately 70%. These patients share with APECED also the correlation of CMC with Th17 autoantibodies. Meager et al. (2008) have proposed that the absence of AIRE from the thymus would create a generally autoimmunizing environment instead of a tolerogenic one, because of absence of AIRE from the APCs, and this would lead to autoantibody production against type 1 IFNs that are widely produced in the thymus. This is intriguing in the light of our results, because high concentrations of type 1 IFNs, produced in this model by the AIRE-deficient over-activated APCs (Meager et al. 2008), could also prime the maturing CD8$^+$ T cells in the thymus to rapid acquisition of effector functions, as reported to happen in nonvirus-specific bystander naïve CD8$^+$ T cells in viral infections (Marshall et al. 2010). Such an effect could take place in a paracrine way despite the neutralizing autoantibodies and their ability to diminish the expression of IFN-stimulated genes (Kisand et al. 2008). Type 1 IFNs can also regulate CD127 expression (Rochman et al. 2009), so they might impinge on our findings even further. Type 1 IFNs also induce increased attrition of CD8$^+$ T cells, although this effect is usually described in memory T cells in the periphery (Bahl et al. 2010). The autoantibodies against type 1 IFNs have even been reported to have an activating effect on endothelial cells, thus partly enhancing immune responses (Moll et al. 2008), and therefore their actual effect inside the thymus would be interesting to investigate.

Criticism for such deductions is warranted, however, as the patients with thymomas rarely develop disease components of APECED – despite the fact that even the expression of peripheral tissue restricted antigens such as insulin is decreased in thymomas – and APECED patients have no susceptibility for myasthenia gravis (Ströbel et al. 2007). These differences have been suggested to be related to the different time of onset of the AIRE defect in APECED and thymomas, and to the different autoantigens present (Meager et al. 2008). The abnormal milieu of the thymus in APECED is not an altogether distorted one either: in Aire$^{-/-}$ mice, T cells with normal functions against pathogens are formed, and the overall process of TCR repertoire formation is normal against pathogens such as the influenza virus (Kedzierska et al. 2010). Our own findings provide only circumstantial evidence that such a thymic state of general alert would be behind the severely altered homeostasis of T cells in the periphery. The Th17 and type 1 IFN autoantibodies in APECED, however, have so far demonstrated that not everything can be directly connected to the lack of AIRE in this disease.
APECED is closely connected to CMC. Chronic exposure to *Candida*, and also inevitably to other microbes because of the altered barrier function resulting from continuous inflammation, renders the mucocutaneous surfaces a challenging environment for T cell homeostasis. Our experiment on the skin of APECED patients demonstrated a deficiency in producing IL-22, which emerged both in the steady-state resident T cells and in the response against PPD. Such a deficiency with an antigen challenge was to be expected from the PBMC stimulation assays performed by others (Ng et al. 2010, Ahlgren et al. 2011), but its presence even without a challenge in the skin was unexpected. The tendency of T cell alterations to present stronger locally was described in type 1 diabetes, where while PBMC appeared normal, inside pancreatic lymph nodes there were severe Treg and Th17 alterations (Ferraro et al. 2011). Our findings further stress the importance of the IL-22 defect, as it is a relevant alteration even *in vivo*.

Tissue infiltrating T cells in Aire-deficient mice are described as hyperactive (Devoss et al. 2008). Our findings from the patients skin are in contrast to this, with a general appearance more of an immunodeficiency. The reason for such a difference might be inter-species related, with the *Aire*<sup>−/−</sup> mice rarely having skin-related disease components (Hubert et al. 2009), or derive from a T cell population especially important in the skin local defence, such as the Th22 cells (Eyerich et al. 2009). Although the differentiation between Th17 cells and Th22 cells is not feasible from our data, there is support for the defective population to be specifically the Th22 cells. Studies on autoantibodies in APECED show that IL-22 is more often the target than IL-17 (Kisand et al. 2010, Puel et al. 2010). Stimulation studies on PBMC have been conflicting in the defect of IL-17 production, but clear on IL-22 (Ng et al. 2010, Ahlgren et al. 2011). In humans, the production of IL-22 in healthy individuals has been attributed to the Th22 cells more than to the Th17 cells (Sonnenberg et al. 2011). In our study, the defect was solid in IL-22 production, and although IL-17 was somewhat affected as well, together these findings favour a Th22 defect. Since the defect appears with stimulation in PBMC and on the other hand resides long-term in the steady-state T cells of the unexposed skin, it is likely to reflect the presence of altered Th22 cells in both the peripheral blood and the skin, and recruitment from the peripheral blood leads to an accumulation of the defect in the skin.

What causes this defect? We find it conceivable that the autoantibodies impinge on the production of IL-22: In IL-17 and IL-22 production there is a special feedback loop, where IL-22 increases the production of IL-6 by the epithelial cells, which then leads to increased differentiation into Th17 and Th22
cells (Sonnenberg et al. 2011). Autoantibodies against IL-22 could therefore imbalance this feedback, resulting in decreased production of IL-22.

Another source of the defect might be altered interaction with Tregs. Th17 cells and Tregs are known to have a close relationship, emphasized by the recent finding that the consumption of IL-2 by Tregs is needed for Th17 differentiation (Chen et al. 2011, Pandiyan et al. 2011). In APECED, the stimulation of PBMC causes a higher level of IL-2 to accumulate in the supernatant than in healthy controls, implying that the uptake of IL-2 might be disturbed (Ahlgren et al. 2011). We briefly analyzed also the frequency of Tregs in the PPD exposed skin of the subjects compared to the peripheral blood, and found that the accumulation of activated Tregs to the skin was apparent, with no significant differences between the patients and the controls (data not shown). The FOXP3 MFI of Tregs in the PPD exposed skin was 1600 ± 887 in the patients vs. 2253 ± 1608 in the controls, and although statistically unsignificant with the current data set, suggested a similar failure of Treg function also in the skin. This might have an impact on the Th22 cells, although further investigation is clearly needed.

Altogether, the IL-22 defect is likely to be involved in the CMC the patients suffer from, and raises interest on mucosal surfaces in APECED as a source of immune dysregulation. On the barrier surfaces, Th differentiation to Th17 and Th22 is especially important, and mishandling of microbial exposure might maintain this dysregulation. Recently, AIRE was shown to associate in monocytes with Dectin-1, an innate immune receptor participating in defence against Candida species, which could offer an additional mechanism of constant Candida exposure in APECED (Pedroza et al. 2012).

Could IL-7 dysregulation contribute even to the defect of IL-22 production? CD127 polymorphism -related susceptibility to MS has been given an explanation through the effects of IL-7 on specifically Th17 cells. In the experimental autoimmune encephalomyelitis -mice, IL-7 -mediated survival was essential for Th17 cells but not for Th1 cells, suggesting that a special relationship in this autoimmune setting existed (Liu et al. 2010). We didn’t determine cytokine levels from the skin, so for APECED this is merely speculation. As to the effects of IL-7 on the CD8+ T cells in the PPD exposed skin, our analysis implicated similarities in CD127 downregulation in CD8+ T cells (median MFI 77 in the patients vs. 357 in the controls) and an interesting accumulation of the naive CD8+ T cells (median frequency 15.2 % in the patients vs. 2.5 % in the controls), but the differences were not statistically significant, most probably due to the small data set. Extending the study might therefore reveal additional factors involved in perturbing naive CD8+ T cell homeostasis in APECED.
Our study on the skin is to our knowledge the first report on \textit{in vivo} \(\text{CD}4^+\) T cell responses in APECED. The number of patients in our study was small, to which the unexpected ophtalmological complications seen in two of the patients contributed. Because of the small number of patients, we probably failed to detect subtle abnormalities affecting the patients’ local responses. However, the consistency of the findings that arose from the data renders them all the more important. We decided to include the one patient with no clinical skin reaction in the data because of the relapse of keratoconjunctivitis, which inferred that the patient had underlying immunity to PPD. The choice of antigen exposure in our experiment, PPD, can be questioned, since in APECED there is no known immunodeficiency against \textit{Mycobacteria}-induced infections. Indeed, we were motivated to model the normal memory response in APECED. Also, this experiment being the first \textit{in vivo} experiment on the patients, we found it safer to start with an antigen unaffected by the disease process. The experimental setup created a memory response because of childhood Bacillus-Calmette-Guérin vaccination, and therefore it was found suitable for studying local memory responses. Our data revealed a constant defect present already in the unexposed skin, which further diminished the role of the antigen choice.

4 KINETIC MODEL OF APECED PATHOGENESIS – IMPLICATIONS FOR MODELLING COMMON AUTOIMMUNE DISEASES

The key question remains, what are the essential requirements for reaching clinical APECED? One study approached this question by investigating the role of the timing of Aire expression in preventing autoimmunity by a doxycycline-controlled \textit{Aire}-transgene system in Aire-deficient mice, and found that neonatal expression of Aire was both sufficient and necessary for preventing autoimmunity (Guerau-de-Arellano et al. 2009). However, a case-report on a 64-year-old woman argues against such a short time-window of significance in AIREs function: this woman developed first hypoparathyroidism, then under follow-ups other symptoms of APECED, and she was diagnosed with an acquired form of this disease. After this the doctors discovered that she had a large thymoma with no functional AIRE detectable within, and concluded that the loss of negative selection even at this age leads to APECED. (Cheng et al. 2010). The size of the tumour here separates this case from thymomas in general, where normal thymic tissue is usually retained despite the tumour. Although this is only one case report, the clear phenotype seems evident enough to defend an impact on tolerance by AIRE in the elderly as well.
Our results together with previous studies and theories allow for an overall hypothesis on the pathogenesis of human APECED (Figure 15). Autoreactive T cell clones escape the negative selection process, and by the summative additional effects of lack of AIRE and IL-7 dysregulation, which could originate from type 1 IFN-mediated downregulation of CD127, the close-to-mature CD8+ T cells are activated inside the medullary thymus and gain features of altered homing and increased effector capabilities. These cells appear in the periphery as autoreactive TEMRA CD8+ cells. The CD4+ T cells are affected by the IL-7 dysregulation as well, but with less apparent consequences. Tregs fail in a cumulative way with aging to hinder the autoreactive T cells, because of the thymically imprinted attrition and failure of peripheral activation, which might be due to thymic alterations alone or contributed to by the absence of peripheral AIRE. Lack of IL-22 further perturbs the peripheral T cell balance, because of the continuous microbial exposure resulting from its absence.
Figure 15. A hypothetical kinetic model of APECED pathogenesis. AIRE deficiency in the thymus affects the developing Tregs that leave the thymus with an imprinted failure to thrive in the periphery, manifesting as increased turnover and a decrease in the frequency of RTE Tregs. Loss of AIRE also gives rise to autoreactive T cell clones escaping negative selection in the thymus, and affects the milieu of the thymus by alarming DCs to produce more type 1 IFNs. These cytokines cause CD127 downregulation on the T cells, and the autoreactive T cells gain a lowered threshold of TCR signalling and proliferate more in response to self-antigens. In the periphery, the emigrated autoreactive T cells are further maintained because of the IL-7 dysregulation and the effects of the IL-22 defect. The IL-22 defect, resulting from the Th17 autoantibodies (marked here as autoAbs), increases microbial exposure, maintains CMC and can imbalance the immune regulation even further. The activated Tregs fail to reach a sufficient suppressive capacity, which might be the result of peripheral AIRE deficiency. As a result, the clinical outcome of human APECED has its onset, as the autoreactive T cells overcome the suppressive effect of functionally declining Tregs, and with the impaired recruitment of functional Tregs because of the disturbed RTE Treg homeostasis, additional disease components emerge with time.

This model is obviously shadowed by the lack of significant correlations between the alterations in CD8+ T cells, Tregs and IL-22. If their absence is not caused by limited data sets, the correlations might arise and the model hold true through a mediating factor yet unmeasured from the perspective of homeostasis, such as the APCs. Support for the kinetic nature of APECED to stem from Treg attrition can be found from a recent study on inducing experimental autoimmune myasthenia gravis in Aire deficient mice: this study detected an age-related increase in susceptibility, which inversely correlated with the frequency of Tregs (Aricha et al. 2011).

Our study identified a homeostatic defect of T cells that is involved in both Tregs and autoreactive T cells. Usually autoimmunity has been explained by factors affecting either side of the pathogenesis separately, and therefore we regard our overall findings rather unique. Although the drasticity of our findings
is probably made possible by the severity of APECED, implications for more common autoimmune diseases are not far-fetched.

CD127 polymorphisms not only have been identified as susceptibility factors in MS (Peltonen 2007), but elevated IL-7 levels are reported in patients with a more severe form of MS. Also rheumatoid arthritis and colitis associate with IL-7 regulation. (Capitini et al. 2009). Our study suggests that the biological consequences of this susceptibility should be sought from the naive T cell and TEMRA homeostasis. The intrathymic effects of IL-7 in CD8+ T cell and Treg homeostasis in MS would also be worth studying, since the RTE Tregs already seem to associate with IL-7 in this disease (Haas et al. 2011). Transciptional profiling of CD8+ T cells in two human systemic autoimmune diseases, namely systemic lupus erythematosus and antineutrophil cytoplasmic antibody-associated vasculitis, identified poor prognosis to be connected with the IL-7/CD127 pathway and TCR signalling, and expansions of the memory population (McKinney et al. 2010). Although their findings have features not evident in our data, the same mechanisms seem to arise in APECED and in these systemic autoimmune diseases, originally perceived quite different in their pathogenesis. Whether Th22 cells are implicated also in other organ-specific autoimmune diseases is another intriguing question. Finally, the role of thymic disturbances in common autoimmune diseases is to be investigated further based on the mechanistic cues presented in APECED homeostasis.
CONCLUDING REMARKS

Despite the clear monogenic background, the pathogenesis of human APECED has remained unsolved. AIRE has been implicated in functions much broader than originally thought possible for a transcriptional regulator of peripheral tissue restricted antigen expression, and the significance of the peripheral lack of AIRE in APECED remains to be established. This study found multiple defects of T cell homeostasis in APECED patients, which are highly conducive to autoimmunity. These homeostatic defects appear to be partially imprinted in the thymus, and partially suggest the influence of peripheral AIRE deficiency, for example in the failure of peripheral activation of Tregs.

To gain comprehensive understanding on disturbances in T cell homeostasis in APECED, further studies on other cytokines, such as IL-2, TSLP and IL-15, are needed. Our study on Tregs indicated that they were not under an altered influence from TCR signalling, and therefore, to understand the peripheral defect of activation, signalling by the key Treg-cytokine IL-2 should be further investigated. On the other hand, studying peripheral conversion of Tregs in APECED, and the possible alterations in this due to the peripheral AIRE deficiency, could bring further understanding on the failure of Tregs. The findings on IL-7 dysregulation were clear, but could still be influenced by other cytokines such as TSLP that uses the same receptor as IL-7, and IL-15, which is also very important for the maintenance of T cells in the periphery. Also, the effects the lack of IL-22 might have on Tregs and autoreactive CD8+ T cells in APECED need to be addressed further.

From the perspective of therapies, this study supports further research on the means of balancing the homeostatic milieu of T cells in APECED, which might be a strategy easier to implement than replacing the dysfunctional AIRE gene or otherwise repairing the process of negative selection.
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