**Nora Pöntynen**

# OF TOLERANCE IN MICE AND MEN – STUDIES IN APECED AND AIRE

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Department of Molecular Medicine, National Public Health Institute
*and*
Department of Medical Genetics, Helsinki
*and*
Helsinki Biomedical Graduate School, University of Helsinki

Helsinki, Finland 2008

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# OF TOLERANCE IN MICE AND MEN -

# STUDIES IN APECED AND AIRE

A C A D E M I C  D I S S E R T A T I O N

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National Public Health Institute, Helsinki, Finland

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*“Of all the mysteries in modern science, the mechanisms of self versus nonself recognition in the immune system ranks at or near the top.”*

D.E.Koshland Jr., *Science,* 1990

*For science,*

*and my grandfathers Olof Ahotupa and Unto Pöntynen who shared my interest in science.*

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# ABSTRACT

Autoimmune diseases affect 5 % of the population but the mechanisms of onset remain unresolved. This thesis work investigates the immunopathology of autoimmunity by studying the APECED disease and the AIRE gene. APECED (autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy) is one of the few monogenic autoimmune diseases. The APECED disease phenotype consists of a multitude of different disease components affecting especially the endocrine glands. The gene mutated in APECED has been identified and is called AIRE (from autoimmune regulator). AIRE plays a role in central tolerance in the control of expression of tissue specific antigens (TSAs) to developing T cells. T cells reactive to these TSAs are eliminated in the establishment of immunological tolerance. AIRE also seems to have a function in peripheral tolerance.

In APECED, a specific set of autoantigens is the target of autoantibodies causing immune destruction. A mouse model of APECED, in which the mouse homologue of AIRE, Aire, is knocked out, has been previously constructed and characterized preliminarily. Using this mouse model, this study established that the Aire deficient mice do not produce autoantibodies against the murine homologues of the human APECED autoantigens. Thus, Aire may have a different immunopathogenic mechanism than AIRE, and care must be taken when interpreting data from this mouse model. The role of AIRE in peripheral tolerance in dendritic cells (DCs) was also a subject of study. APECED patient derived DCs showed decreased cytokine production and analyses of transcript profiles showed differences compared to healthy controls, with reductions in immunologically relevant pathways in APECED DCs. These results show that AIRE does affect DCs and confirms that AIRE is involved in peripheral tolerance, in addition to central tolerance. AIRE affects the development of the major T cell subset, αβ T cells. However, whether AIRE is involved in γδ T cell development is not known. The γδ T cells from Aire deficient mice and APECED patients were analysed and compared to wt mice and healthy controls, respectively. The analyses showed no significant differences in the γδ T cells in absence of AIRE/Aire. Therefore, AIRE/Aire does not affect γδ T cells and further, this T cell subset does not seem to be involved in the loss of tolerance causing autoimmunity in APECED.

This thesis work showed that autoimmunity in Aire deficient mice differs from that seen in APECED patients, as the mouse autoantibodies target different autoantigens. AIRE deficient DCs are defective, but AIRE/Aire does not affect the γδ T cell subset in mice or men.

Keywords: APECED, APS-1, AIRE, autoimmunity, tolerance, gamma delta T cell, dendritic cell, autoantibody, mouse model

Nora Pöntynen, Tolerenssi ihmisissä ja hiirissä – tutkimuksia APECED taudista ja Aire-geenistä
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# TIIVISTELMÄ

Autoimmuunitauteja sairastaa 5% maailman väestöstä, mutta tautien syntymekanismi on tuntematon. Tässä väitöskirjatutkimuksessa selvitetään autoimmuniteetin immunopatologiaa tutkimalla APECED-tautia ja AIRE-geeniä. APECED (autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy) on yksi harvoista monogeenisista autoimmuunisairauksista. APECEDin taudinkuva koostuu monista eri tautikomponenteista, jotka vaikuttavat erityisesti hormonirauhasiin. APECED-taudin tiedetään johtuvan mutaatioista AIRE-geenissä (autoimmune regulator). AIRE toimii sentraalisessa toleranssissa ja kontrolloi monien kudos-spesifisten geenien esittelyä kehittyville T-soluille. T-solut jotka reagoivat omia kudosmolekyylejä vastaan poistetaan immunologisen toleranssin kehittyessä. AIRElla on ilmeisesti funktio myös perifeerisessä toleranssissa.

Autoimmuunitaudit johtuvat autovasta-aineista jotka aiheuttavat kudostuhoa ja APECEDissa esiintyy autovasta-aineita tiettyjä kohdeaineita vastaan. Aire-poistogeenistä APECEDin hiirimallia on tutkittu alustavasti. Tässä väitöskirjatyössä tutkittiin muodostuuko kyseisellä hiirimallilla autovasta-aineita samoille kohteille kuin ihmisten APECED-autovasta-aineet. Mallissa ei muodostunut autovasta-aineita samoja kohteita vastaan kuin APECED-potilailla. Aire aiheuttaa autoimmuuniteettiä ehkä eri mekanismilla hiirissä kuin ihmisissä, joten tämän hiirimallin tulosten tulkinnassa on syytä noudattaa varovaisuutta. Väitöskirjatyössä tutkittiin myös AIREn tehtävää perifeerisessä toleranssissa dendriittisoluissa. APECED-potilaista tuotetut dendriittisolut tuottivat vähemmän sytokiinejä ja genominlaajuinen ekspressioanalyysi näytti, että immunologisesti tärkeiden geenien ilmentyminen oli erilainen kuin terveiden verrokkien. Immunologisesti tärkeitä geenejä tuotettiin potilassoluissa vähemmän kuin verrokkien dendriittisoluissa. Tulokset osoittavat, että AIRE vaikuttaa dendriittisoluihin ja tukee aiempia löydöksiä siitä, että AIRE toimii sentraalisen toleranssin lisäksi myös perifeerisen toleranssin säätelyssä. AIRE vaikuttaa T-solujen kehitykseen. Tähän mennessä on tutkittu vain αβ-T-soluja. Vielä ei kuitenkaan tiedetä vaikuttaako AIRE myös γδ-T-soluihin. Työssä tutkittiin γδ-T-solupopulaatiota Aire-poistogeenisissä hiirissä ja APECED-potilaissa ja verrattiin niitä viilityypin hiirin sekä terveisiin verrokkeihin. Tuloksissa ei näkynyt

merkitseviä eroja γδ-T-soluissa AIREn/Airen puuttuessa. AIRE/Aire ei täten vaikuta γδ-T-solujen kehitykseen eivätkä γδ-T-solut aiheuta myöskään autoimmuuniteettiä joka ilmenee APECEDissa.

Tässä väitöskirjassa havaittiin että autoimmuuniteetti Aire-poistogeenisissa hiirissä eroaa APECED-potilaiden autoimmuuniteetista, sillä hiirten vasta-aineet kohdistuvat eri autoantigeeneihin kuin potilaiden autovasta-aineet. AIREn puuttuessa dendriittisolut toimivat heikosti, mutta sen sijaan AIRE/Aire ei vaikuta γδ-T-soluihin hiirissä eikä ihmisissä.

Avainsanat: APECED, APS-1, AIRE, autoimmuuniteetti, toleranssi, gamma delta T solu, dendriittisolu, autovasta-aine, hiirimalli

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AA | alopecia areata |
| AADC | aromatic L-amino acid decarboxylase |
| AIRE | autoimmune regulator |
| ALPS | autoimmune lymphoproliferative syndrome |
| ANA | anti-nuclear antibody |
| APC | antigen presenting cell |
| APECED | autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy |
| APS | autoimmune polyendocrine syndrome |
| cDNA | complementary DNA |
| CTLA | cytotoxic T lymphocyte antigen |
| CMC | chronic mucocutaneous candidiasis |
| CXCL | chemokine ligand |
| DC | dendritic cell |
| EAE | experimental autoimmune encephalomyelitis |
| FACS | fluorescence activated cell sorter |
| FoxP3 | forkhead box p3 transcription factor |
| GAD | glutamic acid decarboxylase |
| gld | generalized lymphoproliferative disease |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| GO | gene ontology |
| HLA | human leukocyte antigen |
| IA-2 | islet cell antigen-2 |
| ICA | islet cell antigen |
| IFN | interferon |

| | |
|---|---|
| Ig | immunoglobin |
| IL | interleukin |
| IFN | interferon |
| IPEX | immunodysregulation, polyendocrinopathy, and enteropathy, X-linked |
| kb | kilobase |
| kDa | kiloDalton |
| LPS | lipopolysaccharide |
| lpr | lymphoproliferation |
| MHC | major histocompatibility complex |
| mAb | monoclonal antibody |
| moDC | monocyte-derived dendritic cell |
| mTEC | thymic medullary epithelial cell |
| mRNA | messenger ribonucleic acid |
| NALP | NACHT leucine-rich-repeat protein |
| NF-κB | nuclear factor kappa B |
| NK cell | natural killer cell |
| NOD | non-obese diabetic |
| OMIM | online Mendelian inheritance in man |
| PAH | phenylalanine hydroxylase |
| PBL | peripheral blood leukocyte |
| PBMC | peripheral blood mononuclear cells |
| P450c17 | steroid 17-α-hydroxylase |
| P450c21 | steroid 21-hydroxylase |
| P450scc | cholesterol side-chain cleavage enzyme |
| PCR | polymerase chain reaction |
| Q-RT-PCR | quantitative, reverse transcriptase-PCR |

| | |
|---|---|
| SLE | systemic lupus erythematosus |
| SNP | single nucleotide polymorphism |
| SOX | Sry-related HMG-Box |
| SS | Sjögren’s syndrome |
| T1D | type 1 diabetes |
| TCR | T cell receptor |
| TG | thyroglobulin |
| TH | tyrosine hydroxylase |
| TNF | tumor necrosis factor |
| TPH | tryptophan hydroxylase |
| TPO | thyroid peroxidase |
| Treg | regulatory T cell |
| TSLP | thymic stromal lymphopoietin |
| VCAM | vascular cell adhesion molecule |
| wt | wild-type |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

**I** **Pöntynen Nora**, Miettinen Aaro, Arstila T. Petteri, Kämpe Olle, Alimohammadi Mohammad, Vaarala Outi, Peltonen Leena and Ulmanen Ismo. Aire deficient mice do not develop the same profile of tissue-specific autoantibodies as APECED patients. *Journal of Autoimmunity* 2006 Sep;27(2):96-104

**II** **Pöntynen Nora**, Strengell Mari, Sillanpää Niko, Saharinen Juha, Ulmanen Ismo, Julkunen Ilkka and Peltonen Leena. Critical Immunological pathways are Down-Regulated in APECED patient Dendritic Cells. *Journal of Molecular Medicine* 2008 Oct;86(10):1139-52

**III** Tuovinen Heli*, **Pöntynen Nora***, Gyllling Mikhail, Kekäläinen Eliisa, Perheentupa Jaakko, Miettinen Aaro and Arstila T. Petteri. Gamma delta T cells develop independently of Aire. Submitted

*These authors contributed equally to this work.

# INTRODUCTION

The immune system protects organisms against foreign invaders such as bacteria, viruses, and parasites. However, what is foreign and what is part of self is an important distinction to be able to make in order to protect self while fighting off non-self.

Cells of the immune system are taught to learn the difference between self and non-self molecules. Being able to distinguish between these two is termed immunological tolerance. If this process, or education, goes wrong, the immune system can attack its own tissue and cause destruction resulting in autoimmune diseases (AID). Different autoimmune diseases exist and comprise sustained attack against different targets such as certain molecules expressed only in certain tissues, or more systemic targets present in several locations of the body. How tolerance is established is still unknown. It has been a "conundrum, which has irritated immunologists for years" (Hollander 2007).

APECED is an autoimmune disease which is caused by mutations in the AIRE gene. How "malfunction" of one gene can cause such a large variety of symptoms is bewildering and still not fully understood.

The aim of this study has been to shed light on how APECED and further, how autoimmune diseases in general, arise. This question has been attacked by several different approaches. Recent studies have shown that autoimmune regulator (AIRE), the gene mutated in APECED, which is involved in central tolerance, may also play a role in the periphery. First, we have studied whether AIRE affects the development of γδ T cells in the thymus, since it is known AIRE is important for αβ T cell development. Second, we examined the role of AIRE in peripheral monocyte-derived dendritic cells. Third, we have analyzed the autoantigens produced by Aire deficient mice. Aire deficient mice are a murine model of APECED and a valuable tool for studying disease mechanism. We have studied the autoantibodies in order to further characterize the mouse model, to find out whether the same set of genes are targeted by autoantibodies in mice and men, and whether the immunopathology is equivalent in these two species.

# REVIEW OF THE LITERATURE

## 1 THE IMMUNE SYSTEM

"Immunology is the study of the body's defence against infection" (Janeway et al. 2008). The immune system has evolved to take care of the defence against a wide range of invading pathogens and micro-organisms. In multicellular organisms it is a very complex system of immune cells and molecules whose functions are to protect the organism, or host from harmful pathogens and the damage they cause.

The first layer of the immune system is the surface of an organism, such as skin or mucosa. If an invading pathogen can pass this first barrier, it is met by two systems described below.

The immune system is classically divided into two categories: the innate immune system and the adaptive immune system. However, there is overlap between these categories and the innate and adaptive immune systems also work together (Ochsenbein and Zinkernagel 2000). The innate immune response is responsible for the first attack against and elimination of invaders entering the body. Its response is similar to every re-invasion by the same kind of invader, no matter how many times there has been a previous encounter. It is fast and not very specific, whereas the adaptive immune system, whose task is to recognize and destroy invaders which have escaped or survived by the innate system, takes longer to get activated. It is characterized by specificity and memory. The adaptive immune system is able to adapt to re-invasion; it can strengthen and accelerate its defence against towards re-invasion by the same type of invader (Janeway 2008).

### 1.1 Components of the immune system

The immune system contains a multitude of different cells and molecules with specific functions and characteristics. The main cell types of the immune system can be seen in Figure 1.

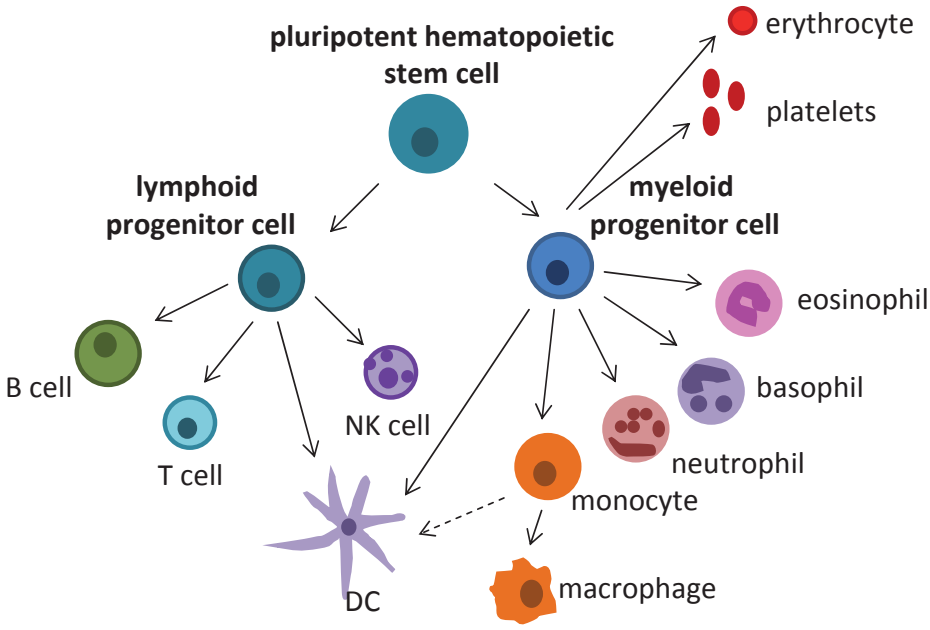



**Figure 1.** ***The human immune cells. Leukocytes differentiate from pluripotent hematopoietic stem cells and divide to lymphoid and myeloid lineages. T and B cells, NK cells and pDCs differentiate from lymphoid lineage progenitors. Monocytes, macrophages, granulocytes and mDCs develop from myeloid lineage progenitors. Modified from Immunobiology (Janeway 2008).***

The innate immune system in made up of cells such as neutrophils, basophils, eosinophils, monocytes, macrophages and natural killer (NK) cells. Additionally, the complement system is important for the innate immune response.

The adaptive immune system comprises lymphocytes; T cells and B cells. These categories are further divided into more specific cell subsets. T cells express different markers according to which they are divided into helper T cells (Th), which are positive for the CD4 marker, into cytotoxic T cells (Tc) which express CD8 and into regulatory T cells. These subsets can be divided even further; for example the Th cells contain Th1, Th2 and Th17 cell populations, of which Th1 produce different set of cytokines than Th2 cells, and while Th1 activate macrophages, Th2 cells generally activate B cells and Th17 are characterized by production of IL-17. B lymphocytes are divided into naïve B cells, memory B cells and plasma cells, which are activated B cells that produce antibodies. B cells got their name from the bursa of Fabricius, a lymphoid organ of chickens. However, it can also stand for “bone marrow-derived”, as bone marrow is where B cells mature. Different cell types act as antigen presenting cells: B cells, macrophages and dendritic cells (DC).

In addition to cells, the immune system contains important secreted components such as complement, antibodies, chemokines and cytokines. Cytokines are small proteins of approximately 25 kDa in size. They are produced by several cell types, usually upon activation of the target cells, and upon binding to specific receptors, induce a multitude of responses in the cells (Janeway 2008). Chemokines, previously included in the interleukins (ILs), are cytokines belonging to a family of chemoattractant cytokines. They can be produced by many cell types as well, and recruit effector cells from the blood to sites of infection by inducing chemotaxis in cells to which they bind via chemokine receptors. Additionally, they can be involved in lymphocyte development and migration (Moser et al. 2004; Janeway 2008).

## 1.2 Antibodies and antigens

The term "antibody" was born in 1901. Previously, an "antibody" referred to an anti-“body” fitting a “body”, which were not known yet. The first comprehensive theory of antibody production was published in 1896 by Paul Ehrlich. The theory suggested that antibody producing cells have surface molecules (or side chains) which can bind to antigens. Binding to a specific side chain causes the cell then to produce more of the same side chain and to release these side chains into the serum as antibodies. Two of these postulates by Ehrlich 1) that antibodies are identical to the antigen receptors, and 2) that antigen binding triggers the synthesis of antibody with the same specificity as the receptor, are now known to be fundamentally correct. Ehrlich won the Nobel prize for Medicine or Physiology in 1908 for his side chain theory together with Elie Metchnikoff, who discovered phagocytosis (http://focosi.immunesig.org/history).

Antibodies are soluble B cell receptors. They are also called immunoglobulins (Igs). Antibodies are made up of two heavy chains and two light chains, which are held together by disulphide bonds (Edelman 1973). Both the heavy and light chains contain hypervariable regions in their amino terminal and constant regions in their carboxy terminal. The antigen recognition site is made up of the hypervariable regions from one light chain and one heavy chain. Therefore there are two antigen recognition sites per antibody. All the antibodies produced by a B cell carry the same specificity due to allelic and isotypic exclusion. Two types of light chains exist: λ and κ. There are five types of heavy chains with different constant (C) domains. These constant domains define the five major antibody isotypes. The C domain can be either: μ, γ, α, ε, or δ and they produce the Ig types IgM, IgG, IgA, IgE and IgD, respectively. Furthermore, IgGs can be IgG1, IgG2, IgG3 and IgG4 in humans and IgAs can be either IgA1 or IgA2.

## 1.3 The thymus

The immune system contains several tissues or organs which are composed of lymphoid cells surrounded by nonlymphoid cells. Lymphoid organs and tissues fall into two categories: primary (central) and secondary (peripheral) organs. Primary lymphoid organs are the sites where lymphocytes are generated and these are bone marrow and the thymus. Secondary lymphoid organs make up the tissues where immune cells are maintained and where the actual immune responses occur. These are the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT) (Janeway 2008).

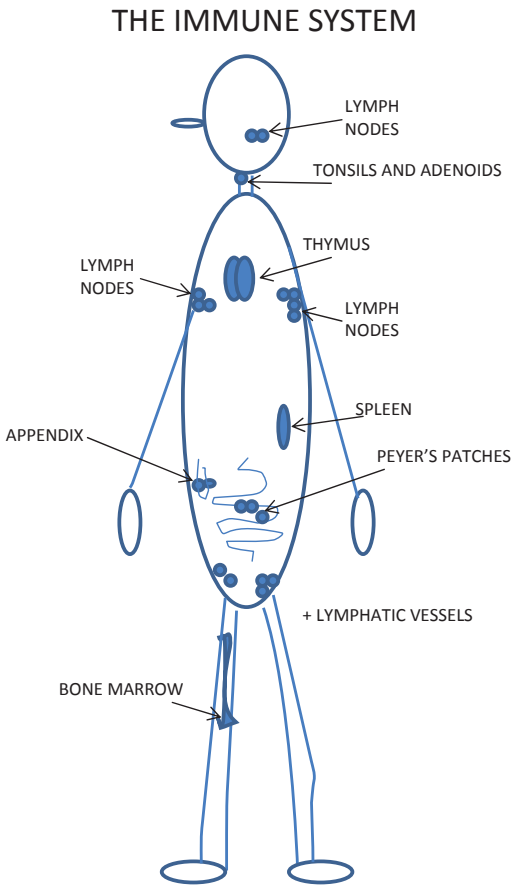


**Figure 2.** ***Central lymphoid organs are the thymus and bone marrow. The others are peripheral lymphoid organs. Lymphocytes originate from stem cells in the bone marrow and differentiate in the thymus, whereas B cells differentiate in the bone marrow. Lymphatic vessels (not shown here) drain extracellular fluid from peripheral tissues, through lymph nodes into the thoracic duct which empties into the subclavial vein. Lymphatic vessels contain fluid called lymph in which antigens and circulating lymphocytes are drained back into the blood. Lymphoid tissue is also associated with other mucosa (not show here).***

The thymus is a central organ of the immune system and it is the site for the establishment of central tolerance. Upon contact with foreign pathogens, the immune system must be capable of mounting a rapid immune response, yet be able to avoid excessive and unwanted responses, as well as reactions against self. This is attained by tolerance (Van Parijs and Abbas 1998). T lymphocytes originate from the bone marrow and migrate to the thymus to mature (hence, they are called T cells). In most mammals, the thymus is located in the thoracic cavity between the heart and the sternum. It is prominent during fetal development, but usually diminishes in size and function in adults (Janeway 2008; Rodewald 2008).

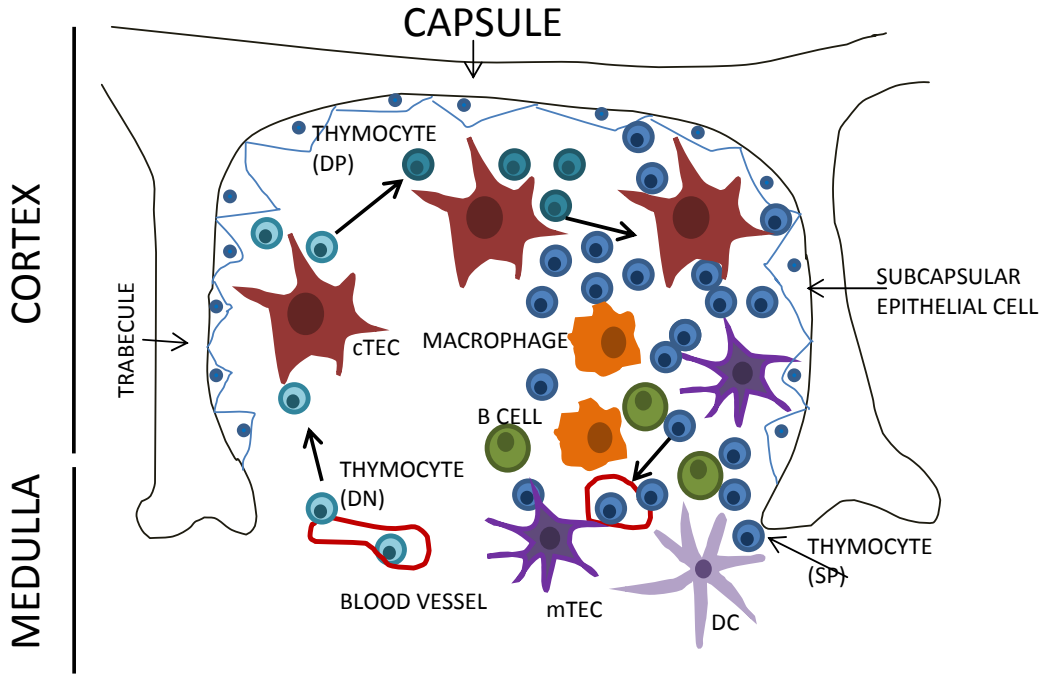
The thymus is made up of several lobules, which are each divided into an outer cortex and an inner medulla (Boyd et al. 1993). Different stages of T cells reside in specific areas of the thymus, although during the process of maturation, they move from one compartment to the other. The different areas of the thymus form distinct microenvironments and are characterized by the presence of specialized thymic stromal cells. The main structure is formed by different types of thymic epithelial cells (TECs), which are surrounded by other stromal cell types, such as macrophages and DCs. Signals from stromal cells are required for T cell development (van Ewijk et al. 1994; Pearse 2006; Takahama 2006).

Developing T cells undergo several steps during maturation, such as positive and negative selection described in more detail in chapter 2.1 on Central tolerance. Immature T cells, or thymocytes, can be found in the cortex, together with some epithelial cells and macrophages with which the T cells communicate. Positive selection occurs in the cortex. Mature thymocytes can be found in the medulla together with abundance of epithelial cells, and some macrophages and DCs. Negative selection is thought to take place in the medulla of the thymus. Thymus-specific structures called the Hassal's corpuscles are also located in the medulla. They are formed by concentric layers of epithelial cells (Boyd et al. 1993) and their importance in central tolerance was demonstrated recently (Watanabe et al. 2004). Before, it was known that Hassal's corpuscles were involved in the maturation of developing thymocytes and removal of apoptotic thymocytes. It is now known that

in humans, a chemokine called thymic stromal lymphopoietin (TSLP) is expressed by Hassal's corpuscles. TSLP activates DCs, which then help in the generation of regulatory T cells (Tregs) in the thymus (Watanabe et al. 2004). However, an identical mechanism does not exist in mice. Both DCs and Tregs are important players in tolerance and will be discussed in more detail in later chapters.

The structure of the thymus plays an important role in the development of T cells, which relocate into, within, and in and out of different thymic compartments. The development of T cells is discussed in more detail below and consists of several steps: entry of lymphocyte progenitors into the thymus, proliferation as double negative ($CD4^-CD8^-$) cells, the generation and positive selection of double positive ($CD4^+CD8^+$) thymocytes in the cortex, their differentiation into single positive cells ($CD4^+$ and $CD8^+$) in the medulla, negative selection in the medulla and finally, export of mature T cells from the thymus (Kyewski et al. 2002; Petrie 2003; Gray et al. 2005; Takahama 2006). In order to make sure this development occurs in the correct order, the developing thymocytes and the thymic stromal cells must communicate with each other. This lympho-stromal interaction is a bilateral coordination or cross-talk between the architectural stromal cells and migrating lymphocytes (van Ewijk et al. 1994; Takahama 2006). The developing thymocytes actually create their own path with these interactions and change their thymic environment in order to ensure further development. In this process, chemokines play a crucial role. They are secreted from the thymic stromal cells and recognized by receptors on thymocytes. Deficiencies in such chemokines or their receptors can cause developmental arrest of T cells (Ueno et al. 2004). Thymic stromal cells also need communication with developing T cells in order to maintain their normal organization and maturation (Shores et al. 1991).

**Figure 3.** ***The cellular composition of the thymus. The major cell types, cell-cell interactions and migratory route of developing thymocytes are shown. Lymphocyte progenitors enter the thymus through the vasculature of the cortico-medullary junction. Double negative (DN) T cells migrate towards the capsule. Conversion to double-positive (DP) T cells occurs in the outer cortex. Positively selected DP cells differentiate into single positive (SP) cells, which move to the medulla. There, autoreactive cells are eliminated by negative selection. Mature thymocytes return to the circulation. Modified from Takahama (Takahama 2006) and Kyewski and Klein (Kyewski and Klein 2006).***




Amazingly, only a few years ago, a second thymus was reported to be found in mice (Terszowski et al. 2006). These cervical lymphoid organs had a structure resembling that of thymic structure with a medulla and cortex, T cells of different developmental stages, expression of genes involved in T cell development (such as Rag1 and Rag2), DCs and endothelial cells as well as expression of Aire and Foxn1. Some differences were the number of T cells and proportion of mature T cells in cervical *versus* classical thymi. Export of functional T lymphocytes bearing different TCRs and transfer of immune competence by grafts consisting of the cervical thymus showed that the cervical thymus not only anatomically resembles a thymus but is also functional (Terszowski et al. 2006).

Cervical thymuses had, however, been known to exist in other mammals such as in marsupials. They can have either a thoracic thymus, a cervical thymus or both. Even in humans cervical thymi have been reported, but it is not known how frequent they really are (Rodewald 2008).

## 1.4 T cells

The lymphocyte has become one of the most studied of all mammalian cells (Gowans 1996). T lymphocytes can be divided into two types: αβ T cells and γδ T cells, according to their TCR. It is the αβ T cells that are divided into $CD4^+$ and $CD8^+$ cells, and subsets such as Th1 and Th2 cells. The lymphocytes that first enter the thymus give rise to both αβ T cells and γδ T cells, out of which the αβ T cells

make up the major T cell population and the γδ T cells the less well-understood, minor population (Reiner 2007).

Lymphocytes are able to recognize a massive amount of targets due to their antigen receptors which are made up of several gene segments assembled by somatic recombination. Several variable (V) gene segments, joining (J) segments and for some chains, diversity (D) segments exist and the recombination events joins any ones of these V, D and J segments together to form the variable part of the TCR. Thus different T cells bear unidentical TCRs since they are composed of different Vs, Ds and Js (*eg.* cell 1: V1D2J3 and cell 2: V5D7J1). Additionally, nucleotides can be added or removed from joining sites, or junctions between these segments, creating even more diversity (Carding and Egan 2002; Janeway 2008).

### 1.4.1 αβ T cells

αβ T lymphocytes are the major T cell population. They are the cells usually referred to when talking about T cells. αβ T cells reside mainly in lymphoid tissue, whereas γδ T lymphocytes reside mainly in epithelial tissues (Moser and Eberl 2007). About half of all T cells reside in connection with the gastro-intestinal tract.

The development of T cells was described previously, as were different types, or subsets of T cells briefly. Three signals are required for activation of naïve T cells: 1) antigen specific signals from the peptide-MHC complex with the TCR of the T cell, 2) activating co-stimulatory signals given to the T cells by the same APC (B7, or CD80 and CD86, recognized by CD28 on T cells), and 3) the cytokine milieu, important in directing the differentiation of the T cells. Activation of T cells leads to changes in their surface marker expression, cytokine production and function. CD8+ cytotoxic T cells (Tc) cause apoptosis in their targets, while CD4+ T cells are divided further into Th1 and Th2 cells, which secrete different sets of cytokines and stimulate antibody production by B cells, Th17 cells help recruit neutrophils and Tregs suppress T cell activity (Tseng and Dustin 2002; Reiner 2007; Appay et al. 2008). A whole book, or several books, could be written about αβ T lymphocytes, the different subtypes and their functions, but in this work, we focused on γδ T lymphocytes described below.

**Table 1.** ***γδ vs. αβ T cells***

| | γδ T cells | αβ T cells |
|---|---|---|
| **antigen receptor** | CD3 complex + γδ TCR | CD3 complex + αβ TCR |
| **number of possible receptors*** | ca. $10^{20}$ | ca. $10^{15}$ |
| **antigen recognition** | protein + non-protein | peptide + MHC |
| **MHC restriction** | rare | yes |
| **phenotype** | most $CD4^-CD8^-$ | $CD4^+$ or $CD8^+$ |
| **frequency in blood** | 1-5% | 65-75% |
| **distribution** | blood, epithelial and lymphoid tissues | blood and lymphoid tissues |
| **effector capacity** | CTLs, cytokine production (Th1>Th2) | CTLs ($CD8^+$), cytokine production (Th1/Th2) |
| **function** | immunoregulation and immune surveillance | immune protection and pathogen eradication |

** theoretical* Adapted from Carding and Egan, 2002

### 1.4.2 γδ T cells

γδ T lymphocytes differ from αβ T lymphocytes in their co-receptor expression pattern (such as CD4 and CD8), the types of antigens that they recognize, their distribution in the periphery and also, their function.

γδ T cells make up only 1-5% of the circulating lymphocytes, but they are more frequent in epithelial tissues, such as the skin, intestine and reproductive tract. In these tissues, they can make up to 50% of the T cells (Carding and Egan 2002). Although they are such a small population, γδ T cells have large effects; even a little population of $10^4$ cells could prevent airway inflammation and hyperresponsiveness (Hahn et al. 2003; Born et al. 2007). However, the numbers of γδ T cells vary with age and differ between the sexes (Moser and Eberl 2007).

γδ T cells develop without MHC restriction, unlike αβ T cells, and they are able to recognize both soluble protein and non-protein endogenous antigens (Carding and Egan 2002). Exactly what determines which cells become αβ T cells and which γδ T cells is not yet known. The signal strength hypothesis suggests that strong TCR signals promote γδ T development, while weaker signals promote αβ T differentiation (Hayday and Pennington 2007). During early T cell development in the thymus, waves of differentiation of different γδ subsets, expressing distinct Vγ and Vδ gene segments takes place (see Picture) (Carding and Egan 2002; Xiong and Raulet 2007). However, the exact pathway of development of γδ T cells remains unclear. They seem to emerge directly from double negative T cells, and seem to require neither a double positive stage, nor a pre-TCR (Eberl and Littman 2004). There is no consensus for whether positive and negative selection is necessary for development of γδ T cells (Xiong and Raulet 2007), although it is beginning to look

like positive selection of γδ subsets does take place in some fashion. It has even been suggested that for the intraepithelial subset, selection would be mediated by a single autosomal gene in the thymic stroma (Lewis et al. 2006). γδ T cells may also be able to leave the thymus as committed precursors and mature in the periphery (Lambolez et al. 2006). An important fact to keep in mind is also that γδ cells produced at fetal and adult stages differ a great deal in repertoire, diversity and function (Xiong and Raulet 2007).

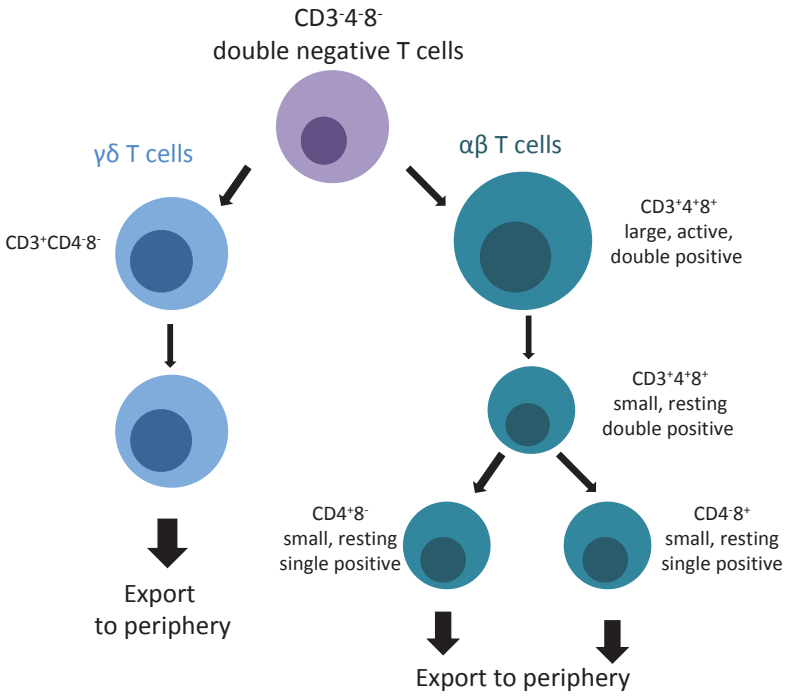



**Figure 4.** ***Two lineages of T cells are produced in the thymus. Double negative precursors give rise to γδ T cells which lack CD4 and CD8 even when they are mature. The development of γδ T cells is not well defined. αβ T cells go through the double positive stage, enlarge and divide. Then they become double positive , before selection to develop into either CD4+ or CD8+ single positive cells and increased TCR expression. Mature T cells are exported from the thymus. Modified from Immunobiology (Janeway 2008).***

There are several different groups of γδ T cells which have different functions, depending on their tissue location, antigen receptors and what part of immune response they act in (Haas et al. 1993; Carding and Egan 2002). For example, skin

γδ T cells, also called dendritic epidermal T cells (DETCs) are one such specific population, and populations of the tongue, lung and reproductive tract epithelium express “canonical” (= identical) TCRs with the same V-D-J chains and junctions. However, these cells are found only in the mouse (Havran and Allison 1990; Itohara et al. 1990). Humans, and certain other mammalian species, have a population of γδ T cells called intraepithelial lymphocytes (IELs) which reside in the epithelium and epidermis (as well as genital tract and tongue) (Hayday and Tigelaar 2003). It seems that γδ T cells belong to the group of “unconventional T cells”, together with NKT cells and mucosa-associated invariant cells (MAIT) (Hayday and Tigelaar 2003).

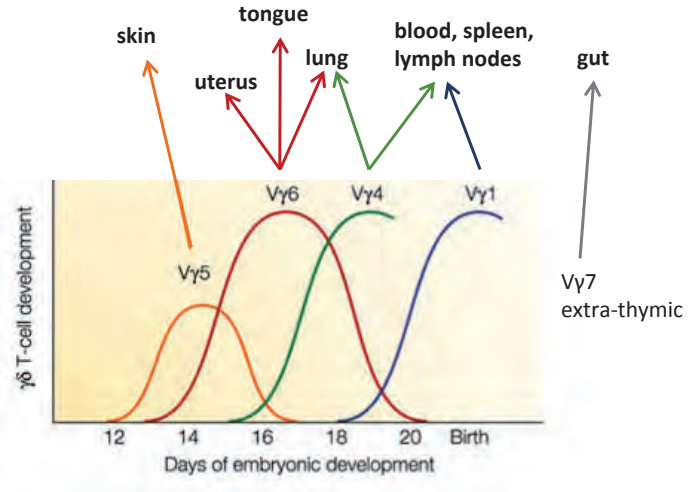



**Figure 5.** ***Mouse γδ T cell generation occurs in waves. γδ T cells with TCRs encoded by specific Vγ gene segments are exported from the thymus at defined periods of development. First, the Cγ1 locus is expressed with its closest V gene Vγ5. After a few days, cells bearing Vγ5 decline, and the next most proximal gene Vγ6 is expressed, and so on. Both rearranged γ chains are expressed with the same δ chain. These subpopulations then migrate to and populate different epithelial tissues in adult mice. The Vγ7$^{+}$ T cells may be independent of the thymus. After birth, the αβ T cell lineage becomes dominant. Modified from Carding and Egan (Carding and Egan 2002).***

γδ T cells reside mainly in epithelial tissue layers such as the skin, intestinal epithelium, lung and tongue. In these locations, they function in innate immune defence (Raulet 1989; Allison and Havran 1991; Haas et al. 1993; Hayday 2000). The TCR of γδ T cells differs in these anatomical locations and most diversity is found in the populations residing in secondary lymphoid organs (Allison and Havran 1991).

Although the developing γδ T cells have a larger genetic capacity for TCR diversity than any other lymphocytes, they do not, for some unknown reason, make use of this potential. Especially γδ T cells in epithelia show limited receptor diversity (Allison and Havran 1991). Instead, γδ T cells are often oligoclonal populations and many populations have no joining segment diversity (Asarnow et al. 1988; Itohara et al. 1990). Also the mechanisms that shape their repertoire remain unrevealed (Carding and Egan 2002).

γδ T cells recognize antigens in a different way than do αβ T cells, since γδ T cells do not need to bind MHC. The manner of recognition seems to resemble that of antibodies. Which antigens γδ T cells bind are not very well characterized, since direct binding has not been shown for many antigens. The few known antigens seem to include both constitutively expressed targets of host cells and microbes as well as inducibly expressed antigens. Especially infected, diseased or stressed cells can be recognized by γδ T cells (Allison and Havran 1991; Hayday 2000; Hayday et al. 2001). Two examples of groups of target antigens are heat shock proteins like HSP 60 (60 kD (O'Brien et al. 1992; Constant et al. 1994) and pyrophosphates and alkylamines (Constant et al. 1994; Tanaka et al. 1995; Bukowski et al. 1999). Intracellular events after the antigen binding resemble those of αβ cells (Lafont et al. 2001; Cipriani et al. 2002). Furthermore, γδ T cells recognize and respond to ubiquitous microbial products, “danger signals”, just as do cells of the innate immune system (Belles et al. 1999; Moser and Eberl 2007). Some studies even suggest that γδ T cells may be able to act as their own APCs (Wen et al. 1998; Belles et al. 1999). In any case, γδ T cells do seem to act as quite professional APCs since they produce markers common to APCs such as CD80/CD86 and CD40. Moreover upon stimulation, γδ T cells express MHC class II, more CD80/86 and CD40 as well as adhesion molecules in similar amounts as seen on DCs. γδ T cells were able to influence naïve T cells, which require APC contact, and additionally, they were able to efficiently process antigens (Brandes et al. 2005). However, how antigen uptake by γδ T lymphocytes takes place and why they act as APCs is currently not understood.

γδ T cells seem to act as a link between innate and adaptive immunity similarly as DCs (Carding and Egan 2002). γδ T cells regulate the activation of for example NK cells (Ladel et al. 1996) and may affect the nature of the αβ T cell response to either a Th1 or Th2 type response, by means of their cytokine production (Ferrick et al. 1995), usually biasing a Th1 type response (Yin et al. 2002). γδ T cells are able to induce effector-cell killing and/or cytolysis (Hayday and Tigelaar 2003) and may be able to induce tissue repair by secretion of growth factors (Jameson et al. 2002). γδ T cells have “considerable plasticity of function” and their function is in many ways dependent on the different types of activation (Carding and Egan 2002). Different γδ

populations may also function differently, even opposingly, for example some promoting and others, preventing inflammation (Hayday and Tigelaar 2003). γδ T cells influence B cells, possibly by cell-cell contact and can influence antibody production, Ig class switching and autoantibody formation in mice (Wen et al. 1994; Pao et al. 1996; Peng et al. 1996; Wen et al. 1996; Maloy et al. 1998). Additionally, γδ T cells have been reported to be in a continuous contact with DCs and other leukocytes (Wands et al. 2005) and induce DC maturation (Ismaili et al. 2002) via Fas-FasL interactions (Collins et al. 2005) (DCs are uniquely resistant to Fas-mediated cell death, and can thus be activated via Fas) (Born et al. 2007). γδ T cells also induced expression of CD86 and MHC class I on DCs (Dieli et al. 2004; Conti et al. 2005). Reciprocally, γδ T cells are also influenced by DCs; DCs induce γδ T cell proliferation and cytokine production (Kunzmann et al. 2004). Cytokine production by γδ T cells may then affect other cells types, as mentioned above. Although all these functions have been suggested for γδ T cells, it is not yet clear what exactly γδ T cells do, and which γδ T cell subset carries out which function/s.

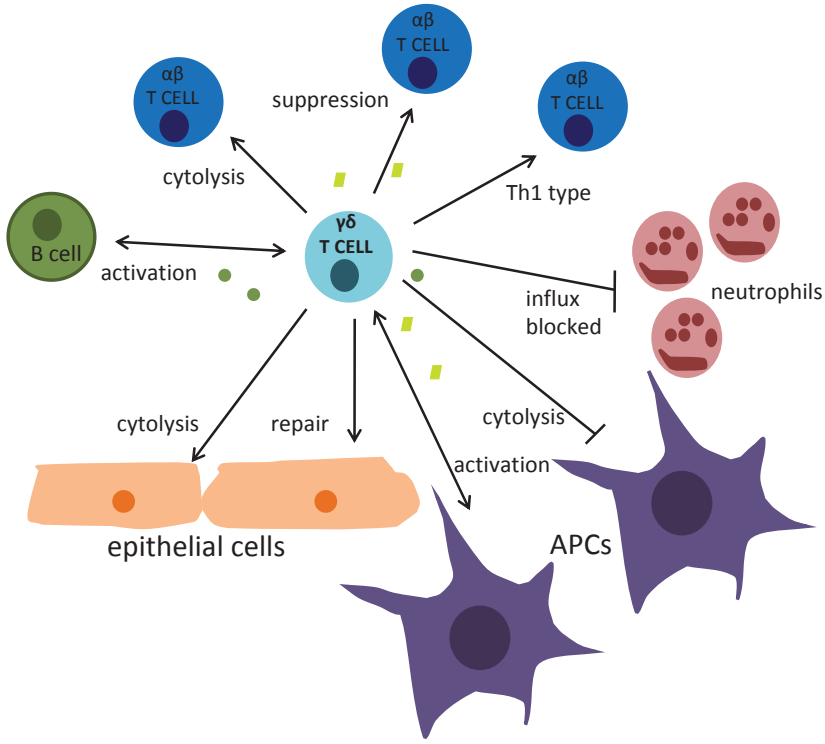


**Figure 6.** ***Mechanisms suggested for immunoregulation by γδ T cells. The functions are based on effector molecules known to be expressed by γδ intraepithelial lymphocytes (IELs), as well as gene-expression profiles of epithelium-associated γδ T cells and known γδ T cell targets. Modified from Hayday and Tigelaar (Hayday and Tigelaar 2003).***

A study on the chemokine, cytokine, and receptor expression on a subset of γδ T cells showed that most γδ T cells lack CCR7 and therefore do not home to secondary lymphoid tissues, unlike αβ T cells. Instead, γδ T cells express markers of inflammatory migration, homing them to sites of inflammation. However, upon TCR stimulation, γδ T cells switched from inflammation homing to secondary lymph node homing to sites of launching of adaptive immunity. They may be among the first cells which influence early adaptive immunity (Moser and Eberl 2007).

Due to similarities of inflammatory responses to pathogens and autoimmune diseases, the role of γδ T cells has been studied in relation to autoimmune diseases. In EAE, a subset of γδ T cells may play a pathogenic role (Olive 1997), although most studies show an immunoregulatory or anti-inflammatory role for γδ T cells (Carding and Egan 2002). In NOD mice, a population of γδ T cells can suppress development of T1D (Harrison et al. 1996). Lpr mice on the other hand, lack γδ T cells and have a more severe phenotype (Peng et al. 1996). One more example is that γδ T cells are accumulated in synovium of rheumatoid arthritis patients (Holoshitz 1999).

Recently, it has been demonstrated that γδ T cells which develop in the thymus and which interact with antigens during this development are not affected in the same way as αβ T cells are during their thymic development. Neither the number of γδ T cells nor their antigen specificity was affected by TCR-ligand interactions in the thymus. However, the γδ T cells that had interacted with antigen in the thymus developed into IFN-γ producing cells, while those that had not interacted with antigen, developed into IL-17 producing cells. In other words, thymic selection seemed to induce expression of distinct cytokine and chemokine receptors, which may further determine location of the cells. Thus, at least two functionally different populations of thymus-derived γδ T cells help control immune response (Jensen et al. 2008).

## 2 TOLERANCE AND AUTOIMMUNITY

The main function of the immune system is to fight off foreign invaders. However, another, equally important feature of the immune system is its capacity to distinguish between self and non-self. Self-tolerance is defined as the stable state in which the immune system does not react self-destructively against self-molecules,

cells or tissues (Pugliese 2004). Self-tolerance operates both centrally and in the periphery. The failure of self-tolerance leads to attack against self; against own molecules, cells and tissues causing destruction of self. Such deleterious actions are called autoimmune responses and can ultimately lead to autoimmune diseases (Pugliese 2004).

## 2.1 Central tolerance

Central tolerance is induced by negative selection of autoreactive cells, *ie.* of cells that can recognize structures of self in lymphopoietic organs.

T lymphocytes originate from the bone marrow and then migrate to the thymus to mature. The aims of T cell development are the following: 1) to commit the cells to become T lymphocytes, 2) to rearrange the T cell receptor genes in order to generate a functional TCR, 3) to determine the αβ T cell line and the γδ T cells, 4) determine T cell subpopulations by expression of CD4 or CD8, 5) to select positively for TCR so that TCRs which bind to self-antigen and present MHC molecules with low affinity and avidity are chosen and, 6) to select TCR negatively in order to delete autoreactive T cell clones (Janeway 2008).

During early maturation, T lymphocytes begin to assemble their T cell receptor variable (V), diversity (D) and joining (J) gene segments by somatic recombination. This results in a T cell population in which each T cell has a unique T cell receptor (Goldrath and Bevan 1999). T cell receptors are able to recognize processed antigenic peptides only when they are bound to MHC molecules on antigen presenting cells, such as DCs. This process is called antigen presentation. DCs determine when T cells become activated, and thus DCs act as a link between innate and adaptive immunity. Once activated, T cells begin to mature and take the shape of either Ths, Tcs or Tregs.

Upon entry into the thymus, thymocytes are double negative (DN), lacking the expression of CD4 and CD8 (Lind et al. 2001). Once the thymocytes have successfully rearranged the β locus of their TCR, they begin to express both CD4 and CD8 and become double positive (DP) cells, expressing both CD4 and CD8 (Lind et al. 2001). Positive selection occurs in the cortex after rearrangement of the TCRα locus. Thymocytes are selected according to interactions between their TCR and self-peptide – MHC ligands on the surface of thymic stromal cells. T cells need to be able to recognize antigens presented by self MHCs. The randomly rearranged TCRs can be non-functional and thus cannot bind to the MHC-peptide complexes presented to them. Thymocytes expressing such non-functional TCRs are eliminated by death by neglect. Only those cells that react weakly are allowed to move on to the

medulla, where self-antigen presentation of TSAs takes place (Starr et al. 2003). Therefore, thymocytes with low to medium affinity for self-peptide-MHC complexes are positively selected. T cells interacting with MHC class II differentiate into $CD4^+$ T cells, while those interacting with MHC class I differentiate into $CD8^+$ single positive (SP) T cells (Starr et al. 2003).

T cells which have undergone positive selection continue their education with a second step called negative selection. The order of these two processes is not necessarily chronological. Also the location of negative selection is unresolved and has been reported to take place in the cortex and medulla, but currently it appears that the medulla is favored (Kyewski and Klein 2006). During negative selection, T cells with high affinity to MHC-self-peptide complex undergo programmed cell death (Palmer 2003). Thymocytes which recognize the self-peptide-MHC complexes very strongly are destroyed, as they are potentially dangerous. Negative selection takes place in order to ensure that self-reactive T cells, which would otherwise attack self components, are eliminated (Ardavin 1997; Farr and Rudensky 1998).

The purpose of negative and positive selection is to educate the T cell population into one that is able to bind to MHC molecules only when the MHC is associated with processed foreign antigenic peptides and thus, to avoid reactions against self-peptides (Farr and Rudensky 1998). Thymic selection finally results in T cells that are both self-MHC restricted and self-tolerant and therefore, able to react against foreign antigens. Altogether, 97-99% of early, immature T cells are eliminated during these rigorous selection processes (Takahama 2006). This negative selection, by clonal deletion, is also called the development of central tolerance.

### 2.1.1 mTECs

Thymic medullary epithelial cells (mTECs) have been characterized as the cell type which express a broad range of tissue-specific genes (Derbinski et al. 2001). Initially, the ectopic expression of peripheral self-antigens in the thymus was not recognized as an important process (Linsk et al. 1989). However, the functional role for this “promiscuous”, thymic expression of tissue-specific antigens (TSA) has been acknowledged in the recent years and is now understood to be very important in the induction of T cell tolerance (Derbinski et al. 2001; Kyewski and Klein 2006). The self-antigens expressed by mTECs represent almost all of the parenchymal organs and tissues of the body and include both developmentally and temporally regulated genes (Sospedra et al. 1998; Derbinski et al. 2001; Bruno et al. 2002; Gotter et al. 2004). Some of the antigens are known to be target autoantigens in autoimmune diseases, such as insulin, glutamic acid decarboxylases (GAD65 and 67) and IA-2 in T1D, thyroid peroxidases and thyroglobulin in autoimmune thyroid disease as well as myelin basic protein in multiple sclerosis (MS) (Gotter et al.

2004). The genes promiscuously expressed by mTECs often co-localize in chromosomal clusters and they might constitute up to 10% of the genome, according to some studies (Gotter and Kyewski 2004). However, mTECs are not the only APC in the thymus. Which APCs present what and how their roles compliment each other are not yet understood. mTECs do present TSAs to T cells, but also thymic DCs obtain self-antigens from mTECs and present them to T cells (Zhang et al. 2003; Gallegos and Bevan 2004; Kyewski and Klein 2006). Furthermore, it is possible that peripheral DCs take up self-antigen and migrate to the thymus to supplement the range of TSAs presented to the developing T cells (Bonasio et al. 2006). Ectopic expression by mTECs is sufficient, but not necessary for the induction of tolerance (Derbinski et al. 2001).

Furthermore, mTECs may play a role in the selection of Tregs in the thymus and thus be involved in induction of peripheral tolerance as well as central tolerance (Klein and Kyewski 2000). Additionally, mTECs can directly eliminate T cells that express TCRs for certain self-antigens (Zhang et al. 2003; Gallegos and Bevan 2004).

Different TEC subsets have different functions. Cortical TECs (cTECs) help attract lymphoid precursors to the thymus from the blood and control the differentiation of T cells up to a point where they express the complete αβ T cell receptor complex. These T cells subsequently enter the positive selection phase, where T cells whose receptor recognizes complexes of MHC and self-peptide displayed on cTECs with enough affinity, are positively selected. Then the T cells move on to the medulla to meet mTECs and undergo negative selection (Hollander 2007).

## 2.2 Peripheral tolerance

Despite the development of central tolerance in the thymus, there is always some leakage of autoreactive cells to the periphery (Walker and Abbas 2002). Therefore, there are various other mechanisms that maintain and strengthen immune tolerance in the periphery. These means are collectively called peripheral tolerance. Peripheral tolerance can be induced by T cell anergy, by Tregs and cell death. In T cell anergy, the T cell becomes inactivated in response to an encounter with antigen. Although the T cell stays alive for some time, it is hyporesponsive (Schwartz 2003). Stimulation of T cells can also be downregulated by soluble factors by immune deviation. In immune deviation, a certain Th cell subset is preferentially activated instead of another in response to antigen (Rocken and Shevach 1996; Gao et al. 1998). For example, activation of Th1 cells instead of Th2 cells, which then produce a different set of cytokines resulting in a different outcome than if Th2 cells had been activated. A more common mechanism is elimination of autoreactive T cells by intrinsic mechanisms. One such method is activation induced cell death (AICD).

AICD is induced by cell-surface death receptors on T cells, which are triggered initially through the TCR (Nagata 1997; Thornberry and Lazebnik 1998) and results in the elimination of T cells of unwanted specificities in order to maintain peripheral tolerance (Budd 2001). Another mechanism is called activated T cell autonomous cell death (ACAD) and results from the loss of survival signals for activated T cells. It attenuates already established T cell responses. Both AICD and ACAD act through apoptosis, a precisely controlled cell death mechanism (Van Parijs and Abbas 1998; Lenardo et al. 1999; Hildeman et al. 2003).

In addition to the T cell intrinsic mechanisms of peripheral tolerance described above, also T cell extrinsic mechanisms exist. While intrinsic mechanisms act directly on responding T cells, the extrinsic regulatory mechanisms affect cells other than T cells; such as DCs or Tregs (Walker and Abbas 2002).

### 2.2.1 Dendritic cells in peripheral tolerance

Dendritic cells (DC) are the most potent of the antigen presenting cells (APC). They have the capacity to capture and process an antigen and present it to naïve T cells. DCs were first characterized in the 1972 (Steinman and Banchereau 2007).

The DCs are quite a heterogenous group of cells (Spits et al. 2000), which originate in the bone marrow from $CD34^+$ progenitor stem cells. These progenitors migrate via the blood stream into peripheral tissues where they can differentiate into either myeloid ($CD14^+$, $CD11^-$, $CD1^+$) or lymphoid precursor ($CD14^-$, $CD11c^-$, $CD123^+$) cells. Which subtype of precursor type the cells differentiate into depends on the essential growth factors that the cells encounter. Growth factors are produced by various cell types, such as endothelial cells, mast cells, keratinocytes and fibroblasts and include GM-CSF, IL-4, IL-15, TNF-α, TGF-β and IL-3 among others (Mohamadzadeh and Luftig 2004; Reis e Sousa 2006). These precursors then differentiate further into myeloid precursors and to myeloid DCs (mDCs), whereas lymphoid precursors differentiate into lymphoid (plasmacytoid) DCs (pDCs). Myeloid DCs constitute the dominant subtype in the periphery and they are considered to be responsible for efficient uptake of antigens and presentation to T cells, whereas lymphoid DCs produce significant amounts of type I interferons (Cella et al. 1999; Banchereau et al. 2000; Penna et al. 2002; Colonna et al. 2004). There are also many other differences between mDCs and pDCs, such as their migratory features. While mDCs are responsive to several chemokines, and thus are rapidly recruited to a site of infection, pDCs localize predominantly in secondary lymphoid tissues and are responsive only to CXCL12 (Penna et al. 2002).

Immature DCs reside in peripheral tissues where they ingest, accumulate and process antigens; sampling their antigenic environment. Immature DCs predominantly express molecules that enable them to recognize and phagocytose

antigens such as the macrophage mannose receptor and pattern recognizing receptors (PRR) (Adams et al. 2005) such as the Toll-like receptors (TLR) (Barton and Medzhitov 2003; Kokkinopoulos et al. 2005). TLRs are a group of transmembrane proteins that recognize pathogen associated molecular patterns (PAMPs) on invading bacteria and viruses. Immature DCs are unable to stimulate T cells (Barton and Medzhitov 2003).

The recognition and ingestion of antigen stimulates DCs to mature. The maturation process leads to the abolishment of endocytic/phagocytic receptors, upregulation of chemokine receptors and the induction of adhesion molecules. This enables DCs to migrate out of nonlymphoid tissue into secondary lymphoid tissues such as the lymph nodes (Banchereau et al. 2000; Sozzani et al. 2000) and to present antigens to naïve T cells in the T cell areas of the lymph node. Mature DCs undergo phenotypic changes of the cell surface, including the up-regulation of HLA class II molecules and several co-stimulatory molecules such as CD40, CD80, CD83 and CD86 (O'Sullivan and Thomas 2003; Adams et al. 2005). The up-regulation of HLA expression enhances antigen presentation to the T cell receptor (TCR) and its co-stimulatory molecules, and induces full activation of T cells through the T cells' CD28 receptor (Guermonprez et al. 2002). In other words, DCs display processed antigenic peptides on MHC class II molecules to $CD4^+$ T cells (Dubois et al. 1997) along with co-stimulatory signals. In addition, T cell – DC interaction is mediated by several accessory receptors and their ligands, for example CD40 and CD40L, as well as adhesion molecules (Cella et al. 1996). Along with DC maturation, the morphology of the DCs also changes. Adhesive structures are lost, the cytoskeleton is reorganized and cell motility increases (Hart 1997; Lipscomb and Masten 2002). Finally, the maturation of DCs induces the cytokine and chemokine production by DCs (Banchereau et al. 2000; Adams et al. 2005). Activated mDCs release for example IL-12, which modulates and stimulates the production of IFN-γ from T cells, whereas pDCs produce high levels of type I interferons (Mackey et al. 1998). The multitude of markers expressed on different subsets of immature DCs, mature DCs and the cytokines and chemokines produced by DCs are reviewed extensively for example by Banchereau et al. (Banchereau et al. 2000).

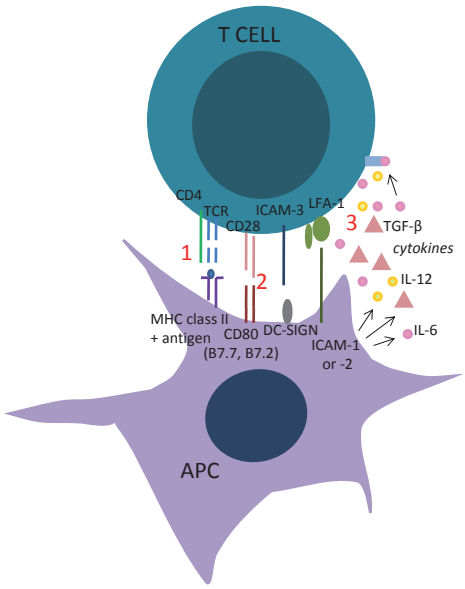



**Figure 7.** ***Three types of signals are required for activation of naïve T cells by APCs, such as DCs. Signal 1 is communicated through the foreign peptide: self MHC complex bound by the TCR and co-receptor (CD4). This signals that antigen has been encountered. Signal 2 is necessary for activation, proliferation and survival. It is mediated via a co-stimulatory signal, for example CD28 on T cells and CD80 on APCs. Signal 3 is composed commonly of cytokines and determines the direction of T cell differentiation. Modified from Immunobiology (Janeway 2008).***

The mature DCs are effective APCs and are able to induce the activation of naïve T cells and NK cells (Godfrey et al. 2000). Naïve T cells circulate in the blood, travelling/trafficking continuously to the lymphoid organs and back to the blood, making contact with thousands of APC every day. A high number of contacts is essential, as only one in $10^4$-$10^6$ naïve T cells is likely to be specific for a particular antigen, and as the T cells depend on signals for survival (Freitas and Rocha 2000). In order for a T cell to become activated and develop into effector cell, naïve T cell requires two signals from APCs in a process called T cell priming (Albert et al. 2001; Shortman and Heath 2001).

In addition to being important cells in initiating an immune response against pathogens, DCs also play a role in the induction of tolerance. Not only do DCs function in the periphery, they also reside in the thymus where they present self-antigens via MHC molecules in the thymic medulla. The importance of DCs in the thymus has been shown in studies where MHC class II molecules were only expressed in the thymic cortical epithelium, not by DCs in the thymic medulla. Such cases resulted in a high susceptibility for autoimmune disease, evidence that also DCs play a critical role in the education of T cells (Reis e Sousa 2006).

Finally and very importantly, DCs can determine when T cells become activated and what type of response they make. DCs can tolerize autoreactive T cells in the periphery by inducing deletion, anergy, or by expanding Tregs (Steinman and Nussenzweig 2002). Accumulating evidence also shows that DCs promote the differentiation of Tregs (Hubert et al. 2007). Exactly how communication between these cells occurs is not known, except that it is complex.

DCs are important cells which function in regulating the magnitude and quality of the immune response. How DCs coordinate central and peripheral tolerance is currently an active field of research. What is clear, is that defects in DC functions, which maintain tolerance, can lead to autoimmune and/or inflammatory diseases (Steinman and Nussenzweig 2002; Manuel et al. 2007).

### 2.2.2 Tregs and peripheral tolerance

Suppressor T cells and cell mediated suppression were described as early as in the 1960s, but both the cells and their function remained controversial for years (Nishizuka and Sakakura 1969; Penhale 1973; Sakaguchi et al. 1982). Finally, little by little, these cells, equipped with a new name: “regulatory T cells” (Tregs) were accepted by researchers. When it was shown that they are able to suppress the activation and proliferation of autoreactive lymphocytes (Sakaguchi 2004), their popularity rose and Tregs became a hot topic in immunology. Now several different subsets of Tregs have been described (Sakaguchi 2004).

In the past years, accumulating evidence has been obtained showing that a subset of the medium- to high-affinity, self-recognizing, developing T cells are positively selected and such cells differentiate into Tregs (Sakaguchi 2004). This distinct population of T lymphocytes usually expresses the IL-2 receptor α chain (CD25) (Sakaguchi et al. 1995) and the transcription factor FoxP3 (Fontenot et al. 2003; Hori et al. 2003; Sakaguchi 2004), in other words they are usually $CD4^+CD25^+FOXP3^+$ T cells, although new Treg markers are being found as well. New markers are needed, since it is becoming increasingly clear that FoxP3 is not a pure Treg marker, but may be expressed on non-regulatory cells as well. Tregs play a distinct role in the suppression of autoreactive T cells in the periphery (Sakaguchi 2004).

Why Tregs are so important was shown by Sakaguchi et al. (Sakaguchi et al. 1995). Their research showed that removal of the $CD4^+CD25^+$ T cell subpopulation from normal animals resulted in spontaneous autoimmune disease and importantly, that restoration of this T cell population prevented autoimmunity (Sakaguchi et al. 1995).

$Foxp3^+$ cells originate in the thymus, but the mechanisms of their differentiation are still unknown. One suggested hypothesis is that they develop in the medulla of the thymus and have high affinity to self-antigens, yet they are able to escape negative selection due to Foxp3 expression (Watanabe et al. 2005; Liston and Rudensky 2007). Additionally, other costimulatory factors and cytokines are important for Treg development. One model of the steps of Tregs development is first recognition of self-peptide with appropriate affinity, followed by exposure to cofactors leading to up-regulation of Foxp3 expression. Later, the reinforcement of Foxp3 expression serves as protection from negative selection (Liston and Rudensky 2007).

Actually, there are several subsets of Tregs (Bach 2003; Bluestone and Abbas 2003; Liu and Leung 2006), though a consensus for these has not been reached. So-called *natural* Tregs such as the $CD4^+CD25^+$ T cells develop during T cells development in the thymus (La Cava et al. 2006). *Adaptive* Tregs can be induced into Tregs from Th cells or from natural Tregs in the periphery by antigenic stimulation and cytokines (TGF-β) (Bluestone and Abbas 2003; Chen et al. 2003; Shevach et al. 2008). Adaptive Tregs are subpopulations such as IL-10 producing Tr1 cells and TGF-β producing Th3 cells (Wing et al. 2006). How each subpopulation exerts its suppressive or regulatory role is not yet clear, but one hypothesis is that naturally occurring Tregs would prevent immune responses to self-antigens and adaptive Tregs would regulate adaptive immune responses (Bluestone and Abbas 2003).

## 2.3 Autoimmunity – breakdown of tolerance

In autoimmune diseases, target organs are attacked by autoantibodies and/or by T cells (Santamaria 2001). The targets of T cells can be a single or several antigens which are restricted to a single, or multiple organs (Marrack et al. 2001; Santamaria 2001). As the autoimmune disease progresses epitopes of target molecules multiply in a process called determinant spreading (Sercarz 2000). How autoimmune destruction occurs is quite complex and varies, but it involves a range of different immune cell types and killing pathways. For example T1D seems to result from an attack of autoreactive $CD4^+$ and/or $CD8^+$ T cells on the insulin producing β-cells of the pancreas, while in myasthenia gravis (MS) and systemic lupus erythematosus (SLE) autoreactive Th2 cells induce B cells to produce autoantibodies, which cause the autoimmunity (Santamaria 2001).

Autoimmune diseases are caused when tolerance breaks down. This means that a responsiveness to self-antigens arises and sustained adaptive immune reactions result in pathology. In other words, a body's tissues are attacked by its own immune system When a T cell meets an APC, like a DC, it decides whether to initiate autoimmunity, immunity or tolerance. This decision is mediated by co-stimulatory factors which help to decide which type of reaction will follow. In the absence of co-stimulation, tolerance is induced. If APC express self-antigens and maturation signals, autoreactive T cells become activated (Lesage and Goodnow 2001; Ohashi 2002).

Autoreactive lymphocytes that have escaped the thymus are present in everyone, but autoimmunity arises only in some cases, due to a sustained attack against target antigens. See Chapters 2.2 and 2.2 for more about central and peripheral tolerance.

## 3 AUTOIMMUNE DISEASES

The failure of self-tolerance can lead to autoimmune responses, cellular or tissue destruction and ultimately manifest as an autoimmune disease (Pugliese 2004).

Paul Erlich called the phenomenon of self-tolerance "horror autotoxicus", whereby the body somehow abhorred the production of harmful "autotoxins" against self that could otherwise cause tissue damage or dysfunction. However, it took a while – several decades – until the existence of autoimmune reactions were recognized (Maslloréns 2000).

The prevalence of autoimmune diseases is estimated to be about 5% of the general population (Marrack et al. 2001). Usually, autoimmune diseases are chronic, life-long and require immune-modulatory as well as pharmacological/ replacement therapies for treatment. In 2000 there were at least 40 known or suspected autoimmune diseases (Mackay 2000). Autoimmune diseases are usually more common in women than in men (Lleo et al. 2008).

In brief, autoimmunity results from abnormal recognition of self-antigens by T cells and B cells. This usually leads to autoantibody production. The classical definition of autoimmune disease included four criteria and were later divided into three groups (Witebsky et al. 1957; Rose and Bona 1993; Marmont 1994): 1) direct evidence that the disease can be transmitted from human to human or from human to animal (by antibodies, T or B lymphocytes) 2) indirect proof by isolation of autoantibodies or autoreactive T lymphocytes, or reproducing the disease in an animal model and 3) circumstantial evidence via clinical signs such as lymphocyte infiltrates in target tissue or association with other autoimmune diseases in the same

person, or family. These criteria have been revised later, but in principle, are still valid (Rose and Bona 1993).

Normally, the immune system does not react to self-antigens. However, when immunological tolerance breaks down, autoimmune disease arises. Immune and autoimmune responses both involve the same mechanism, which are: 1) an antigen or autoantigen 2) a response by immune cells (such as T cells, B cells and APCs) 3) "messenger molecules" such as cytokine, chemokines and receptors for them and 4) costimulatory molecules on the cells involved (Mackay 2000).

Autoimmune diseases can be divided into two main categories: systemic and organ specific diseases. Organ-specific autoimmune diseases can be either organ specific destructive or organ-specific non-destructive (Davidson and Diamond 2001).

Organ-specific autoimmune diseases are characterized by the immune response being directed against a specific organ or organs, and even a certain part or cell type of an organ. For example, in T1D beta cells of pancreatic islands are affected (Marrack et al. 2001). Other examples are Grave's disease and Hashimoto's thyroiditis, both of which affect the thyroid gland. Although any or every organ can be the target of autoimmune disease, the endocrine glands are more susceptible to autoimmunity than other organs. It is not known why this is the case. The thyroid, the pancreas and adrenal cortex are examples of these autoimmune prone organs and when considered together as a group, they are responsible for half of the organ-specific autoimmune cases in Europe and in the USA (Anderson 2002).

Non-destructive autoimmune diseases involve autoantibodies which meddle with the function of an organ, but do not induce destruction (Gazda et al. 1995). Such diseases are Graves' disease and myasthenia gravis.

Systemic autoimmune diseases are characterized by the immune response directed against ubiquitous self-antigens causing destruction of a wide range of tissues. Examples of systemic autoimmune diseases are systemic lupus erythematosus (SLE) and primary Sjögren's syndrome in which tissues such as the kidney, skin and brain are affected (Coppo et al. 2003).

When a person, or other organism, is sick or under stress, lymphopenia, meaning the loss of a large number of T lymphocytes, may occur. This is followed by quick T cell proliferation in order to reconstitute the immune system. This phenomenon is known as homeostatic proliferation (Rocha et al. 1983). However, not all T cell populations necessarily expand in similar amounts and this may cause a distortion of cell populations favouring expansion of self-reactive T cell populations (Ernst et al. 1999; La Gruta et al. 2000). This in turn could be a possible explanation for

autoreactivity seen in autoimmune diseases. States of lymphocyte deprivation may therefore lead to autoimmunity (King et al. 2004).

One theory explaining the induction mechanism of autoimmune diseases is the "danger hypothesis", which states that tissue injury itself cannot cause an autoimmune reaction, but also a "danger" signal is required to be presented by APCs in order to initiate an immune response (Matzinger 1994; Matzinger 2002). Therefore, it would be more important for an organism to recognize what is dangerous and what is not, instead of what is self and non-self (Matzinger 2002).

## 3.1 Genetics of autoimmune diseases

Genetic susceptibility to, or protection from polygenic autoimmune diseases (*i.e.* autoimmune diseases with multiple causative genes) are conferred, with a few exceptions, by polymorphisms in multiple genes acting in concert. This has been shown by differences in concordance rates between monozygotic and dizygotic twins (Wandstrat and Wakeland 2001). Monozygotic twins are genetically identical, except for their BCR and TCR genes, which are subject to somatic mutation and inactivated genes on the X chromosomes in female twins (Strachan 2003). Dizygotic twins share about half of their genes with their twins, just like siblings. By comparing disease rates in monozygotic and dizygotic twin pairs, assuming they share the same environment, one can find out the proportion of a disease that is due to genetic factors. The proportion of a random sample of pairs that are concordant for a trait of interest is called concordance rate. The concordance rate, *i.e.* how much of the disease is due to genetic factors, varies between different autoimmune disorders. For example, concordance rates for monozygotic twins are 12-15% for rheumatoid arthritis (RA), 24-57% for SLE and 30-50% for T1D. In contrast, concordance rates in dizygotic twins are 3-4% for RA, 2-5% for SLE and 0-13% for T1D (Wandstrat and Wakeland 2001). Familial clustering of different autoimmune diseases and co-association of several autoimmune diseases is often observed (Vyse and Todd 1996).

Susceptibility genes can be either such that direct the response to particular autoantigens or those influencing tolerance, apoptosis, or inflammatory responses. Many common autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and type 1 diabetes also show strong association to specific MHC alleles (Theofipoulos 1995). A multitude of other genes such as cytokines, immunoglobulins, adhesion molecules and genes encoding T cell receptors have been shown to be involved in autoimmune diseases (Theofipoulos 1995). Some genes are common such as CTLA-4, the polymorphisms of which can be linked to susceptibility to Graves disease (Yanagawa et al. 1995), type 1 diabetes (Marron et

al. 1997), Addison's disease (Kemp et al. 1998) and multiple sclerosis (Ligers et al. 1999). Sex differences also exist (Lleo et al. 2008) and most autoimmune disease are more common in women.

MHC alleles and haplotypes, particularly of MHC class II genes, are associated with many autoimmune diseases (Nepom and Kwok 1998). The most known example is T1D for which there are both risk alleles and protective alleles (Redondo and Eisenbarth 2002). The mechanism/s by which MHC alleles influence disease susceptibility are unclear, but they are likely to be related to antigen presentation in the thymus and/or the periphery.

## 3.2 Non-genetic factors

In addition to genetic factors, also non-genetic factors contribute to the development of autoimmune diseases. One piece of evidence for this is that the concordance rates for monozygotic twins are far from 100% in polygenic autoimmune diseases (as mentioned above). Furthermore, the penetrance in spontaneous animal models of autoimmune disease, such as inbred rodent strains, is not complete and can be affected by changes in their environment (Shepel and Gould 1999; Lam-Tse et al. 2002). Stochastic events may also be of importance for the development of autoimmunity. For example inbred rodent strains kept under identical conditions can still display a variable phenotype. Some explanations for these stochastic factors could be differences in the randomly generated antigen receptor repertoires, variability of intracellular signaling proficiency and spatial and temporal elements (causing the "right" lymphocyte to be in the right place at the right time or not) (Germain 2001).

## 3.3 Autoantibodies in autoimmune diseases

Autoantigens are self-structures with which autoantibodies or autoreactive T cells react and which are associated with autoimmune diseases. Antibodies that react in the absence of immunization with the target are called natural antibodies and can also be found in autoimmune diseases (Lacroix-Desmazes et al. 1998; Quintana and Cohen 2004). The effector mechanisms in autoimmune diseases are mediated both through autoantibodies, as well as through T cells. These mechanisms can be classified into types II-IV with analogy to hypersensitivity reactions (Coombs 1968).

The mechanism of autoantibodies resembles the common response of an organism to antigens. Autoantibodies, like antibodies, show class-switch from IgM to IgG and other classes, the antibody genes mutate over time, they show strong specificity to their target and they undergo affinity maturation. This means that the autoantibodies

present late in a disease are more strongly reactive with their targets than early antibodies (Miller et al. 1990; Shlomchik et al. 1990). The number of targets of autoantibodies is surprisingly small; only 1-2% of the human proteins (Plotz 2003). Additionally, there is no common principle or feature that links this group of autoantibody molecules together. Also, why specific epitopes, or autoepitopes, the antigenic determinants of autoantibodies, are recognized is another key question of autoimmunity (Mackay et al. 1986, Mackay et al. 2004). Despite new knowledge on the properties, structural features and of certain groups of autoantibodies, the reasons and mechanisms for autoantibody synthesis remain a mystery (Plotz 2003).

The pathogenesis and etiology of autoimmune diseases are not understood and therefore, researchers have turned to autoantibodies in order to elucidate autoimmune mechanisms and to find out clues as to why autoimmune disease arise (Plotz 2003).

## 3.4 Monogenic autoimmune diseases

Generally, autoimmune diseases are caused by complex interactions of several different genes and environmental factors. Only a few monogenic autoimmune diseases have been described (Ulmanen et al. 2005). These include APECED (discussed in detail later), ALPS, IPEX and IL-2Rα deficiency. In these rare diseases, the malfunction of one gene is the cause of autoimmune tissue destruction. The analyses of the molecular pathology of these diseases are more straightforward than of diseases with polygenic backgrounds. Not only are monogenic diseases severe and important *per se*, what's more, studying monogenic diseases is a good way to learn more about complex diseases as well (Ropers 2007).

The disease called immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) (OMIM 304790) is caused by mutations in FOXP3 (Bennett et al. 2001; Brunkow et al. 2001; Wildin et al. 2001). As the name indicates, IPEX is inherited in an X-linked fashion and in addition, it is a recessive disease (Wildin et al. 2002). IPEX is also called XLAAD for X-linked autoimmunity-allergic dysregulation syndrome. The disease is very severe and leads to early death. The phenotype is highly variable with T1D, severe enteropathy, hypothyroidism and autoimmune skin disease among other symptoms. Additionally, for example severe allergic inflammation, secretory diarrhea, hemolytic anemia and thrombocytopenia have been described (Powell et al. 1982).

The phenotype of the disease suggested a defect in Tregs and study of IPEX indicated that Foxp3 was required for thymic development of Tregs (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). Peripheral naïve T cells may also be

induced to express Foxp3 and gain Treg function in the periphery (Kretschmer et al. 2005). Foxp3 (from forkhead box P3) is a forkhead transcriptional factor essential for Treg differentiation and function. Although the exact functional mechanisms of Foxp3 have not been specified yet, it has been shown to interact with nuclear factor of activated T cells (NFAT) and to bind directly to other target genes (Zheng and Rudensky 2007). However, FoxP3 may not be the master regulator of Tregs, but other transcriptional events may also be involved (Hill et al. 2007). Treg deficiency has been also associated with diseases other than IPEX. Such examples include rheumatoid arthritis, MS and T1D (Ehrenstein et al. 2004; Viglietta et al. 2004; Lindley et al. 2005). Although lack of Foxp3 expression alone is not sufficient to explain Treg function (Vignali 2008), studies of IPEX and its mouse model have unravelled important knowledge on Treg function and maintenance of tolerance.

Mutations in either the *Fas* (*TNFRSF6)* or *FasL* genes cause the autoimmune lymphoproliferative syndrome (ALPS-1) (Behrmann et al. 1994; Fisher et al. 1995; Wu et al. 1996) and mutations in *caspase 10* gene cause ALPS-2 (Wang et al. 1999). Fas, Fas-L and caspase 10 all belong to the same Fas-mediated pathway for apoptosis (Wu et al. 1996; Wang et al. 1999; Chun et al. 2002; Sprick et al. 2002; Walsh et al. 2003) which is important for T cell homeostasis. A mutation in *caspase 8* has recently been detected in two children of one family with ALPS (Chun et al. 2002). ALPS type III has also been described (Van Der Werff Ten Bosch et al. 2001). Type I can be divided into types Ia and Ib according to slightly different disease components (Van Der Werff Ten Bosch et al. 2001); (Rieux-Laucat et al. 2003). ALPS (OMIM 601859) is inherited in an autosomal, dominant fashion (Straus et al. 1999) and is characterized by lymphocytosis, *i.e.* accumulation of double negative T cells ($CD3^+$ TCRαβ $CD4^-CD8^-$), nonmalignant lymphadenopathy, splenomegaly and hypergammaglobulinemia. Autoimmune manifestations include autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura and autoimmune neutropenia (Straus et al. 1999). ALPS-3 is milder than the other types of ALPS (Van Der Werff Ten Bosch et al. 2001) and mutations in other than Fas-linked pathways have been suggested as causes for this disease subtype (Rieux-Laucat et al. 2003). Fas is an important factor in the regulation of T cell homeostasis, self-tolerance and stopping immune responses. Thus, defective Fas leads to deficient apoptosis, the accumulation of lymphocytes in lymphoid organs and an excess of autoreactive naïve T cells as they are not eliminated as they should be (Kischkel et al. 1995). In one SLE patient, a mutation in FasL causing defective apoptosis was found. However, FasL mutations were not found to be a common cause of SLE (Wu et al. 1996). Studying ALPS has taught us more about how the death receptor cascade functions in immunological processes (Worth et al. 2006).

IL-2Rα deficiency (OMIM 606367) causes an impairment of peripheral tolerance. Also IL-2Rα deficiency is rare and causes a multitude of immunological deficiencies and autoimmune symptoms (Sharfe et al. 1997; Ulmanen et al. 2005). IL-2Rα is also called CD25 and is important for Tregs (Sakaguchi 2005), proliferation of T cells as well as being an activation marker for T cells. In the absence of a functional CD25, Tregs were not able to maintain peripheral tolerance in the IL-2Rα deficient patients (Sharfe et al. 1997; Roifman 2000; Caudy et al. 2007). Studies of IL-2Rα deficiency have shown that CD25 is important for expression of IL-10 by $CD4^+$ T cells (Caudy et al. 2007) and have confirmed that $CD25^+$ Tregs are needed for peripheral tolerance through keeping autoreactive cells in the periphery in control (Sharfe et al. 1997; Roifman 2000).

## 3.5 Autoimmune polyendocrine syndromes

Autoimmune polyendocrine syndromes/polyglandular syndromes (APS) make up a group of rare, but unique autoimmune diseases (Schmidt 1926; Eisenbarth and Gottlieb 2004). The autoimmune polyendocrine syndromes are APS-1, -2, -3 and -4 (Neufeld et al. 1980; Neufeld et al. 1981; Brun 1982; Obermayer-Straub and Manns 1998; Betterle et al. 2002; Betterle and Zanchetta 2003). APSs tend to be associated with each other and with other organ-specific autoimmune disorders (Neufeld et al. 1980; Riley 1992).

**Table 2.** ***APS classification***

| APS SUBTYPE | DEFINING DISEASE COMPONENTS | CONDITIONS |
|---|---|---|
| APS-1 | chronic candidiasis, chronic hypoparathyroidism, Addison's disease | at least two present |
| APS-2 | Addison's disease + autoimmune thyroid diseases and/or T1D | Addison's disease always present |
| APS-3 | autoimmune thyroid disease associated with other autoimmune diseases | excluding Addison's disease and/or hypoparathyroidism |
| APS-4 | combinations not included in the groups 1-3 | |

APS-1 and APS-2 share features of their clinical phenotype, but differ in their prevalence, time of onset and inheritance (Eisenbarth and Gottlieb 2004). APS 1 will be discussed in more detail in the following sections.

APS-2 is also called Schmidt's disease, according to the discoverer of two patients with non-tuberculous Addison's disease and chronic thyroid disease by M.B. Schmidt in 1926 (Schmidt 1926). APS-2 is a polygenic disease with a combination of Addison's disease with autoimmune thyroid disease and/or T1D as the disease components. The patients do not have hypoparathyroidism or candidiasis, the onset is at an adult age and the etiology is multifactorial (Carpenter et al. 1964; Obermayer-Straub and Manns 1998; Eisenbarth 1999). APS 2 patients also have circulating autoantibodies (Song et al. 1996). APS-2 is more common than APS-1 (Guisan et al. 1969). APS-2, as well as isolated Addison's disease, show strong association with the MHC locus (HLA-DR3 and DR4 alleles) and both diseases are more common in females, as opposed to APS-1 (Eisenbarth and Lebovitz 1978; Partanen et al. 1994; Huang et al. 1996).

APS-3 is a heterogenous disease with autoimmune thyroid disease and at least one other autoimmune disorder, excluding Addison's disease. The mode of inheritance and background are unknown (Neufeld et al. 1980; Neufeld et al. 1981; Obermayer-Straub and Manns 1998). APS was classified earlier into three subtypes (Neufeld et al. 1980), but the classification has recently been modified into four subtypes (Betterle and Zanchetta 2003). The classification of APS-1 and APS-2 remain as they were before, but APS-3 is now classified into four subtypes APS-3A, APS-3B, APS-3C and APS-3D according to the different organ-specific and nonorgan-specific autoimmune diseases associated with autoimmune thyroiditis (excluding Addison's disease and hypoparathyroidism). APS-4 is a group of all the clinical associations not included in the other APS subtypes (Betterle and Zanchetta 2003). The mechanisms for the molecular pathology behind these diseases remain unknown.

## 4 APECED

APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; OMIM 240300), also called autoimmune polyglandular syndrome type 1 (APS1), is a rare, monogenic autoimmune disease caused by mutations in the Autoimmune Regulator (AIRE) gene (Consortium 1997; Nagamine et al. 1997). APECED belongs to APS disease group (Eisenbarth and Gottlieb 2004) and is an organ specific AID affecting several endocrine and ectodermal tissues and organs (Perheentupa 2002).

A patient with APECED-like symptoms was described for the first time in the literature in 1929 (Thorpe 1929). Back then, APECED was called APS1 or Whitaker's syndrome and after that, several different names were applied to this disease (Leonard 1946; Whitaker 1956; Neufeld et al. 1981; Ahonen 1985). Nowadays, only the names APECED or APS1 are used.

## 4.1 Genetics of APECED

APECED is an autosomal, recessively inherited disease (Ahonen 1985; Vogel et al. 2002). Initially, the APECED locus was mapped to chromosome 21q22.3 in Finnish patients (Aaltonen et al. 1994). In a study consisting of a multinational group of APECED patients, the same chromosomal locus was found responsible for the disease in both the Finnish patients as well as in patients of other nationalities (Bjorses et al. 1996). However, the haplotype segregation predicted different mutations in patients of different origin (Bjorses et al. 1996). The causative gene of APECED was found in 1997. The same gene was found both in Finnish patients as well as in patients of other nationalities (Consortium 1997; Nagamine et al. 1997). It was a novel gene named AIRE for Autoimmune Regulator. Confirmation for defects in AIRE causing APECED came from studies which identified *AIRE* mutations in APECED patients (Consortium 1997; Nagamine et al. 1997) (Aaltonen and Bjorses 1999).

APECED is quite a rare disease. However, it is enriched in certain isolated populations such as in the Finns (lifetime prevalence 1: 25,000) (Perheentupa 1972; Norio et al. 1973), Iranian Jews (1:9,000)(Zlotogora 1992) and Sardinians (1:14,500) (Rosatelli et al. 1998). APECED is also relatively common in Norway (Myhre et al. 2001) (1:90,000) (Kisand et al. 2008) and in Northern Italy (Betterle et al. 1998). Additionally, numerous cases have been reported in Great Britain (Pearce et al. 1998), North America (Scott et al. 1998), Eastern and Central European countries (Cihakova et al. 2001) and Japan (Kogawa et al. 2002). Among the Finnish and Iranian Jewish populations, the disease is presumably inherited from just one or two founder individuals (Bjorses et al. 1996). The phenotype differs between Finns and Iranians somewhat, as well as in other populations, partly due to the differing patient mutations in AIRE. However, different mutations do not have a great impact on disease phenotype, hence other factors, such as possible additional genes and environmental factors also affect the phenotype (Bjorses et al. 1996).

Altogether, there are about 90 patients who have been diagnosed with APECED in Finland. This is the largest population of patients and has been thoroughly analyzed and the course of their disease has been extensively followed-up (Perheentupa 2006). Due to its relative abundance in Finland, APECED belongs to the Finnish disease heritage (Nevanlinna 1972; Perheentupa 1972; Norio et al. 1973; Norio 2000).

## 4.2 Clinical phenotype

What makes this monogenic disease so fascinating is the vast and variable phenotype due to mutations in just one gene. APECED is characterized by a triad of main manifestations of which at least two must be present for diagnosis: Addison's disease (adrenocortical failure), hypoparathyroidism and chronic mucocutaneous candidiasis (CMC) (Perheentupa 2002). Additionally, as the name of the disease indicates, APECED patients develop a variable number of other symptoms affecting endocrine and ectodermal organs and tissues.

Endocrinopathies include type 1 diabetes (T1D), gonadal atrophy (testicular and ovarian failure), autoimmune thyroid disease (hypothyroidism), and gastric parietal cell atrophy (Ahonen et al. 1990; Betterle et al. 1993; Perheentupa 2002). Testicular failure occurs more commonly in older patients, whereas ovarian failure affects over 50% of female APECED patients aged over 20 (Perheentupa 2006). Autoimmune hepatitis is an additional, serious disease component that can lead to acute liver failure and even death if untreated (Perheentupa 2006).

Ectodermal disease components are typically alopecia, vitiligo, nail dystrophies (eg. enamel hypoplasia 77% prevalence), rash with fever and keratoconjunctivitis (and other eye problems even leading to blindness in 11% of the Finnish patients with eye problems) (Wagman et al. 1987; Ahonen et al. 1990; Lukinmaa et al. 1996; Perniola et al. 1998).

Mucocutaneous candidiasis can infect the oral, oesophageal and the vaginal mucosa as well as the nails. Candidiasis may even lead to squamous cell carcinoma of the mouth or of the oesophagus (Perheentupa 2006; Rautemaa et al. 2007). All Finnish APECED patients get candidiasis at some stage of their life and it is usually their first symptom. Other APECED patient populations also have high prevalences of CMC (Betterle et al. 1998; Collins et al. 2006; Stolarski et al. 2006; Wolff et al. 2007). An exception is Iranian Jews who usually do not get CMC (Zlotogora 1992).

A number of rare disease components and individual disease cases have also been described (Ahonen et al. 1990; Friedman et al. 1991; Betterle et al. 1998; Franzese et al. 1999; Makitie et al. 2006). The main clinical features of APECED are summarized in Table 3 (Ahonen et al. 1990; Myhre et al. 2001; Perheentupa 2002). Usually autoimmune diseases are more common in women than in men (Lockshin 2006). However, APECED is an exception, because it affects both men and women equally (Perheentupa 2006).

**Table 3.** ***Disease components of APECED.***

| DISEASE COMPONENT | PREVALENCE % |
|---|---|
| **Triad** | |
| Mucocutaneous candidiasis | 100 |
| Hypoparathyroidism | 79 |
| Addison's disease | 72 |
| **Other endocrine components** | |
| Gonadal failure* | 60 |
| Hypothyroidism | 4 |
| Type 1 diabetes | 12 |
| Malabsorption | 18 |
| Autoimmune hepatitis | 12 |
| Autoimmune gastritis | 13 |
| Pernicious anemia | 13 |
| **Other ectodermal components** | |
| Enamel hypoplasia | 77 |
| Alopecia | 72 |
| Vitiligo | 13 |
| Nail dystrophy | 52 |
| Keratopathy | 35 |
| *Adapted from Ahonen et al. 1990 and Perheentupa 2002* | |
| ** postpubertal individuals* | |

The course of the disease varies among patients. The onset of APECED occurs usually in childhood, by the age of five in Finnish patients (0,2-18 years) (Ahonen et al. 1985) and at the average age of 9 years in Norwegians (Wolff et al. 2007). Candidiasis appears at a very young age (peak incidence is over the first two years of life, although it may not appear until adulthood) (Perheentupa 2002), hypoparathyroidism at the age of 3-5 years, followed by adrenal failure at the age of 11-15 years (Ahonen et al. 1990). Hypoparathyroidism is more common in female APECED patients with a prevalence of 98% vs. 71% in men and also appears earlier in women (Gylling et al. 2003). Several deaths due to APECED have been described in Norwegians (Wolff et al. 2007).

Most adult patients have up to seven different disease components (some with combinations of over ten components!). Disease symptoms may manifest even more

as the patients age, since the prevalence of most components increases with age at least until the age of 60 yrs (Ahonen 1985; Ahonen et al. 1990; Perheentupa 2002; Perheentupa 2006).

The clinical picture varies from one patient to another and even siblings within one family may have a very different phenotype. Also the occurence of the different disease components vary between populations (Ahonen et al. 1990; Betterle et al. 1998; Perheentupa and Miettinen 1999; Myhre et al. 2001; Perheentupa 2002). The reasons for this complexity are not yet understood, but one suggestion is the involvement of genetic factors other than AIRE (Ahonen et al. 1990).

## 4.3 Diagnosis and treatment

In short, diagnosis is based on the clinical detection of the triad of disease components, sequencing of AIRE mutations (although this usually only covers the major mutations) and testing for APECED autoantibodies in the serum. After diagnosis, follow-up and therapy should be arranged in addition to regular monitoring of new symptoms and disease components. The major forms of treatment for APECED patients are antifungal drug therapy when required and adequate replacement therapy for endocrine organ failures, as well as specific treatment for other disease components as required. Additionally, it is important to inform patients and their families of how the disease may evolve (Perheentupa 2002; Perheentupa 2006).

## 4.4 Genetic factors affecting the APECED phenotype

In monogenic disease, the main cause for the disease is a mutation in a single causative gene, rendering it unfunctional. However, the nature of the mutation may affect the causative disease phenotype. Yet, even in monogenic diseases there are often other modifying factors that have a small or a bit larger impact on the precise disease phenotype. These factors can be other genes, for example HLA.

### 4.4.1 Correlations between mutations and phenotype

To date, over 60 patient mutations have been reported in the *AIRE* gene (Consortium 1997; Nagamine et al. 1997; Heino et al. 2001; Halonen et al. 2002; Meloni et al. 2002; Buzi et al. 2003; Harris et al. 2003; Vogel et al. 2003; Peterson et al. 2004; Meloni et al. 2005; Podkrajsek et al. 2005; Dominguez et al. 2006; Stolarski et al. 2006; Ulinski et al. 2006; Wolff et al. 2007). A mutation registry has been set up at bioinf.uta.fi/AIREbase. Mutations occur throughout the protein coding region of *AIRE* and include different mutation types: single nucleotide substitutions, small

insertions and deletions. Additionally, mutations affecting the splicing consensus sequences have been identified.

In isolated populations, where APECED is enriched, an apparent founder effect results in one common mutation in the majority of the disease alleles. The major Finnish mutation is R257X (called $Finn_{Major}$), substituting a C to T and changing arginine to a premature STOP codon in exon 6. 77% of Finnish patients analyzed are homozygotes and 17% are compound heterozygotes for R257X (Perheentupa 2006). Furthermore, about 30% of non-Finnish patients also have the R257 mutation (Bjorses et al. 2000). Thus, the $Finn_{Major}$ mutation is also the most common mutation in non-Finnish populations (Scott et al. 1998). In Sardinian APECED patients the mutation R139X dominates, comprising 90% of their mutant alleles (Rosatelli et al. 1998). Amazingly, all the Iranian Jews with APECED are homozygous for Y85C. This mutation has not been reported in any other patients from other populations (Zlotogora 1992). Another mutation common in several different APECED patient populations is the deletion (967-979del13bp) in exon 8 (Nagamine et al. 1997; Pearce et al. 1998; Scott et al. 1998; Wang et al. 1998; Heino et al. 1999; Bjorses et al. 2000; Dominguez et al. 2006; Wolff et al. 2007).

No strong correlations have been shown between the genotypes and phenotypes of APECED patients. Only an association between the lack of the $Finn_{Major}$ mutation and a decrease in the frequency of candidiasis has been shown (Halonen et al. 2002). Additionally, CMC is rare in the Iranian Jews with APECED, all of whom carry the Y85C mutation (Zlotogora 1992). The variability of APECED and the differences in the range of disease components between patients suggest that factors other besides AIRE, genetic and/or environmental, affect the disease phenotype (Halonen et al. 2002). An interesting note on the heritability of APECED is that although APECED appears to be inherited in an autosomal, recessive fashion, one Italian family with APECED carrying mutation G228W has shown a dominant negative mode of inheritance (Cetani et al. 2001). A mouse model in which the same mutation was knocked into mice caused autoimmunity in the mice and also showed a dominant negative effect (Su et al. 2008).

Most mutations in the coding region of *AIRE* are nonsense mutations which lead to a premature STOP codon. Missense mutations are also common and reside in functional protein domains of AIRE. These mutations have been utilized to analyze the functions of the different AIRE domains (Bjorses et al. 2000; Pitkanen et al. 2000; Pitkanen et al. 2001; Halonen et al. 2004; Uchida et al. 2004; Pitkanen et al. 2005).

### 4.4.2 APECED and HLA

Unlike in many common autoimmune diseases, APECED, or increased risk for APECED, has not been shown to be associated with polymorphisms in the human

leukocyte antigen (HLA) gene locus. However, individual HLA class II alleles have been shown to affect the phenotype of APECED. The strongest associations were for alopecia and Addison's disease and T1D. For T1D the association was found for a protective allele (Halonen et al. 2002). The same alleles have also been found to be associated with these disease components in non-APECED patients (Maclaren and Riley 1986; Weetman et al. 1991; Halonen et al. 2004).

## 4.5 Autoantibodies in APECED

Many of the clinical manifestations of APECED patients are associated with the presence of circulating autoantibodies directed against specific autoantigens expressed in the affected tissue. They may be a cause of target tissue destruction and these autoantibodies also often precede specific manifestation. Therefore, they are valuable in diagnosis of APECED as well as in predicting the course of the disease to some degree (Ahonen et al. 1987; Betterle et al. 1998; Betterle et al. 2002).

APECED patients produce high-titer IgG autoantibodies with high affinity for tissue-specific antigens. This has made the use of these autoantibodies valuable for identifying autoantigens important in also more common autoimmune diseases, since patients with similar individual disease components may harbor autoantibodies against the same autoantigen targets (Mirakian et al. 1998).

Using a set of three autoantibodies: P450c21, P450scc and AADC, the sensitivity of diagnosis of APECED was 89% (Soderbergh et al. 1996). Presence of antibodies against 21-hydroxylase predict the development of adrenal insufficiency (Ahonen et al. 1987; Betterle et al. 2002). Some of the APECED autoantibodies are specific for APECED, whereas others are found in other autoimmune diseases as well. A specific autoantigen is, for example, anti-tryptophan hydroxylase (TPH), whereas eg. anti-GAD-65 autoantibodies are found in T1D and anti-21-hydroxylase antibodies are typical for Addison's disease as well (Baekkeskov et al. 1990; Winqvist et al. 1992; Ekwall et al. 1998; Ekwall et al. 1999).

Many of the APECED autoantibodies are directed against intracellular key enzymes in the affected organs such as the adrenal gland, gonads and placenta (Peterson et al. 1998). The major autoantigens in APECED belong to the steroidogenic P450 superfamily. They are the steroidogenic enzymes 17-α-hydroxylase (P450c17), 21-hydroxylase (P450c21) and cholesterol side-chain cleavage enzyme (P450scc) (Krohn et al. 1992; Winqvist et al. 1992; Uibo et al. 1994; Winqvist et al. 1995), catalyzing the chemical reactions required for the production of steroid hormones like aldosterone, progesterone and cortisole (Chen et al. 1996). P450c21 and P450scc are associated with Addison's disease in APECED (Soderbergh et al.

2000), as is also P450c17 (Uibo et al. 1994). P450c21 is expressed in the adrenal cortex, while P450c17 and P450scc are expressed in the gonads as well (Peterson et al. 1995). However, P450scc alone associates with hypogonadism (Soderbergh et al. 2000). The presence of P450c17, P450scc and P450c21 precede adrenocortical and ovarian failure and therefore can be used as predictive markers for APECED (Ahonen et al. 1987).

Another group of APECED autoantigens is pteridine dependent hydroxylases and includes autoantibodies against tryptophan, tyrosine and phenylalanine hydroxylase (TPH, TH and PAH, respectively) (Ekwall et al. 1998; Ekwall et al. 2000; Hedstrand et al. 2000), although the role of PAH as an autoantibody for APECED is disputed (Ekwall et al. 2000). They are involved in the catalysis of serotonin and dopamine together with aromatic L-amino acid decarboxylase (AADC), which is also an APECED autoantigen (Husebye et al. 1997; Ekwall et al. 2000). TPH correlates with gastrointestinal dysfunction (malabsorption) and targets serotonin-secreting enterochromaffin cells of the small intestine (Ekwall et al. 1998).

Autoantibodies against cytochrome P450 enzymes 1A2 and 2A6 are present in APECED patients with chronic, autoimmune hepatitis (Clemente et al. 1997; Clemente et al. 1998), as well as AADC (Gebre-Medhin et al. 1997).

GAD65 (Bjork et al. 1994; Velloso et al. 1994) and AADC (Husebye et al. 1997) are pancreatic autoantibodies, react against islets of Langerhans and generally predict T1D. Yet, in APECED their value in this prediction is not high (Tuomi et al. 1996). In the case of GAD65 this may be due to the autoantibodies targeting different epitopes in APECED than in T1D patients (Velloso et al. 1993; Bjork et al. 1994). Additionally in APECED patients with T1D, autoantibodies against tyrosine phosphatase, islet cell antigen-2 (IA-2), insulin and/or islet cell autoantibodies (ICA) are present (Ahonen et al. 1985; Tuomi et al. 1996; Gylling et al. 2000; Klemetti et al. 2000). However, only IA-2 was shown to have a predictive value for T1D in APECED (Soderbergh et al. 2000).

Autoantibodies against hair follicles associate with alopecia totalis in APECED (Hedstrand et al. 1999). The transcription factors SOX9 and SOX10 are APECED autoantigens expressed in melanocytes and have been associated with vitiligo in APECED (Hedstrand et al. 2001).

Various other tissue-specific autoantibodies: GAD67, insulin, thyroid peroxidase, thyroglobulin and calcium-sensing receptor (Li et al. 1996; Betterle et al. 1998; Heino et al. 2001; Peterson et al. 2004) have also been identified as targets for autoimmune response in APECED. Surprisingly, APECED patients may have organ-specific autoantibodies even if they show no signs of the diseases (Soderbergh

et al. 1996). Why these specific antigens are targets of APECED autoantibodies is not understood.

# 5 MOUSE MODELS OF AUTOIMMUNITY

Mice are used in several branches of biological research in order to study different aspects that cannot be studied in humans in order to gain insight to what happens on a whole organism level. In order to study disease mechanisms, such as those for autoimmunity, animal models are valuable tools in addition to cells and *in vitro* experiments. Although lower organisms such as bacteria, worms and flies can be used for some aspects, mammalian models are needed in order to understand disease mechanisms of higher level organisms. For example one cannot study tolerance in worms lacking an adaptive immune system and no thymus is present in species which are more primitive than vertebrates (Rodewald 2008). The mouse is a widely used mammalian model animal, due to its small size, short lifespan, short generation time and similarity in some respects, and to some extent, to humans (Haley 2003).

Most human genes have mouse homologues. These homologous genes can be manipulated, for example stably or conditionally knocked-out, in order to produce mouse models of human disease. For example the Aire knock-out mouse is a model for the human APECED disease, in which the human AIRE gene is rendered unfunctional due to mutations. Genetic manipulation of the mouse has been made possible by developments in different transgenic technologies for targeted gene manipulation and the availability of embryonic stem cell lines (Glaser et al. 2005). Availability of inbred mouse strains, phenotypic databases and the mouse genome sequence are important tools for current biomedical research (Waterson et al. 2002; Guenet 2005). Analyses of immunological mouse models have helped understand more about our immune system. For example intracellular signalling pathways have been elucidated and regulatory mechanisms of autoimmunity have been illuminated (Mak et al. 2001). However, valuable as mouse models are, one must bear in mind when converting these data to humans that mice are not men (Leiter 2002; Haley 2003; Mestas and Hughes 2004).

## 5.1 Selected mouse models of autoimmunity

There are probably hundreds, if not thousands of different mouse models of some form of autoimmunity or immune deficiency. This chapter will concentrate on some interesting and relevant autoimmune mouse models. These include models for MS, SLE and T1D, which are among the most common autoimmune diseases (Morel 2004) as well as some monogenic mouse models relevant to this study.

Animal models, particularly mice in the last few decades, have become the best tool in which to study disease mechanisms and particularly those of autoimmune diseases (Morel 2004). Organ-specific autoimmune diseases contain autoantibodies against antigens confined to a particular organ, whereas systemic autoimmune diseases are characterized by antigens widely distributed throughout the body (Van Parijs and Abbas 1998). Many mouse models of autoimmunity are induced models, for example several models of gastritis (Sakaguchi et al. 1985; Field et al. 2005). Autoimmunity in mice may be induced by several methods such as by thymectomy, manipulation of thymus, or of their T cells, by immunization etc. Induced mouse models will not be discussed here.

### 5.1.1 NOD mice

Type 1 diabetes (T1D), also called insulin-dependent diabetes mellitus or juvenile diabetes, is one group of glucose intolerance syndromes. The non-obese diabetic (NOD) mouse is probably one of the most well-known models of autoimmune disease. This mouse is used as a model of T1D although many papers have reported differences with human T1D (Velloso et al. 1994; Leiter 2002; Roep and Atkinson 2004). The model was established by Makino et al. in 1980 (Makino et al. 1980). The mice develop insulitis already at 5 weeks of age. Onset occurs usually around 12-14 weeks of age, slightly earlier in females than in males (Andre et al. 1996). The cumulative incidence of diabetes reaches 80% of females and 35% of males at 30 weeks of age (Makino et al. 1980). In a dirtier environment, the mice are less sick (Singh and Rabinovitch 1993; Bach 1994; Bowman et al. 1994) due to unknown environmental factors.

Several loci, MHC and others seem to control the susceptibility to T1D in NOD mice. Exact genes causing the dysregulation seen in NOD mice have not been identified so far and absence of protective genes has been hypothesized to affect the NOD phenotype (Anderson and Bluestone 2005). It is only known that NOD mice have several defects in central tolerance, in the process of thymic selection (Kishimoto and Sprent 2001). These include defects in apoptosis during thymic deletion (Lesage et al. 2002; Liston et al. 2004), morphological lesions (Delovitch and Singh 1997; Atlan-Gepner et al. 1999), and global defects in thymic negative selection (Kishimoto and Sprent 2001). Yet the molecular mechanisms for these defects remain unclear, though a multitude of different hypotheses have been suggested by different groups. NOD mice usually also develop other autoimmune syndromes besides T1D. These include autoimmune inflammation of salivary glands or ducts (sialitis) (Hu et al. 1992), autoimmune thyroiditis (Many et al. 1996), autoimmune peripheral neuropathy (Salomon et al. 2001) and a SLE-like disease if exposed to killed mycobacterium (Silveira and Baxter 2001). T lymphocytes are key players in these diseases, but the other factors such as antigens recognized and role

of costimulation vary (Anderson and Bluestone 2005). Transfer of islet-specific $CD4^+$ T cells accelerates the onset of T1D in NOD mice (Haskins and McDuffie 1990). However, both $CD4^+$ and $CD8^+$ T cells are required for β cell destruction (Gerling et al. 1992). NOD mice show a multitude of immune defects and defects in several leukocyte subsets (Anderson and Bluestone 2005). There are however, differences between different NOD colonies (Pozzilli et al. 1993). In the NOD mice, autoantigens are targeted against at least insulin, GAD and IA-2 (Lieberman and DiLorenzo 2003). Proinsulin is the essential driver autoantigen in T1D (Harrison et al. 2008). In T1D there is a strong influence of the MHC and a multitude of other loci each having additional weak effects on the phenotype, as opposed to the case of APECED (discussed below in 5.2 Mouse models of APECED). However, similarly to APECED, there are defects in DC maturation in NOD mice (Prasad and Goodnow 2002; Prasad and Goodnow 2002).

Thanks to NOD mice, although the molecular pathology remains incompletely resolved, progress has been made and we have learnt more about organ-specific autoimmune diseases, T1D and some mechanisms behind a complex disease (Atkinson and Leiter 1999; Adorini et al. 2002; Anderson and Bluestone 2005).

### 5.1.2 EAE mice

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system in which chronic inflammation, demyelination, and axonal damage occur. Why MS occurs is proposed to be due to the breakdown of tolerance to CNS self-antigens in genetically susceptible individuals (Frohman et al. 2006; Zozulya and Wiendl 2008). Experimental autoimmune encephalomyelitis (EAE) is the rodent model of MS. First it was shown that EAE mice can be protected from disease by injection of $CD4^+$ splenocytes or thymocytes (Furtado et al. 2001). By studying EAE mice further, important mechanisms in the immunopathology of MS and CNS have been revealed. Now it is known that EAE can be alleviated by transfer of Tregs and that certain Treg populations play a key role in MS by suppressing T cell mediated “autoaggression” against structures of the central nervous system (CNS). Also DCs may play an important role in MS, in modulating Treg responses (Zozulya and Wiendl 2008).

In addition to the EAE mice, many other mouse models of MS have been used as well. An example of one that has been around for a long time as a model of demyelination induced by a virus. This is Theiler's murine encephalomyelitis virus (TMEV) infection of mice, which stimulates a MS-like disease in susceptible strains of mice. However, TMEV does not cause disease in humans and is thus 'merely a disease of mice' (Drescher and Sosnowska 2008). Other models are different types of transgenic and induced mice (Morel 2004; Bettelli 2007).

### 5.1.3 Scurfy mice

Scurfy is an X-linked, recessively inherited mouse mutant model (Brunkow et al. 2001). The phenotype of scurfy mice resembles human IPEX (Lyon et al. 1990). With the help of scurfy (*sf*) mice, the human Scurfin or FOXP3 was found after identifying mouse Foxp3. The human FOXP3 is homologous to mouse scurfin, or Foxp3 (Brunkow et al. 2001). The name scurfy comes from the meaning of the word scurfy: scaly or shredded dry skin. This phenotype or disease "scurfy" applies to the appearance of the scurfy mice (Brunkow et al. 2001).

Absence of functional FOXP3 leads to IPEX in humans (discussed in section 3.4) and the corresponding defect leads to a similar disease in scurfy mice. Disease is due to Treg deficiency, due to the lack of FOXP3 which is a transcriptional enhancer (Marson et al. 2007; Zheng et al. 2007) necessary for normal function of Tregs (Yagi et al. 2004). In the absence of FOXP3, T lymphocytes still develop markers for Tregs, but the Tregs are not able to function as suppressors (Gavin et al. 2007).

Scurfy mice have fulminant systemic autoimmunity, like IPEX patients, but they also exhibit lymphoproliferative infiltrations of the liver, spleen and lymph nodes, which does not usually manifest in IPEX patients (Torgerson and Ochs 2007). In mice, in addition to the infiltrations, the $CD4^+$ T cells hyperproliferate, and an elevation in cytokine levels is notable (Brunkow et al. 2001).

### 5.1.4 lpr and gld mice

Deficiency of the *lpr* and *gld* genes causes very similar phenotypes. The lpr phenotype resembles human systemic lupus erythematosus (SLE). SLE is an autoimmune, inflammatory disease of connective tissue with variable features and it is able to affect nearly every organ. Additionally, high titers of systemic autoantibodies are produced in SLE patients (Liu and Mohan 2006).

The mutated gene in ALPS type 1a was found with the help of the spontaneous mouse model for ALPS called the *lpr/lpr* or lpr mouse, in which lpr stands for lymphoproliferation (Watanabe et al. 1991). These mice were found to lack expression of Apo-1 (CD95) (Watanabe-Fukunaga et al. 1992). Apo-1 is also called Fas and mediates apoptosis (Trauth et al. 1989; Yonehara 1999). In other words, the lpr mouse carries a spontaneous mutation in Fas rendering it unfunctional (Watanabe-Fukunaga et al. 1992) (Kuhtreiber et al. 2003).

The lpr mice have been bred into different backgrounds and these background strains affect the disease phenotype (Cohen and Eisenberg 1991), similarly as in other mouse models. Lpr mice develop numerous autoimmune traits and accumulation of double-negative ($CD4^-CD8^-$) T cells, just like in ALPS (Cohen and Eisenberg 1991). The lpr mice produce autoantibodies to target antigens

homologues for the human SLE autoantigens, such as against chromatin and DNA. However, mice produce additional autoantibodies against targets not found in human patients, such as IgG rheumatoid factors (Eisenberg et al. 1979). The autoantibodies common in mice and men are believed to reflect the essential immunoregulatory abnormalities of SLE, but how they generate the pathology is not yet understood (Cohen and Eisenberg 1991). In addition, it is important to keep in mind that mouse strains, or background genes may have an important impact on the phenotypes and autoimmune features of SLE mouse models (Bygrave et al. 2004).

FasL-deficient mice are called *gld/gld* or gld mice, in which gld stands for generalized lymphoproliferative disease (Roths et al. 1984). Deficiency in gld is also autosomal, and recessively inherited. It leads to lymphoproliferation and autoimmunity resembling ALPS type 1b, which is caused by mutations of the human FasL. The phenotype of gld mice is nearly identical to that of lpr mice (Allen et al. 1990; Cohen and Eisenberg 1991), since the two mice have genes defective in the same Fas (CD95) – FasL (CD95L) pathway of apoptosis (Kuhtreiber et al. 2003).

Studies using these two mouse models have given new insights into systemic immunity and control of lymphocyte proliferation (Cohen and Eisenberg 1991). Additionally, they have taught us that autoimmune disease can be mediated by defects in apoptosis genes (Nagata and Suda 1995; Kuhtreiber et al. 2003). Interestingly, lupus patients do not commonly seem to have mutations in FasL and show accelerated apoptosis – exactly the opposite to the situation in the mouse models (Kuhtreiber et al. 2003; Xue et al. 2006).

#### 5.1.5 Other selected mouse models

Mice with a defect in IL-2R (CD25) have a severe phenotype of autoimmunity, including splenomegaly, lymphadenopathy, colitis and hemolytic anemia, similar to the phenotype of scurfy mice (Willerford et al. 1995). Studies on mice lacking CD25 have implied defects in Tregs, but exact mechanisms are not yet known. Further studies are required to verify the mechanism of the corresponding pathology in the human disease (Torgerson and Ochs 2007).

Alopecia areata (AA) develops spontaneously in several animal species (McElwee et al. 1998). For example C3H/HeJ mouse colony manifests clinical and immunological features that resemble those of human AA (Sundberg et al. 1994). The mice produce antibodies against hair follicle structures and react with keratins similarly as do human AA antibodies (Tobin et al. 1994). These mice are an appropriate model for human AA and support the hypothesis that AA results from an abnormal autoimmune response to hair follicles (Tobin et al. 1994).

New Zealand mice are a spontaneous animal model of human SLE (Andrew et al., 1978, Borchers A Ansari AA et al. 2000 Semin Arthritis Rheum). Other, less well-known autoimmune models are for example mouse models of autoimmune ovary disease (AOD) and experimental autoimmune uveitis (Caspi et al. 2008), as well as new models such as for ocular myasthenia gravis (oMG) (Yang et al. 2007) and autoimmune gastritis (Alderuccio et al. 2002).

Animal models of other APS's than APS 1 include other animals than just mice. There are the spontaneous models of obese strain White Leghorn chicken (OS chicken) for APS 2, the BioBreeding (BB) rat for APS 3 and the NOD mouse, described above, for APS 4 (Ikegami 2002). In addition, induced models can be found for APS2 and 3. For example, the administration of cyclosporin A and subsequent thymectomy lead to APS 2 in mice (Sakaguchi and Sakaguchi 1989).

Another experimental model worth mentioning is the Smyth line chicken model. Spontaneous vitiligo and AA like symptoms are manifested in these birds. Furthermore, these chickens express autoantibodies against melanocytes (Smyth and McNeil 1999).

## 5.2 Mouse models of APECED

Aire-deficient mice have been valuable tools in learning more about Aire function and the pathogenesis of APECED (Anderson et al. 2002; Ramsey et al. 2002; Kuroda et al. 2005).

The first two mouse models of APECED were published in 2002 and were both Aire knock-outs made in 129/Sv ES cells and backcrossed into the C57/BL6 background (Anderson et al. 2002; Ramsey et al. 2002). Both of the mouse models showed general signs of autoimmunity while lacking clinical symptoms resembling those of APECED patients. However, in the first Ramsey paper, the knock-out mice had not yet been backcrossed into congenity in the B6 strain and infertility, as well as some other symptoms were greater before the line was congenic in B6 mice (own observations and Goodnow, personal communication).

Autoimmune manifestations of these mice, which were kept in sterile conditions and which developed normally, include lymphocyte infiltrates of the target organs, circulating autoantibodies and infertility (Anderson et al. 2002; Ramsey et al. 2002).

The Ramsey mouse was constructed by the Peltonen group using targeted disruption of exon 6 of murine Aire in order to mimic the $Finn_{Major}$ mutation (Ramsey et al. 2002). The size, weight and maturation of the Aire-deficient mice were normal compared to wild type (*wt*) mice, *i.e.* Aire-positive controls. However, Aire-

deficient mice did not reproduce in normal numbers and 85% of the males and females were found to be infertile. This feature resembles the clinical phenotype of human APECED, as APECED patients develop ovarian failure in 39% to 72% of cases (at 15 years and 36 years respectively). Hypogonadism occurs also in male APECED patients, but their testicular failure occurs at a later age and lower prevalence than the ovarian failure in women with APECED (Perheentupa 2002; Perheentupa 2006).

Histological analyses of the Ramsey mouse showed atrophy of the thymus and adrenal glands as well as lymphocyte infiltrations in the liver, in accordance with autoimmune hepatitis in APECED patients (prevalence 12%). Organ atrophy varied between mice, for example one atrophied ovary was found. This variance resembles the variable phenotype between patients. In the Aire-deficient mice, circulating autoantibodies were found against liver, spermatogonia/spermatids, exocrine pancreas, adrenal cortex and in one case, β cells of islets of Langerhans. This also resembles the panel of autoantibodies found in APECED patients (Halonen et al. 2002).

Analyses of the Aire deficient mice revealed that lymphocyte populations were normal, with the sole differences in hyperproliferation. Hyperproliferation of Aire deficient T cells occurred in response to immunisation and in three out of the 24 splenic TCR vβ chain families of the Aire knock-out mice as compared to Aire-positive mice. These defects were suggested to imply at least a partial role for Aire in regulation of peripheral tolerance, since the overrepresentation of certain TCRs may induce imbalance in T cell homeostasis. The authors hypothesized that environmental stimuli could also be a cause of the autoimmune reactions seen in the Aire knock-out mice, since in the mouse model, as with APECED patients, disease manifests after birth and thus after contact with the outside world (Ramsey et al. 2002). However, a fresh report showed that no environmental stimulation is needed for autoimmunity in Aire deficient mice (Gray et al. 2007).

The mouse model constructed by Anderson et al. (Anderson et al. 2002) was produced by conditional targeted disruption of exon 2, including parts of the surrounding intronic sequences.

This induced defect may not cause marked differences from the Ramsey mouse in its outcome, since no truncated form of Aire could be detected in the Ramsey model, nor was it reported in the Anderson mouse (Anderson et al. 2002; Ramsey et al. 2002).

Histological analyses showed, again, lymphocytic infiltrations in the salivary glands, ovarian follicles, retina of the eye. Autoantibodies were observed against oocytes of the ovary, parietal cells of the stomach and the outer layer of the retina of the eye. Thus a broad, yet specific defect of tolerance was observed. No faults with retina or salivary gland have been shown in APECED patients (Lambe et al. 2007). Further,

the phenotype of the two mouse models also differs slightly with their findings of target organs. This may of course be due to the difference in gene disruption.

Further analyses of the mice showed that the number of mTECs was twice as much as that in the *wt* mice and the number of activated memory T cells was also twice as high in the Aire deficient mice compared to the *wt* mice. The results of gene expression studies suggested that Aire functions in non-hematopoietic cells. Follow-up experiments showed that Aire functions in the radio-resistant stromal cells of the thymus. Furthermore, it was shown by cell transplantation experiments that Aire-deficient lymphocytes were sufficient to cause autoimmunity in alymphoid $Rag^{0/0}$ recipients.

Finally, using microarrays, Aire was shown to function as a transcriptional regulator of 200-1200 genes and thus control the expression of ectopic antigens in the thymus by mTECs. However, not all of the previously established ectopically expressed genes (Derbinski et al. 2001) were found to be down-regulated in Aire deficient mice. Thus, Aire appears to regulate only a fraction of these genes.

After these two mouse models had been characterized, a few more Aire deficient mice strains were published. First, another Aire knock-out was constructed using gene targeting of exons 5 to 12 into TT2 embryonic stem cells and then ICR 8 cell embryos which were mated into the C57/BL6 and BALB/c backgrounds. Also these C57/BL6 $Aire^{-/-}$ mice showed a reduction in reproductive capacity and presence of lymphocytic infiltrates in lacrimal glands, parotid glands, submandibular glands, and in addition, a reduction in tear secretion. No other pathologic changes were found. A new finding was autoreactivity against α-fodrin, although the expression of α-fodrin in the thymus was unrepressed. In the BALB/c background, additional features were lymphoid cell infiltrations of the gastric mucosa and autoantibodies against gastric mucosa. The genetic background was concluded to clearly influence the target-organ specificity of the autoimmune attack caused by Aire deficiency (Kuroda et al. 2005).

The mouse constructed by Anderson et al. in 2002 was backcrossed into different mouse strains (Jiang et al. 2005). The comparison showed that different backgrounds did affect the phenotype of the mice, just as individual APECED patients show variation in their disease phenotype. The pattern of organs targeted by the autoantibodies varied between backgrounds and the severity of the targeting differed from mild in the C57BL/6 to strong in the NOD strain. The other strains analyzed were Balb/c and SJL/J. Autoantibodies were found in line with the pattern of autoimmune manifestations (Jiang et al. 2005). NOD mice are known to have defects in their central induction of tolerance by clonal deletion (Kishimoto and Sprent 2001), so a more severe phenotype in this background was expected. A significant finding was that the NOD background did not broaden the range of protein targets in the Aire deficient NOD mice, although the intensity was stronger

in the Aire$^{-/-}$ NODs (Jiang et al. 2005). Explanations for the varying phenotypes in the different mouse backgrounds and why such a limited array of antigens are targeted if Aire controls thymic ectopic expression of hundreds of genes (Anderson et al. 2002; Johnnidis et al. 2005) remain to be resolved.

Later, another Aire knock-out mouse was published by a Japanese group (Niki et al. 2006). This time the mouse constructed was back-crossed into the NOD mouse strain (Kuroda et al. 2005). Not surprisingly, considering the complicated NOD background, their Aire$^{-/-}$NOD$^{-/-}$ mice exhibited both expected and unexpected autoimmune characteristics. Homozygous offspring did not quite follow the Hardy-Weinberg equilibrium, as less Aire$^{-/-}$ homozygotes were born than expected. The Aire deficient NOD mice showed a decrease in body-weight, with one third also eliciting growth retardation and gaunt appearance. A reduction of pancreatic size was observed together with lymphocytic infiltrates. Infiltrates were also present in the pancreatic lymph nodes, submandibulary and axillary lymph nodes, which were moreover enlarged in size. The degree of infiltrations was more severe in the Aire deficient NODs than in the pure NOD strain and infiltrations were present in more numerous organs, such as the liver, lung, thyroid gland and prostate and seminal vesicle of the male mice. Furthermore, the intra-pancreatic autoantibody specificity had switched from targeting β cells to acinar cells in the Aire deficient NODs and the degree of pancreatic lesions was augmented, as was the acceleration of the onset of the lesions. Small numbers of mice showed a reduction in thymocyte number and the CD44$^{high}$ and ICOS-expressing cell populations of splenic CD4$^{+}$ cells, activated T cells, were increased in Aire deficient NOD mice. Unlike NOD mice, Aire$^{-/-}$ NOD mice did not develop diabetes, implying a role for Aire in resistance to diabetes, probably due to the alteration of the pancreatic target molecule in Aire$^{-/-}$ NODs. Finally, serum IgM and all subclasses of IgG except IgG3 were elevated in Aire$^{-/-}$ NOD mice, associating with the augmented production of autoantibodies against various organs. The mice rarely survived more than 5 months probably due to the severe autoimmune phenotype (Niki et al. 2006). Although it is difficult to differentiate the role of Aire in the complicated NOD background, the alteration of pancreatic target from β cells to acinar cells is an interesting finding (offering an explanation for absence of diabetes in the Aire$^{-/-}$ NODs noted already by (Jiang et al. 2005)) and deserves further focus.

A fifth Aire deficient mouse was published in 2008 (Hubert et al. 2008). This was the third knock-out mouse in the C57BL/6 background this time disrupting exon 8. This mouse was used for expression analyses of Aire using a new monoclonal antibody and no new/unexpected phenotypes or findings were published in this mouse strain (Hubert et al. 2008).

Su et al. (Su et al. 2008) constructed a knock-in mouse by introducing a dominant-negative Aire mutation (G228W) into C57BL/6 and NOD mice. These mice showed global TSA suppression, and these decreased expression levels caused autoimmunity, showing that normal expression levels of TSAs are important in development of central tolerance. These mice also exhibited a novel autoimmune phenotype consisting of autoimmune thyroiditis and spontaneous peripheral neuropathy (Su et al. 2008).

Only one double knock-out mouse model including the Aire knock-out has been reported. In these mice, both Aire and Foxp3 were deficient. FoxP3 deficient scurfy mice in the B6 background were crossed with Aire deficient mice in either the B6 or NOD background (Chen et al. 2005). The double knock-outs died, or had to be terminated before 28 days of age. Before about 10 days after birth, their phenotype seemed approximately normal. After that, infiltrations appeared in their lungs by day 14. The liver was also invaded by infiltrations and mild pancreatitis was noted. More severe pathology was noted, not surprisingly, in the NOD background compared to the B6 background. Aire seemed to accelerate pathology caused by FoxP3, since the same targets as in scurfy mice were affected in the double knock-outs, only more severely. It was surprising that while the lung and liver suffered severe damage, many tissues remained very healthy. Autoantibodies did not seem to be the cause of the "fulminent autoimmune disease". Furthermore, defective genes in NOD and Aire seem to function via different pathways in central tolerance, which may be due to their expression in the thymocytes and stromal cells, respectively (Chen et al. 2005).

To summarize, in mice, Aire deficiency results in almost complete failure to delete self-specific lymphocytes in the thymus (Liston et al. 2003) and the mice have served as a valuable tool in studying the function of Aire *in vivo*. Aire deficiency resulted in quite a mild autoimmune phenotype in the various C57BL/6 strains (Anderson et al. 2002; Ramsey et al. 2002; Kuroda et al. 2005). A few more features were seen in the Balb/c background (Kuroda et al. 2005) and a more severe model of autoimmunity was reported in the NOD background than expected (Jiang et al. 2005; Niki et al. 2006). The Aire deficient phenotype is inducible by transferring either $Aire^{-/-}$ lymphocytes to immunodeficient mice, or $Aire^{-/-}$ thymic stromal cells to normal mice (Anderson et al. 2002; Anderson et al. 2005; Kuroda et al. 2005).

These mouse models provide interesting and valuable models for studies of immunological tolerance and its breakdown, but whether they are good models of APECED is dubious; the similarity between the clinical effect of Aire deficiency in mouse and men is debatable (Kekalainen et al. 2007).

**Table 4.** ***Aire deficient mouse models***

| MOUSE STRAIN | GENE CONSTRUCT | REFERENCE |
|---|---|---|
| C57BL/6 | exon 4/5 | Ramsey et al. 2002 |
| C57BL/6 | exon 1 | Anderson et al. 2002 |
| C57BL/6, Balb/c | exon 5 | Kuroda et al. 2005 |
| C57BL/6, Balb/c, SJL & NOD | *back-cross* | *Jiang et al. 2005 (Anderson mouse)* |
| NOD | *back-cross* | *Niki et al. 2006 (Kuroda mouse)* |
| C57BL/6 | exon 8 | Hubert et al. 2008 |

# 6 Autoimmune Regulator (AIRE/Aire)

Autoimmune Regulator (AIRE) was found to be the causative gene for APECED in 1997. It was cloned independently by two collaborations to chromosome 21, to locus 21q22.3 using positional cloning (Consortium 1997; Nagamine et al. 1997). Two years after finding AIRE, its mouse homologue, Aire, was cloned and characterized by several groups in 1999 (Blechschmidt et al. 1999; Mittaz et al. 1999; Wang et al. 1999). Murine Aire is located on chromosome 10 and is highly homologous in structure and sequence with the human AIRE. The Aire protein is 71% homologous with the human AIRE protein (Blechschmidt et al. 1999; Mittaz et al. 1999; Wang et al. 1999).

## 6.1 AIRE/Aire, the gene and the protein

The *AIRE* gene contains 14 exons spanning 12 kb of genomic DNA. The exons have boundaries that follow the GT-AG rule (Mount 1982). The last exon of *AIRE* seems to overlap with the promoter area of the gene *PFKL* (which stands for phosphofructokinase). PFKL is transcribed from the same strand of DNA as AIRE (Levanon et al. 1995). The *AIRE* cDNA was originally isolated from an adult human thymus cDNA library and contains an open reading frame (ORF) with a high GC content (69%). The *AIRE* promoter contains a TATA box, a GC box and the 5' end of the gene also harbours a CpG island (Consortium 1997; Nagamine et al. 1997; Aaltonen and Bjorses 1999; Meriluoto et al. 2001).

AIRE encodes for a protein of 545 amino acids and has a predicted molecular mass of 58 kDa (Consortium 1997; Nagamine et al. 1997). The AIRE protein contains several functional domains which are suggestive of a role as a transcriptional regulator. The N-terminus contains a conserved nuclear localization signal (NLS) and a homogenously staining region (HSR) domain. In the middle lies a Sp100, AIRE, NucP41/75 and DEAF-1/supressin (SAND) domain, four nuclear receptor-binding LXXLL motifs (in which L = leucine and X is any amino acid) are

interspersed along *AIRE* and the C-terminus harbours two plant-homeodomain (PHD) -type zinc fingers (Consortium 1997; Nagamine et al. 1997).

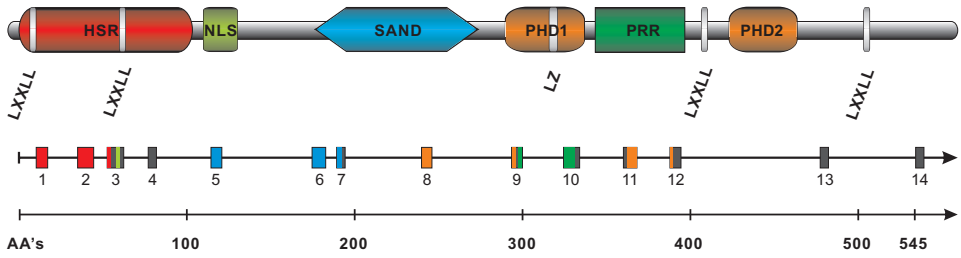



**Figure 8.** ***The AIRE gene. A schematic representation showing the functional domains (above) and exons (below) of the AIRE gene. Modified from Peterson and Peltonen (Peterson and Peltonen 2005).***

Since AIRE is quite difficult to express as a whole *in vitro*, and stable cell lines have also been rather unsuccessful in the past, the functional domains have been studied using *in vitro* assays in order to shed light on the function of AIRE. The NLS directs AIRE to the nucleus and a nuclear export signal (NES) may direct it out again (Pitkanen et al. 2001). The SAND domain is involved in DNA binding of AIRE (Kumar et al. 2001; Purohit et al. 2005). The HSR domain is responsible for the homomultimerization of AIRE (Halonen et al. 2004) and AIRE can appear as homomers (Pitkanen et al. 2000; Kumar et al. 2001). The HSR domain has also been reported to be involved in the attachment of AIRE with cytoplasmic filaments (Pitkanen et al. 2001; Ramsey et al. 2002) and in the targeting of AIRE to nuclear bodies and to molecular complexes (Halonen et al. 2004). The PHD zinc fingers are important for AIRE, since the major patient mutations occur in them (Heino et al. 2000). The PHDs mediate the transactivation ability of AIRE, especially the second PHD (Bjorses et al. 2000; Pitkanen et al. 2001; Uchida et al. 2004; Meloni et al. 2008). The first zinc finger may also harbour E3 ubiquitin ligase activity (Uchida et al. 2004), but this has not been conclusively proven and instead, it may also mediate protein-protein interaction (Bottomley et al. 2005). There is also a leucine zipper (LZ) motif in the first PHD zinc finger (Consortium 1997; Nagamine et al. 1997; Mittaz et al. 1999) and this possibly helps AIRE form dimers (Kumar et al. 2001). A proline rich area in AIRE (Consortium 1997; Nagamine et al. 1997; Mittaz et al. 1999) may mediate protein-protein interactions (Kay et al. 2000). The last LXXLL in the COOH terminal end of *AIRE* is important for transactivation as well (Meloni

et al. 2008). Other roles for domains in AIRE, and for areas between them, have also been suggested, but need yet to be confirmed. AIRE has many domains similar to Speckeld protein 100 kDa (Sp100) proteins, which are involved in transcriptional activation and repression (Seeler et al. 1998; Yordy et al. 2005).

A recent study also revealed that the homogenously staining region (HSR) domain of *AIRE* may actually resemble a caspase recruitment domain (CARD). The CARD structure has six alpha helices whereas the HSR model of AIRE has four, but otherwise they are rather similar in their 3D structure. Furthermore, CARD has the same function as it is also involved in the transactivation activity of AIRE, leading to AIRE and CBP accumulation in the nucleus. This results in alterations in the mTEC transcriptome, since co-expression of CBP and AIRE seems to increase the transactivation potential of AIRE, possibly leading to induction of TSA expression (Ferguson et al. 2008).

The same AIRE domains are also found in the mouse homologue of AIRE (Blechschmidt et al. 1999; Mittaz et al. 1999; Wang et al. 1999).

## 6.2 Associations of AIRE with other autoimmune diseases

To resolve whether AIRE is involved in the susceptibility of complex autoimmune diseases, several association studies of the AIRE gene with the different disease components included in APECED, like T1D and hypoparathyroidism, have been recently reported. Linkage disequilibrium was found between HLA class II region and autoimmune hepatitis (AIH) in pediatric patients (Djilali-Saiah et al. 2004) and one intronic polymorphism in AIRE (G11107A) correlated with systemic sclerosis (Ssc) and with Ssc associated with thyroiditis (Ferrera et al. 2007). Recently, AIRE has been shown to be strongly associated with vitiligo in a case-control study. This raises the possibility of defective skin antigen selection in the thymus being involved in the resulting melanocyte destruction in vitiligo (Tazi-Ahnini et al. 2008).

A group who studied whether AIRE is involved in the pathogenesis of alopecia areata (AA), also showed that there was a strong association of one potentially functional AIRE polymorphism, the 961G allele, with the most severe form of AA and with early-onset AA. Furthermore, they suggest that this allele may decrease the DNA-binding activity of AIRE, offering an explanation of the high frequency of AA in APECED (Tazi-Ahnini et al. 2002). Surprisingly, another group could not replicate this result in their AA patients (Pforr et al. 2006). The first group continued their studies and analyzed six SNPs in AIRE. In this new analysis, the allele previously reported was no longer significantly associated. However, two other

haplotypes were associated with AA. The authors suggest this may represent one genetic risk factor for AA (Wengraf et al. 2008).

A study analyzing AIRE SNPs suggested that AIRE does not associate with T1D (Turunen et al. 2006), and another study indicated that there was no association of the mutations in exons 6 and 8 of the AIRE gene with inflammatory bowel disease (Torok et al. 2004). AIRE gene polymorphisms in exons 6, 8 and 10 did not contribute to the development of isolated autoimmune hepatitis (AIH) either, nor did AIRE SNPs associate with patients with sporadic idiopathic hypoparathyroidism (Goswami et al. 2005).

In this context it is interesting that mmutational analysis of the AIRE gene first showed that patients with sporadic autoimmune Addison's disease (AAD) may actually have APECED although they had not been diagnosed. One such patient (out of 40) was found, with mutations in AIRE but with AAD as his only disease component of APECED (Boe et al. 2002). Nonetheless, no association of the common United Kingdom mutation (964del13bp) in the AIRE gene with AAD or APSII was found (Vaidya et al. 2000). Following this, negative results were published by another group analyzing whether AIRE gene polymorphisms (9 SNPs) or copy number variations were associated with AAD and with APS II (Boe Wolff et al. 2008).

## 6.3 Expression of AIRE/Aire

AIRE is localized in cells mainly in the nucleus appearing mainly as nuclear bodies (NBs) (Bjorses et al. 1999; Heino et al. 1999; Rinderle et al. 1999). The nuclear bodies resemble promyelocytic leukemia (PML) nuclear bodies which are known to be associated with different transcriptionally active proteins (Bjorses et al. 1999; Heino et al. 1999; Rinderle et al. 1999). When AIRE cDNA is transfected into cultured cells, the recombinant AIRE is expressed in the nucleus either in NBs, or evenly distributed, lacking NBs (Akiyoshi et al. 2004; Ilmarinen et al. 2005; Tao et al. 2006). In addition, AIRE colocalized with intermediate filaments or microtubules and with vimentin in the cytoplasm, as well as was present in aggregates of different sizes (Bjorses et al. 1999; Heino et al. 1999; Rinderle et al. 1999). Also human thymus tissue sections contain expression of AIRE in the cytoplasm (Cavadini et al. 2005).

The earliest expression of Aire mRNA was seen after E14 (Zuklys et al. 2000). However, according to another study, Aire was detected at E14.5 (Adamson et al. 2004) and in a third, at E16 (White et al. 2008) probably reflecting the sensitivity of the detection methods used. Aire mRNA is expressed in different forms, suggestive of splice variants, in the thymus and secondary lymphoid organs. In addition to the

full *Aire* gene, eleven splice variants of murine Aire have been described (Ruan et al. 1999). So far, the functional relevance of the different RNA variants is open.

The highest expression level of AIRE/Aire is found in the thymus, in the medulla and corticomedullary junction, in cells called thymic medullary epithelial cells (mTECs) (Consortium 1997; Nagamine et al. 1997; Bjorses et al. 1999; Rossi et al. 2007; Martins et al. 2008). AIRE has also been reported to be expressed in another type of antigen presenting cell (APC) in the thymus, namely in cells of the monocyte-dendritic cell lineage (Heino et al. 1999). Lower levels of AIRE/Aire expression have been reported in other immunological tissues; the spleen, lymph nodes and fetal liver (Consortium 1997; Nagamine et al. 1997; Bjorses et al. 1999) and cells of the immune system; differentiated DCs and peripheral blood monocytes (Heino et al. 1999) (Kogawa et al. 2002; Sillanpaa et al. 2004). Additionally, very low quantities were detected in thyroid gland and heart, but none in the pancreas, liver or spleen in one study (Ruan et al. 1999). Furthermore, no expression of Aire in any other cell types tested except mTECs was reported by yet another study (even in DCs vs. previous reports (Kogawa et al. 2002; Sillanpaa et al. 2004)) (Klamp et al. 2006). So, whether or not Aire is expressed in tissues other than thymus was disputed (Mathis and Benoist 2007). Furthermore, AIRE seems not to be expressed in target organs such as the adrenal cortex, adult liver and pancreas (Bjorses et al. 1999; Heino et al. 1999).

The cell type in which Aire is expressed in thymus is clear. Expression of Aire in the lymph nodes has been shown quite convincingly (see Tables 4 and 5), but the cell type is not clear, as expression has been reported in lymphocytes, neutrophilic granulocytes, DCs, medullary and paracortical cells (Bjorses et al. 1999; Heino et al. 1999). In any case, expression of Aire in secondary lymphoid organs suggests a role in peripheral tolerance. No expression in any other cell types tested (even in DCs vs. previous reports (Kogawa et al. 2002; Sillanpaa et al. 2004)) (Klamp et al. 2006), before a fresh report indicating expression of AIRE in B cells and possibly in DC/macrophages and granulocytes (Suzuki et al. 2008). An interesting theory to why Aire may be expressed in very low levels in tissues such as testis, spleen and lymph nodes, at mRNA level, is that Aire is expressed in $CD11c^+$ DCs, and these cells migrate into all the aforementioned tissues (Heino et al. 2000), but in limited amounts.

Making use of a new AIRE antibody and flow cytometry, it was newly shown that $Aire^+$ cells were restricted to the thymus, to the thymic medulla and was extremely low in DCs (Hubert et al. 2008).

**Tables 4 and 5** ***below show expression of Aire and AIRE in humans and mice, respectively.***

**Human AIRE**

| Organ | Cell type | mRNA | Protein | References for mRNA | References for protein |
|---|---|---|---|---|---|
| thymus | whole | X | | Consortium 1997, Nagamine et al. 1997, Klamp et al. 2006 | |
| | medulla | | X | | Björses et al. 1999 |
| | margin of Hassall's corpuscles | | | Kekäläinen et al. 2007 | |
| | cortex | | | Cavadini et al. 2005 | |
| | corpuscle cells | | X | | Björses et al. 1999, Heino et al. 1999 |
| | thymic DCs | X | X | Gotter et al. 2004 | Heino et al. 1999 |
| | mTECs | X | X | Gotter et al. 2004, Klamp et al. 2006 | Heino et al. 1999 |
| | medullary thymocytes, reticular epithelial cells | | X | | Björses et al. 1999 |
| spleen | whole | X | *X?*[*] | Consortium 1997, Klamp et al. 2006 | Heino et al. 1999 |
| | lymphocytes of red pulp | | X | | Björses et al. 1999 |
| | neutrophils and basophils | | X | | Björses et al. 1999 |
| lymph node | whole | X | X | Consortium 1997, Nagamine et al. 1997, Klamp et al. 2006 | |
| | lymphocytes | | X | | Björses et al. 1999 |
| | neutrophils | | X | | Björses et al. 1999 |
| | DCs, medullary and paracortical cells | | X | | Heino et al. 1999 |
| PBL | whole subset | *X?*[*] | *X?*[**] | Consortium 1997, Björses et al. 1999, Klamp et al. 2006 | Rinderle et al. 1999 |
| | $CD14^+$ monocytes | X[£] | X | Kogawa et al. 2002 | Kogawa et al. 2002 |
| | lymphocytes, monocytes, neutrophils | | X | | Björses et al. 1999 |
| | DCs | [£]X | | Kogawa et al. 2002, Sillanpää et al. 2005 | |
| | B cells | X | X | Suzuki et al. 2008 | Suzuki et al. 2008 |
| bone marrow | | X | | Consortium 1997, Nagamine et al. 1997 | Heino et al. 1999 |
| fetal liver | | *X?*[*] | *X?*[**,§] | Consortium 1997, Nagamine et al. 1997 | |
| adrenal cortex | | *X?*[*] | [*,**,¤] | Consortium 1997, Nagamine et al. 1997 | |
| adrenal medulla | | X | | Consortium 1997 | |
| pancreas | | *X?*[*,¤] | [**] | Consortium 1997 | |
| testis | | *X?* | [**] | Consortium 1997 | |
| thyroid gland | | X | [**,§] | Consortium 1997 | |
| appendix | | X | | Nagamine et al. 1997 | |

*Modified from Klam et al. 2006 using the references shown*

*X? - conflicting data*

* not found by Nagamine et al. 1997

** not found by Heino et al. 1999

¤ not found by Björses et al. 1999

§ not found by Rinderle et al. 1999

£ not found by Klamp et al. 2006

**Mouse Aire**

| Organ | mRNA | Protein | References for mRNA | References for protein |
|---|---|---|---|---|
| thymus | X | X | Ruan et al. 1999, Hubert et al. 2008, Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004, Hubert et al. 2008 |
| lymph nodes | X | X | Heino et al. 1999, Halonen et al. 2001, Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004, Lee et al. 2007 |
| heart | X | | Blechschmidt et al. 1999, Adamson et al. 2004 | |
| lung | X | X | Blechschmidt et al. 1999, Adamson et al. 2004 | Adamson et al. 2004 |
| testis | X | | Blechschmidt et al. 1999, Ruan et al. 1999 | Adamson et al. 2004 |
| spleen | *X?* | X | Heino et al. 1999, Blechschmidt et al. 1999, Ruan et al. 1999, Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004 |
| kidney | *X?* | X | Blechschmidt et al. 1999, Halonen et al. 2001, Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004 |
| brain | *X?* | X | Blechschmidt et al. 1999, Halonen et al. 2001, Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004 |
| liver | *X?* | X | Ruan et al. 1999, Halonen et al. 2001, Adamson et al. 2004 | Halonen et al. 2001 |
| ovary | X | X | Ruan et al. 1999, Adamson et al. 2004 | Adamson et al. 2004 |
| adrenal gland | X? | X | Ruan et al. 1999 | Halonen et al. 2001 |
| thyroid | X | X | Ruan et al. 1999 | Halonen et al. 2001 |
| bone marrow | | X | | Halonen et al. 2001 |
| pancreas | | X | | Halonen et al. 2001 |
| intestinal canal/gut | X | X | Adamson et al. 2004 | Halonen et al. 2001, Adamson et al. 2004 |
| gonads | | X | | Halonen et al. 2001 |
| pituitary | | X | | Halonen et al. 2001 |
| cells of lymphoid lineage | | X | | Halonen et al. 2001 |
| cells of myeloid lineage | | X | | Halonen et al. 2001 |
| reticular epithelial cells | | X | | Halonen et al. 2001 |
| corpuscle cells of thymus | | X | | Halonen et al. 2001 |
| epithelial cells of lymph vessels | | X | | Halonen et al. 2001 |
| DCs | | X | Hubert et al. 2008 | |

*X?* = conflicting data

Mouse Aire is expressed in similar locations as the human AIRE. As mice are more readily available for analysis, expression studies have been broader in mouse tissues and have revealed Aire expression in several more tissues than in humans, albeit at low levels. Expression has been observed at RNA level in the brain, liver, kidney, pancreas, intestine, gonads, thyroid gland and adrenal glands (Blechschmidt et al. 1999; Heino et al. 2000; Halonen et al. 2001) and at protein levels in thymus, spleen and lymph nodes (Adamson et al. 2004).

The differences in methods, antibodies and probes probably explain the differences in expression found between different studies. For example using Northern blot, Aire expression was undetectable, but using RT-PCR Aire could be detected in the thymus, ovaries, lungs, testes, kidney and adrenal glands of the mouse (Ruan et al. 1999). Now the consensus concerning Aire expression seems to be shifting towards expression in restricted lymphoid organs (Klamp et al. 2006).

A fascinating fresh report only just released (Saltis et al. 2008), shows that Aire is expressed in all gnathostome classes except in cartilaginous fish. The sequence of Aire was compared in mouse, human, opossum, chicken, Xenopus frog, zebrafish and puffer fish. All species had the first PHD domain but the other domains were absent or divergent in these species. Other conserved domains were the NLS and HSR. The second PHD domain showed more sequence similarity to Ring finger domains than to PHD domains. Still, the conserved regions convey the nuclear localization, transport and dimerization of Aire. This evolutionary comparison shows that certain parts of Aire, like the SAND domain, has evolved very quickly and may be more random in its binding. Aire-dependent T cell tolerance probably dates back to the emergence of bony fish (Saltis et al. 2008).

## 6.4 Interactions of AIRE/Aire

As it is most likely that AIRE is located in larger molecular complexes in cells, it is most important to study what other molecules may reside in these complexes and/or interact with AIRE. Not many interaction partners of AIRE have been found yet, considering the amount of research done on AIRE. The common transcriptional coactivator CREB-binding protein (CBP) was shown to interact *in vitro* with AIRE (Pitkanen et al. 2000) and to activate the transcription of self-antigens as a regulator of transcription together with CBP (Pitkanen et al. 2005). How AIRE and CBP collaborate on the molecular level remains to be resolved, although a fresh report suggests AIRE is needed for CBP to localize from the cytosol to the nucleus and CBP may induce AIRE's transactivation potential (Ferguson et al. 2008).

AIRE has been shown to interact with several members of the nuclear transport receptor importin α family. These interactions proposed that AIRE is imported into the nucleus using the classical importin α/β pathway (Ilmarinen et al. 2005). Why exactly AIRE is transported in and out of the nucleus is not yet known.

Interactions have been shown with PIAS proteins as well (Ilmarinen et al. 2008). AIRE and PIAS proteins were found in common complexes, but AIRE was not found to be SUMOylated by PIAS. PIAS1 probably affects the SUMOylation of molecules other than AIRE in the complex in which they reside and by interactions with SUMO moieties in proteins included in the complex (Ilmarinen et al. 2008).

Additionally, AIRE interacts with multiple components of the transcription complex including a novel interaction with the protein GBDR1 (glialblastoma cell differentiation factor-related protein) and functions in a novel manner, differing from classic transcription factors (Tao et al. 2006). The ubiquitin-associated (UBA) domain in GBDR1 often associates with components of the ubiquitin pathway (Tao et al. 2006), in line with the first PHD finger in AIRE having E3 ligase activity (Uchida et al. 2004). As this finding has not been confirmed, the role of ubiquitination in AIRE function is currently not known (Bottomley et al. 2005).

The newest interaction partner found for AIRE is a heterotrimeric complex of DNA-dependent protein kinases (DNA-PK), which once again, affects the transactivation capacity by phosphorylating AIRE. This modification may affect other features of the functional regulation of the AIRE protein as well (Liiv et al. 2008). Chromatin modifications may also be affected by AIRE as it was just shown that AIRE may directly bind to histones and sense epigenetic chromatin modifications (Org et al. 2008).

## 6.5 Functions of AIRE/Aire

The fact that AIRE is expressed in mTECs was the first hint of its role in central tolerance (Blechschmidt et al. 1999; Heino et al. 1999), since mTECs express a vast range of tissue-specific antigens (TSAs) (Derbinski et al. 2001). Additionally, *in vitro* studies had shown that AIRE has transcriptional activation potential (Bjorses et al. 2000; Pitkanen et al. 2001).

### 6.5.1 Aire in central tolerance

The current hypothesis for the function of Aire is that AIRE regulates the ectopic transcription of certain genes encoding peripheral TSAs in mTECs in the thymus (Klein and Kyewski 2000; Anderson et al. 2002). Absence of AIRE causes a defect in the thymic expression of ectopic antigens and subsequently loss of tolerance to these antigens resulting in the widespread autoimmunity in APECED patients. Proof

for this hypothesis comes from studies mainly in Aire deficient mice, described below.

The function of Aire in negative selection was proven by two groups (Anderson et al. 2002; Liston et al. 2003; Liston et al. 2004) using TCR transgenic mouse models. They provided direct evidence that Aire deficiency causes a disruption in the process of T cell deletion in the thymus. Liston et al. also showed the first proof of central tolerance affecting TSAs as well as ubiquitous antigens (Liston et al. 2003).

First it was considered that AIRE may be involved in transcription complexes, not directly contacting DNA but modulating the promoters of the TSA genes (Derbinski et al. 2005). A recent study showed that Aire binds directly to DNA and to the promoters of some of its target genes *in vivo*, directly regulating their expression in the thymus. Thus, Aire seems to regulate the expression of autoantigens (Ruan et al. 2007). The set of genes directly regulated by Aire is not defined, but Ruan et al. reported some target genes downstream of Aire. These included some autoantigens, post-translational modifiers, transcription factors and growth factors, of which three are involved in immunologically relevant pathways. Studying these further may help explain the mechanism of Aire function (Ruan et al. 2007). Furthermore, AIRE may constitute a part of a transcriptional complex together with IRF8 (Giraud et al. 2007) and possibly other proteins. Global chromatin modification may be used by Aire to silence TSA expression (Ferguson et al. 2008). Excitingly, it was recently found that AIRE may be necessary for regulation of transcription at elongation phase (Oven et al. 2007; Org et al. 2008). More research is required for the elucidation of the molecular mechanisms of Aire.

Microarray and expression studies using mTECs isolated from Aire knock-out mice have shown that the expression of a sizeable subset of hundreds to thousands of tissue-specific antigen genes in the mTECs is lost in the absence of Aire (Anderson et al. 2002; Derbinski et al. 2005). Some of these genes seem to encode proteins which are known targets of APECED autoantibodies, such as insulin (Gylling et al. 2000).

Many transcription factors have dual activating and silencing capabilities (Gabellini et al. 2003). Therefore array studies showing both up-and down-regulated genes in absence of Aire may not be so surprising. Up-regulation of some TSAs occurs in absence of Aire (Derbinski et al. 2005). The genes dependent on Aire are found in clusters, where single clusters can contain both Aire up- and down-regulated genes. No single explanation for this has been established, but epigenetic regulation mechanisms (such as methylation) have been suggested (Gotter and Kyewski 2004).

Analysis of the chromosomal clustering of Aire-controlled genes showed, further, that Aire controls antigen presentation of TSAs and other genes. Further, AIRE is not likely to function merely by chromatin remodeling (Johnnidis et al. 2005) since

Aire-controlled and Aire-independent genes were often interspersed. Using slightly different analyses, Gotter and Kyewski came to the similar conclusions (Gotter and Kyewski 2004).

Hughes and Friedman (Hughes and Friedman 2006) extended the Aire gene expression analysis reported by Anderson et al. (Anderson et al. 2002) by analyzing the up-and down-regulated, Aire-dependent genes. The aim was to see whether Aire-activated genes are disproportionately tissue-specific. Particularly they studied the genes down-regulated by Aire. They found differences among the genes both activated and repressed by Aire, as well as those unaffected by Aire, confirming Anderson's results. Expression levels of Aire activated genes were lower than those of other genes. Phylogenic studies showed that evolution of Aire may have occurred long ago, although the proteins activated by Aire may have changed with evolution (Hughes and Friedman 2006).

A loss of tolerance to two Aire-dependent antigens, a stomach antigen (mucin 6) and an eye antigen (interphotoreceptor retinoid-binding protein, IRBP) has been reported in Aire deficient mice. Absence of the expression of these antigens in the thymus caused autoimmunity against the eye and the stomach, showing a direct link between TSA expression controlled by Aire and an autoimmune phenotype in Aire knock-out mice. Identification of these specific antigenic targets show that the lack of expression of these targets in the thymus causes the generation of harmful antibodies against them and that this autoimmunity is dependent on Aire (DeVoss et al. 2006; Gavanescu et al. 2007).

Another finding supporting the hypothesis of AIRE regulating TSA expression in the thymus came first from a study showing that proinsulin expression in the thymic epithelium was correlated with the gene dosage of functional Aire alleles (Liston et al. 2004). Later, direct correlation between AIRE and insulin expression in the thymus was shown by another group (Sabater et al. 2005). This would imply that AIRE regulates insulin expression (Sabater et al. 2005). Levels of AIRE may, according to the authors, also influence T1D (Sabater et al. JAutoim 2005). Autoimmune susceptibility may in fact arise from small differences in expression levels of genes involved in the pathway from TCR to cell death, called quantitative variation of negative selection (Liston and Rudensky 2007). Variable expression levels of AIRE and TSAs in the thymus of different individuals were shown by Taubert et al. by examining human mTECs. This was evidence for the regulation of TSAs by AIRE (Taubert et al. 2007).

Opposingly, Aire-deficient mice develop autoimmunity against some antigens that are expressed in thymus despite the absence of Aire. These genes are not Aire-dependent. One of such antigens is α-fodrin and an other is pancreas specific protein

disulphide isomerase (PDIp) (Kuroda et al. 2005; Niki et al. 2006). A possible explanation for this could be that Aire is involved in the processing and presentation of these antigens. Further the suggested function of Aire as an ubiquitin E3 ligase may play a role in this function (Uchida et al. 2004). Further clues to the other/alternative roles of Aire come from expression array analyses which have shown for example down-regulation of a gene involved in MHC class II peptide loading. These studies also hint at Aire deficient mTECs having poorer antigen presentation efficiency than Aire-positive mTECs (Anderson et al. 2005). Why the autoimmune phenotype of APECED patients and Aire deficient mice are limited to a certain set of tissues is not known.

Not long ago, it was confirmed that Aire functions in tolerance by the negative selection of T effector cells, not positive selection of Tregs (Anderson et al. 2005). The authors showed that Aire is involved in negative selection, as had been shown by previous reports. Moreover, they demonstrated that Aire may be involved in negative selection processes other than just expression of TSAs, perhaps in the interaction between cells presenting TSAs and thymocytes. In any case, the function of Aire is critical for inhibiting AID (Anderson et al. 2005).

Finally, while Aire had been shown to have an allele dose-dependent effect on TSA expression in the thymuses of mice (Liston et al. 2004), no effect on TSA expression in the lymph nodes was observed in this new study. In the thymus, Aire and TSAs were both localized in the medulla. Aire could directly induce the expression of TSAs and a correlation between expression of Aire and TRAs was suggested (Kont et al. 2008).

Thus, accumulating evidence shows AIRE is important in central tolerance. The exact mechanism for how AIRE functions in TSA expression and which TSAs it targets and why, still need to be confirmed. The recent active research in the field has already provided many hypotheses and pieces of knowledge that will need to be put together and tested in the near future.

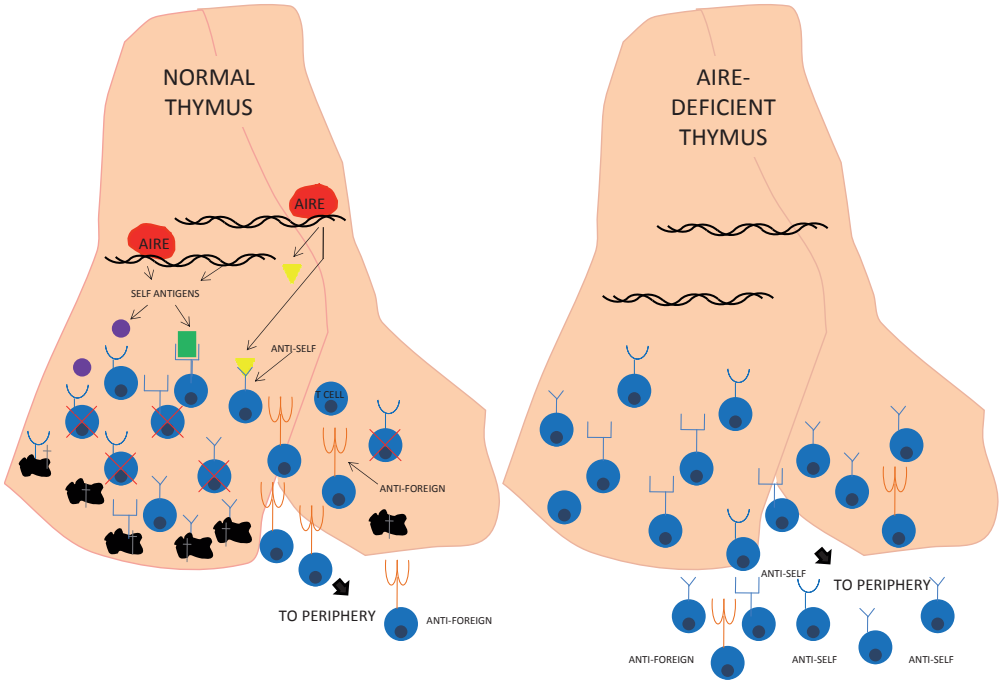



**Figure 9.** ***The role of Aire in central tolerance. Aire drives expression of self-antigens presented to developing T cells. Self-specific T cells are eliminated, while microbe-specific T cells survive and leave the thymus to fight infection. In the absence of Aire, some self-antigens are not expressed, self-reactive T cells are not eliminated and autoreactive t cells are allowed to escape the thymus, into the periphery, where they attack organs and cause autoimmune disease. Modified from Heath and Scott (Heath and Scott 2002).***

### 6.5.2 Aire, mTECs and thymic structure

Aire deficiency may cause changes in the organization and composition of the medullary epithelium (Gillard et al. 2007). This could explain some functions of Aire. However, previous work has not shown serious defects in thymic stromal structure in Aire knock-out mice (Anderson et al. 2002; Kuroda et al. 2005). In any case, Aire plays a role in thymic epithelial cell differentiation and in "terminal differentiation" *i.e.* mTECs maturation enabling them to express TSAs (Derbinski et al. 2005).

Earlier, it has been shown that Aire is expressed in cells and anatomical sites important for negative selection and that this expression is modulated by T cells undergoing negative selection. Additionally, the role of Aire as a transcriptional regulator and correlation of Aire expression with the structural organization of the thymus, suggest that Aire may play a role in determining the architectural organization of the microenvironment of the thymus and thus, affects T cell selection and self tolerance in this way. It is known that the disruption of the thymic architecture causes defects in the expression pattern and function of the thymus (van Ewijk et al. 1994). Indeed, absence of Aire did lead to some unorganization of the thymic stroma in one mouse model. Aire was also differently expressed during positive and negative selection in the thymus. Aire was localized to sites of negative selection and modified by cells in these locations (Zuklys et al. 2000).

Aire has previously been suggested to be regulated by lymphotoxin beta receptor (LtβR) signaling, by the lymphotoxin-pathway (Chin et al. 2003; Chin et al. 2006; Zhu et al. 2006). Surprisingly, two groups have recently published papers showing the contrary: that the lymphtoxin pathway or LtβR do not affect the mTECs or regulation of TSA expression by Aire (Venanzi et al. 2007; Martins et al. 2008) (at least not directly). Using whole genome transcriptome analysis, it was shown that absence of LtβR does not have an effect on Aire expression, or on Aire-controlled tissue specific antigen (TSA) expression. The elimination of LtβR did not perturb Tregs or the allocation of DCs in the thymus. Contrarily, LtβR signaling did affect the 3D structure of mTECs, but in an Aire-independent manner. The 3D structure of the thymic medulla was disturbed in LtβR deficient mice leading to an abnormal distribution of mTECs and T lymphocytes, causing autoimmunity. Therefore, the correct structure of the thymic medulla guarantees negative selection by providing an environment facilitating T cell-epithelial cell interaction (Martins et al. 2008).

These findings are in line with Boehm et al. (Boehm et al. 2003) who showed that $Lt\beta R^{-/-}$ mTECs express normal levels of AIRE. Also Rossi et al. (Rossi et al. 2007) proposed that another pathway concerning the TNF superfamily receptor, through activator of NFκB (RANK) may explain the phenomena previously suggested to be due to LtβR, concerning promotion of $AIRE^{+/+}$ mTEC development (Venanzi et al. 2007). RANK signals from intrathymic lymphoid inducer cells induce the development of $Aire^{+}$ mTEcs (Rossi et al. 2007). This theory was newly confirmed. White et al. suggest that initial differentiation of $Aire^{+}$ mTECs requires RANK with inducer cells and then mTEC turnover/survival via CD40 interactions with lymphocytes expressing CD40L in the thymus (White et al. 2008).

Absence of Aire and/or of mTECs causes blockage of T cells between the SP3 and SP4 stages of T lymphocyte development (Li et al. 2007). Although these SP3 T cells

were not yet fully mature, nevertheless, some were exported to the periphery and may have contributed to the autoimmunity in Aire deficient mice (Li et al. 2007).

Although the stroma in Aire deficient mice was intact (Anderson et al. 2002; Kuroda et al. 2005), alterations in the thymic medullary epithelium were noted. These alterations caused changes in thymic environment suggesting Aire has a role in mTEC differentiation and/or survival. This would imply a broader role for Aire than just in regulation of TSA expression, maybe as a "randomizer" of gene expression (Gillard et al. 2007). New studies concerning mTECs have shown that $Aire^{+}$ mTECs arise from a unique mTEC cell lineage and express tight-junction components claudin 3 and claudin 4 (Cld3,4) (Hamazaki et al. 2007). The mTECs that express Aire only make up a small proportion of all mTECs (Derbinski et al. 2001) and this subset expresses a lot of MHC class II and costimulatory molecules as well as Aire (Hamazaki et al. 2007). Studies on AIRE have therefore also taught us about the development of the mTEC lineage (Hollander 2007). However, whether the terminal differentiation model holds and how exactly Aire functions in mTECs and mTEC – lymphocyte interactions remains to be seen.

### 6.5.3 Aire in the periphery

While it is clear that AIRE plays an important role in central tolerance, it has been suggested that it has additional functions outside of the thymus. Aire is expressed in the periphery and could act there in the control of peripheral tolerance. This means controlling self-reactive T cells which have escaped thymic negative selection. Three mechanisms of function for Aire and cell types involved in development of tolerance have been the focus of studies so far: 1) DCs, 2) thymic development of Tregs and 3) functions in the stromal cells of lymph nodes.

Several publications have reported expression of Aire in the periphery, in secondary lymphoid organs and DCs (Halonen et al. 2001; Adamson et al. 2004; Sillanpaa et al. 2004; Ramsey et al. 2006). First, AIRE/Aire was shown to be involved in DC differentiation (Sillanpaa et al. 2004). Later, it was shown that Aire-deficient DCs activate naïve T cells more efficiently than Aire-positive DCs and that Aire may thus control T cell activation by DCs in the periphery (Ramsey et al. 2006). However, loss of Aire in DCs alone was insufficient to break tolerance (Anderson et al. 2002).

It has been shown that TSA expression in the thymus can drive positive selection of Tregs (Jordan et al. 2001; Walker et al. 2003). Thus, Aire-positive mTECs may be involved in the development of Tregs in the thymus (Aschenbrenner et al. 2007). However, numbers and functions of Tregs were normal in Aire deficient mice (Anderson et al. 2002; Kuroda et al. 2005). Double knock-outs of Aire and FoxP3 had a more severe phenotype than the individual knock-outs, although they

resembled more of a scurfy type than a mixture of Aire deficiency and scurfy (Chen et al. 2005). Nevertheless, it was shown that when Aire-positive mTECs were targeted to present an antigen, this resulted in production of Tregs specific for the antigen. This occurred independently of antigen transfer to DCs, indicating that mTECs themselves were able to present the antigen, and that mTECs are important for Treg development (Aschenbrenner et al. 2007). Thus, the absence of Aire may lead to defects in Tregs acting in the periphery. This study, together with a few other recent reports, shows that Aire is involved in both clonal deletion and differentiation of Tregs. Tregs were also found to be impaired in function in APECED patients, although these defects were not found in Aire deficient mice (Kekalainen et al. 2007). These findings begin to link Aire, FoxP3 and Treg selection (Nomura and Sakaguchi 2007). Still, it has not been directly shown that Aire promotes thymic development of Tregs. Since Tregs develop in the cortex whereas Aire is expressed in the medulla of the thymus, the role of Aire in development of Tregs requires further investigation.

Aire was recently shown to be expressed in the stromal cells of lymph nodes. Aire controls the development of tolerance to retinal and pancreatic antigens. However, peripheral tolerance plays an important role in the "decision to commence" autoimmunity. Induction of anergy by antigen presentation in for example lymph nodes in the periphery is required in addition to central tolerance in order to avoid autoimmune attack (Lambe et al. 2007). Stromal cells of lymph nodes are also involved in TSA presentation to $CD8^+$ T cells in a similar way to thymic TSA presentation, leading to clonal expansion and subsequent elimination of the T cells. This mechanism possibly reinforces peripheral tolerance induced by cross presentation by DCs (Lee et al. 2007; Zehn and Bevan 2007). Although this would be a tempting role for Aire in the periphery, the role of Aire in stromal lymph node cells remains at present unclear, as for example insulin, an Aire-dependent TSA, is not expressed in these cells (Lee et al. 2007).

Just before sending in this thesis, an intriguing study by Gardner et al. (Gardner et al. 2008) identified cells in peripheral lymphoid tissues, in the T and B cell area interfaces, expressing Aire and mediating deletion of autoreactive T cells. These data suggest peripheral lymphoid tissues act as a "safety net", stopping autoreactive T cells, specific for other antigens than those expressed in thymus, from escaping elimination in the periphery. Further, this group showed that the number of Aire-controlled genes in the periphery is only about 1/10 of those controlled in the thymus and that these gene pools do not overlap much, although both include many TSAs (Gardner et al. 2008; Kyewski 2008). This study adds to the list of questions to be answered about Aire and the role of Aire, especially in the periphery.

### 6.5.4. Other functions of AIRE/Aire

***Aire and apoptosis***

Apoptosis plays an important role in autoimmunity such as in T cell development, T cell repertoire, immune responses etc (Kuhtreiber et al. 2003). Aire has been shown to interact with DNA-PK. DNA-PK activates apoptosis, therefore indicating a possible role for AIRE in apoptosis. Furthermore, AIRE contains a CARD domain which may interact with apoptotic molecules, thus indicating a possible role for Aire in apoptosis (Kuhtreiber et al. 2003).

Centrally, it has been hypothesized that Aire may induce apoptosis in mTECs. Aire-positive mTECs have a high turn-over rate (10% per day!). Additionally, Aire promotes changes that ensure efficient presentation of TSAs, termed "terminal differentiation" since Aire-induced apoptosis is not immediate, but leads to changes in mTECs culminating in their death. Whether these effects of Aire are direct or indirect is not known. Aire could therefore facilitate cross-presentation of self-antigens produced to DCs before the mTECs are programmed to die (Gray et al. 2007).

Expression of AIRE in the testis may be required for the scheduled apoptosis of germ cells (Schaller et al. 2008). This conclusion came from a finding where Aire deficient mice had reduced levels of scheduled apoptosis, but reciprocally, higher levels of sporadic apoptosis. This lead to the hypothesis that scheduled apoptosis provides a "counterselection mechanism" keeping the germline stable. So, cells with mutated genes would apoptose spontaneously and prematurely if they acquire mutations (Schaller et al. 2008).

***Aire in thymoma and lymphoma***

Marginal zone B cell lymphoma has been reported in Aire deficient mice over 1 year to 2 years of age, as well as liver infiltrates consisting of B cells. These symptoms are suggestive of antigen exposure and lymphocyte overactivation. A peripheral role for Aire in control of APC development and marginal zone B cell activation is thus implied by this study. Aire may therefore have a peripheral regulatory role by controlling the development of APCs and marginal zone B cell activation, or modulation of their phenotype (Hassler et al. 2008).

A mysterious lack of Aire expression has been noted in thymomas, possibly due to gene silencing or maturational arrest of epithelial cells (Offerhaus et al. 2007). Another study of Aire in relation to cancer has been reported where AIRE expression was noted to be decreased in thymomas, compared to healthy thymuses (Suzuki et al. 2008). This is in agreement with findings by Ströbel et al. (Strobel et al. 2007), although Ströbel. et al. noted a total absence of AIRE.

Finally, an important note is that there are some differences in the role of AIRE/Aire in mice and men. For example FoxP3 Tregs are defective in APECED patients (Kekalainen et al. 2007), but such defects have not been observed Aire-deficient mice (Anderson et al. 2005; Kuroda et al. 2005; Niki et al. 2006; Kekalainen et al. 2007).

To summarize, Aire plays an essential role in central tolerance. It regulates development of tolerance to several autoantigens and functions also in the negative selection of autoreactive T cells, by regulation of TSAs in the thymus (reviewed by Peterson et al. 2004). Aire may also play a role in the periphery, perhaps in peripheral tolerance, but whether this is the case and how Aire functions outside of the thymus is not yet established (Cheng et al. 2007).

# 1 AIMS OF THE STUDY

APECED is a monogenic autoimmune disease and, therefore, in addition to being an intriguing disease of which the mechanisms are not yet understood, also a unique ”simple model” enabling the study of the onset of autoimmune diseases in general. The specific aims of this study were:

1. To use the Aire-deficient mouse model of APECED as a tool in order to study the APECED autoantibodies
2. To study the possible role of AIRE in peripheral tolerance
3. To find out whether Aire affects the development of γδ T cells

# 2 MATERIALS AND METHODS

### Published materials and methods

The materials and methods used in this study are described in the original publications (I-III) in more detail.

| Material or method | Original publication |
|---|---|
| Aire-deficient mice | I, III |
| APECED patients | II, III |
| preparation of tissues and blood | I, II, III |
| DNA extraction | I |
| PCR/RT-PCR | I |
| SDS-PAGE and Western blotting | I |
| Cloning of autoantigens and constructs | I |
| In vitro transcription and translation (ITT) | I |
| Radioimmunoprecipitation (RIPA) | I |
| Assays for nuclear antibodies | I |
| RIA for detection of insulin | I |
| Genotyping of mice | I, III |
| Lymphocyte proliferation assay (thymidine incorporation) | I, III |
| extraction/homogenization of cells | I, III |
| Cell culture | II |
| Culture of *Candida albicans* | II |
| MoDC stimulations and infections | II |
| Cytokine assays | II |
| Affymetrix arrays | II |
| Genome-wide expression analyses | II |
| Pathway analyses | II |
| RNA extraction | II, III |
| cDNA synthesis | II, III |
| Taqman (Q-RT-PCR) | II, III |
| Flow cytometry (FACS) | II, III |
| TCR repertoire analysis | III |
| Statistical analyses | I, II, III |
| Ethical considerations | I, II, III |

## Ethical considerations

This study has been evaluated and approved by the Laboratory Animal Committees of the National Public Health Institute and of the University of Helsinki, Finland, as well as the State Provincial Offices of Southern Finland. The study has been conducted according to good practise in laboratory animal handling and the regulations for handling genetically modified organisms have been followed.

# 3 RESULTS

## 1 No typical APECED autoantibodies in Aire deficient mice (I)

A characteristic attribute of APECED is a spectrum of well-characterized, circulating, tissue-specific autoantibodies. The presence of autoantibodies targeted to endocrine organs has been reported in Aire deficient mouse models using indirect immunofluorescence analyses of tissue sections by three independent mouse models, all of the B6 background (Anderson et al. 2002; Ramsey et al. 2002; Jiang et al. 2005). This suggests that autoimmune processes against these tissues might be present in Aire deficient mice despite the absence of clinical disease. The discrepancies between the symptoms of Aire deficient mice and patients with APECED led us to investigate whether the Aire deficient mice produce autoantibodies similar to human APECED. This issue is important not only for evaluating the relevance of Aire deficient mice as a model of APECED, but for autoimmunity in general and for comparison of the mechanisms of autoimmunity between mice and man.

In the first study we tested the presence of autoantibodies against eleven human APECED antigen homologues in Aire deficient mice, listed in bold in Table 7.

**Table 7.** ***Autoantigens and autoantibodies in APECED.***

| AUTOANTIGEN | PREVALENCE | CLINICAL DISORDER | TARGET ORGAN | REFERENCE |
|---|---|---|---|---|
| **P450c21** | 66 % | Addison's disease | adrenal cortex | Krohn et al. 1992, Uibo et al. 1994, Chen et al. 1996, Söderbergh 2000 |
| **P450c17a** | 44 % | | | |
| **P450scc** | 52 % | | | |
| **P450scc** | | failure of gonads | gonads | Söderbergh 2000, Uibo et al. 1994 |
| **Thyroid peroxidase (TPO)** | 36 % | hypothyroidism | thyroid gland | Perheentupa et al. 1998 |
| **Thyroglobulin (TG)** | 36 % | | | Perheentupa et al. 1998 |
| **GAD65**, GAD67 | 37 %** | T1D | pancreas | Gylling et al. 2000 |
| **IA-2** | | | | |
| insulin* | | | | |
| **Tyrosine hydroxylase (TH)** | 40 % | alopecia | scalp | Hedstrand et al. 2000 |
| **Cyp1A2** | | autoimmune hepatitis | liver | Clemente et al. 1997, 1998, Gebre-Medhin et al.1997, Husebye et al. 1997, Söderbergh 2000 |
| **Cyp2A6** | | | | |
| **AADC** | 51 % | | | |
| **Tryptophan hydroxylase (TPH)** | 45 % | intestinal dysfunction/ malabsorption | gastrointestinal tract | Tuomi et al. 1996, Ekwall et al. 1998, Söderbergh 2000 |
| GAD65, GAD67 | | | pancreas | |
| **SOX9** | | vitiligo | skin | Husebye et al. 1997, Hedstrand et al. 2001 |
| **SOX10** | | | | |
| AADC | | | | |
| ICA | | T1D | pancreas | Ahonen et al. 1985 |
| *IFN-α, IFN-ω, (IFN-β, IFN-λ1¤)* | *100 %* | | | Meager et al. 2006 |
| *NALP* | *49 %* | *hypoparathyroidism* | *parathyroid gland* | Alimohammadi et al. 2008 |
| *TDRD6* | *49 %* | | | Bensing et al. 2007 |
| *enolase* | *58 %* | | | O'Dwyer et al. 2007 |

**bold - analyzed in our study**
*grey, cursive - new autoantibodies described after our antibody screen*
*controversial data
*** GAD65*
¤ = also called IL-29

Modified from: Meriluoto et al. 2001, Heino et al. 2001, Peterson et al. 2004, and Peterson and Peltonen 2005

## 1.1 Cloning of APECED antigens and their murine homologues

In order to determine whether APECED autoantibodies found in Aire deficient mice correspond to their human equivalents, we screened the sera of individual Aire deficient mice for antibodies against autoantigens previously identified in APECED patients.

First, the cDNAs of murine homologues for eleven human APECED autoantigens (see Table 1.) were amplified by RT-PCR. Then, they were cloned into an appropriate expression vector. Correctness of the clones was verified using DNA sequencing, followed by production of the antigens by *in vitro* transcription-translation (ITT). Analysis of the radiolabelled ITT products in SDS-PAGE confirmed their expected molecular masses. Finally, these radiolabelled human or mouse polypeptides were used as antigens in immunoprecipitation assays.

## 1.2 Screening for APECED autoantibodies in Aire deficient mice

Initially, sera from individual Aire deficient mice were tested for antibodies against the human autoantigens 21OH, 17OH, P450scc, TPH, AADC, GAD65, IA-2, and Cyp1a2 and Cyp2a5. Unexpectedly, neither $Aire^{-/-}$ nor $Aire^{+/+}$ mice displayed antibodies against any of the human APECED antigens tested. In order to rule out the possibility that the murine autoantibodies do not react with the human homologues, we went on to repeat the assay using the mouse homologues of human APECED autoantigens, cloned as explained above. The mouse genes were: 21OH, 17OH, P450scc, TPH, AADC, IA-2, PAH, Sox9, Cyp1a2, Cyp2a5 GAD1 and GAD2 (and later, insulin).

Sera from 20 individual wt and 20 Aire deficient mice were tested for reactivity against the *in vitro* synthesized, $^{35}S$-labeled mouse antigens using, again, immunoprecipitation assays. No autoantibodies were found in these mouse sera. As positive controls and in order to confirm our assay worked, we showed that *in vitro* produced human P450scc and GAD65 antigens could be immunoprecipitated with sera from APECED patients. Furthermore, we verified that *in vitro* synthesized mouse AADC was precipitated with an AADC positive serum from an APECED patient (Figure 10).

Negative results must be thoroughly verified. We therefore went on to analyze sera from $Aire^{+/+}$ or $Aire^{-/-}$ mice by immunoblotting using the eleven *in vitro* translated mouse antigens. None of the sera which we had obtained from the Aire knock-out mice (3-7 $Aire^{+/+}$ and 3-7 $Aire^{-/-}$ sera were tested per antigen, as well as human serum controls) reacted against any of the *in vitro* synthesized antigens tested.

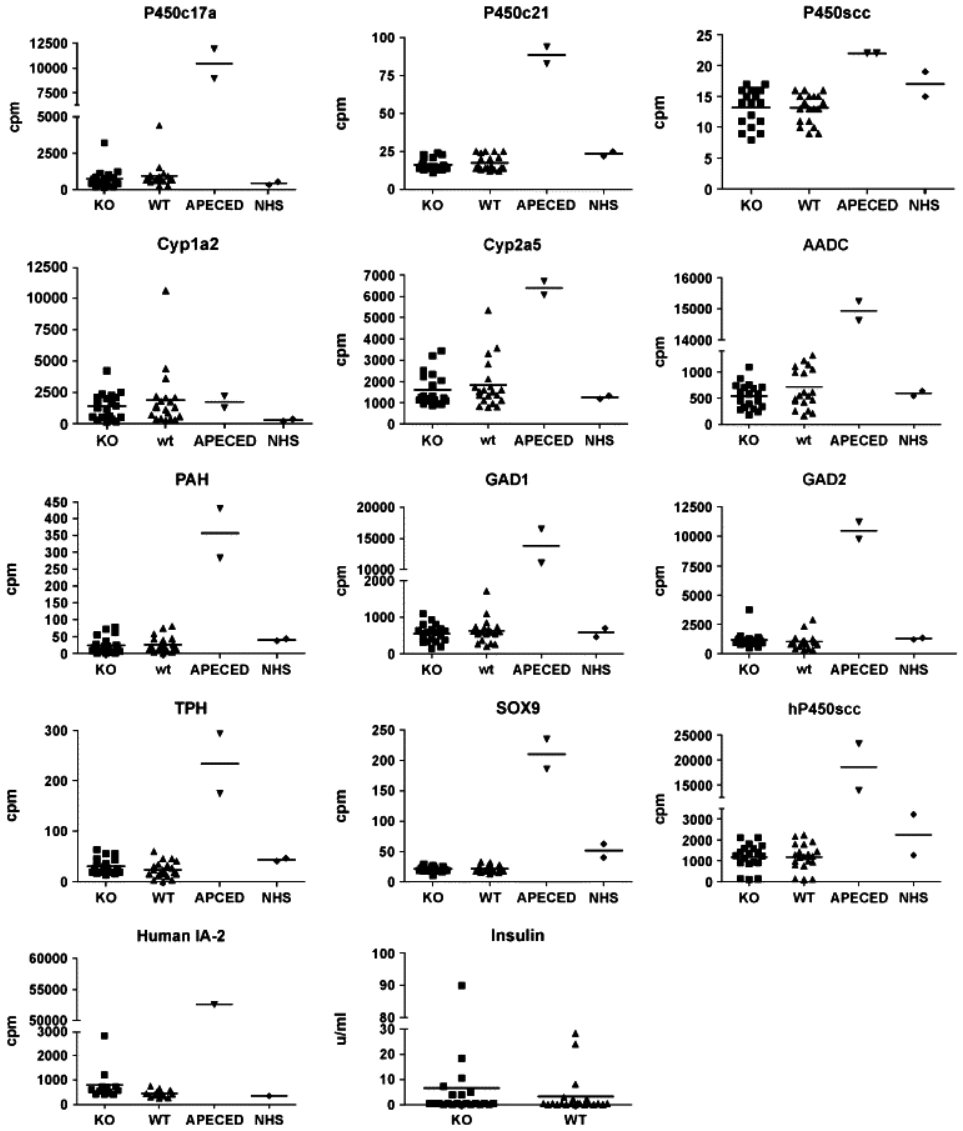



**Figure 10.** ***Autobodies against the APECED antigens tested were not detected in Aire deficient mice, or were not present in levels higher than in wt mice.***

However, some reactivity against different unidentified polypeptides of the rabbit reticulocyte lysate, in both wt and the Aire deficient mouse sera were revealed. Yet no distinct knock-out specific immunoreactive bands could be detected. The

immunostaining pattern with the serum from the same mouse remained the same in spite of the antigen used. Interestingly, the staining patterns seemed to be serum specific, showing individual reactivity patterns for the mice.

It has been reported (Anderson et al. 2002; Liston et al. 2004) that Aire controls the expression of insulin in the thymus in mice. Proinsulin is the driver antigen in T1D in NOD mice and likely to be the primary antigen in human common T1D (Nakayama et al. 2005; Harrison et al. 2008; Kobayashi et al. 2008). Insulin is also a well-known autoantigen in T1D in both humans and mice, as well as in APECED patients (Maruyama et al. 1988; Gylling et al. 2000). A previous study concerning 60 Finnish patients with APECED had shown that 12 of them developed type 1 diabetes and insulin autoantibodies were detected in 36% of the prediabetic APECED samples (Gylling et al. 2000). Furthermore, insulin antibodies were not found in the sera of non-diabetic patients. Taking these findings into consideration, we wanted to determine the presence of insulin autoantibodies in Aire deficient mice. Since ITT did not provide the post-translational modifications to the synthesized insulin polypeptide, an immunoprecipitation assay using $^{125}$I human insulin was used instead for this antigen. We could not detect a significant difference in insulin reactivity between the 21 Aire$^{-/-}$ and the 21 Aire$^{+/+}$ mouse sera tested.

## 1.3 Systemic autoantibodies

Systemic autoimmune diseases such as SLE and Sjögren's syndrome (SS) are characterized by anti-nuclear autoantibodies (ANAs) in both mice and men. SS is also a disease component of APECED. A few groups have reported that APECED patients have low-titer systemic autoantibodies as well as circulating ANAs (Perniola et al. 2000; Obermayer-Straub et al. 2001). Therefore, we wanted to test Aire deficient mouse sera for presence of ANAs. Commercial Hep 2 cell slides were used to test sera from 18 Aire$^{-/-}$ and 18 Aire$^{+/+}$ mice individually.

Nearly equal amounts of ANA-positive titers (≥100) were detected in both knock-out and wt sera (in eight and six mice, respectively), whereas higher numbers of mouse sera: 12 Aire$^{-/-}$ and 14 Aire$^{+/+}$, tested negative. Positive reactions of high titers (≥ 500) were detected in six Aire knock-out sera and in two wt sera, but this difference was not statistically significant ($p = 0.241$).

Five sera from Aire$^{-/-}$ and wt mice each which had tested ANA positive were tested further by line blotting for extractable nuclear antigens. Three of the five sera and 1/5 wt serum gave a faint reaction with histones and 1/5 of the Aire$^{-/-}$ sera reacted in addition with RNP-A, RNP 70kd, CenpB and Scl70-proteins. None of these autoantibodies are typical for SS.

### 1.4 Proliferation of lymphocytes in response to autoantigens

Lastly, we wanted to determine whether the Aire deficient mice elicited T cell responses against the autoantigens studied. Spleen cells isolated from 7 $Aire^{-/-}$ and 5 $Aire^{+/+}$ mice were harvested and then cultured for six days in the presence of reticulocyte lysates with or without addition of *in vitro* produced autoantigen, or in the presence of medium only (control). A stimulation index of two, compared with the control wells, was used as threshold. Proliferative responses against altogether seven antigens in $Aire^{-/-}$ mice and against six antigens in $Aire^{+/+}$ mice were detected with no apparent pattern. Two mice, one $Aire^{-/-}$ and one $Aire^{+/+}$, demonstrated T cell responses also against the plain reticulocyte lysate. Knock-out mice did not elicit higher levels of proliferative responses versus wt mice. Thus, none of the autoantigens studied produced proliferative responses more often in lymphocytes from the $Aire^{-/-}$ mice than in lymphocytes from the $Aire^{+/+}$ mice.

## 2 APECED patient DCs are defective (II)

AIRE plays an important role in central tolerance. However, AIRE is also expressed in the peripheral monocyte/dendritic cell lineage (Heino et al. 1999; Kogawa et al. 2002; Sillanpaa et al. 2004; Zheng et al. 2004)) and the expression has been shown to be increased during the differentiation process of human monocyte to dendritic cells (Sillanpaa et al. 2004). Moreover, it has been proposed that AIRE is required for normal development and function of dendritic cells (Sillanpaa et al. 2004; Ramsey et al. 2006). The tolerogenic activity of AIRE not only takes place centrally, in the thymus, but AIRE is also involved in the regulation of peripheral tolerance, at the level of T cell – peripheral DC interaction (Ramsey et al. 2006). Still, the functions of AIRE in DCs are presently not known.

In order to further investigate the role of AIRE in host immune functions and DC biology, we compared the properties and transcript profiles in *in vitro* monocyte-differentiated dendritic cells (moDCs) obtained from APECED patients carrying the most common human *AIRE* mutation (R257X) and in healthy controls.

The high prevalence of chronic mucocutaneous *Candida albicans* infections (CMC) in APECED patients is a prospective sign of a defect in peripheral tolerance and/or innate immunity. Currently, the molecular basis of the increased susceptibility for CMC in APECED patients remains elusive.

## 2.1 AIRE is expressed in human DCs and its expression remains unchanged in response to microbial stimulation

We compared the expression of AIRE in moDCs generated from six APECED patients and six controls as well as in the plasmacytoid DC (BDCA $4^+$) and myeloid DC ($CD1c^+$, BDCA 1) subsets which we isolated directly from the blood of three healthy control individuals. The expression levels of AIRE mRNA were assessed using Q-RT-PCR. They were relatively low and nearly equal in all three DC populations studied. We also studied whether the expression level of AIRE mRNA would change during stimulation of moDCs with different microbes or their components. No significant changes in response to Sendai virus infection or stimulation with *Candida albicans* were observed.

## 2.2 Monocytes from APECED patients differentiate into immature moDCs and express typical DC specific genes

First, we studied whether monocytes isolated from peripheral blood of APECED patients are able to differentiate normally *in vitro* and do they show typical features of moDCs. Microscopical examination showed that moDCs from APECED patients and controls looked very similar during the 6 day differentiation culture *in vitro* with GM-CSF and IL-4 stimulation. Infection of cells with Sendai virus or stimulation with *Candida albicans* produced cellular processes typical for differentiated moDCs in both patient and control moDCs.

Further analysis of differentiation of AIRE-deficient moDCs using established DC markers and flow cytometry showed that the mean fluorescent intensity of HLA-II-DR of APECED moDCs was slightly lower than the corresponding levels of the controls. However, upon stimulation with *E. coli*-derived lipopolysaccharide (LPS) or infection with Sendai virus, HLA- II-DR was expressed at higher levels in patient moDCs than in control moDCs. Similarly, CD86 was expressed at a similar basal level, whereas expression levels were somewhat higher in stimulated patient DCs compared to control moDCs. However, there was a lot of individual variation in the expression of these markers and none of the differences were statistically significant.

The transcript levels of moDC-specific genes characteristic of differentiated immature DCs (Lehtonen et al. 2007) (CD1α, DC-SIGN, IL-21 receptor, IRF4, FcεR1a, SOCS1, WNT5a, and TLR2) were measured using Q-RT-PCR and calculated from two to six patients (depending on the marker) and from six controls. Slightly higher expression of DC-SIGN (not significant) and IL-21R ($p = 0.04$) and a lower expression of FcεR1A ($p = 0.014$) were found in APECED moDCs as

compared to control moDCS. The other marker genes showed no significant differences between patients and controls.

## 2.3 Impaired expression of inflammatory cytokine genes in AIRE deficient moDCs in response to microbial stimulation

In order to detect functional defects in patient DCs we analyzed microbe-induced cytokine gene expression in APECED moDCs using, again Taqman Q-RT-PCR. Prior to stimulation, the expression of most cytokine and chemokine mRNAs appeared similar in APECED and control DCs, suggesting normal monocyte-DC differentiation with the exception of CXCL8 transcript levels which were lower in patients versus controls (Article II, Figure 3; $p \leq 0.01$).

For stimulation, we used live *Candida albicans* and Sendai virus, which have been widely used as microbial stimuli in DC research. Control moDCs showed clear induction of most cytokines transcript levels tested in response to Sendai virus infection ($p \leq 0.01$). Conversely, APECED moDC responses remained at levels that were at least a hundred fold lower. Nonetheless, these differences were not statistically significant ($p > 0.05$) in our study sample. *C. albicans* was quite a poor inducer of IL-29 in both patients and controls. TNF-α and CXCL8 transcript levels were similar in APECED and control moDCs in response to *C. albicans* stimulation. CXCL10 was induced more weakly in APECED moDCs ($p \leq 0.001$). CXCL8 and TNFα were up-regulated significantly in both APECED and control moDCs in response to *C. albicans*, whereas in control cells IL-12p35, IFN-β and CXCL10 mRNA expression was also stimulated significantly ($p \leq 0.01$ or $p \leq 0.001$) by C. albicans.

## 2.4 Reduced cytokine production by APECED patient moDCs in response to microbial stimuli

We further analyzed cytokine concentrations in cell culture supernatants of moDCs that were infected with Sendai virus or stimulated with *C. albicans* or LPS for 24h. This was done using a FACS-based, multiplex bead assay. The basal expression level of TNF-α, IL-1β, IL-6, CXCL8 and IL-12 in unstimulated cells was lower in APECED moDCs than controls (Figure 11). The production of TNF-α, IL-1β, IL-6, CXCL8, IL-10 and IL-12 was clearly higher in controls in response to stimulation with *C. albicans*. The differences were statistically significant for TNF-α, IL-1β, IL-6 and CXCL8. Likewise, there was a clear difference in the ability of APECED moDCs and control moDCs to produce cytokines in response to Sendai virus infection or LPS stimulation (Figure 11). Of the stimuli used, Sendai virus infection

induced the highest levels of TNF-α, IL-1β and IL-12, while LPS was the best inducer of IL-6 and IL-10.

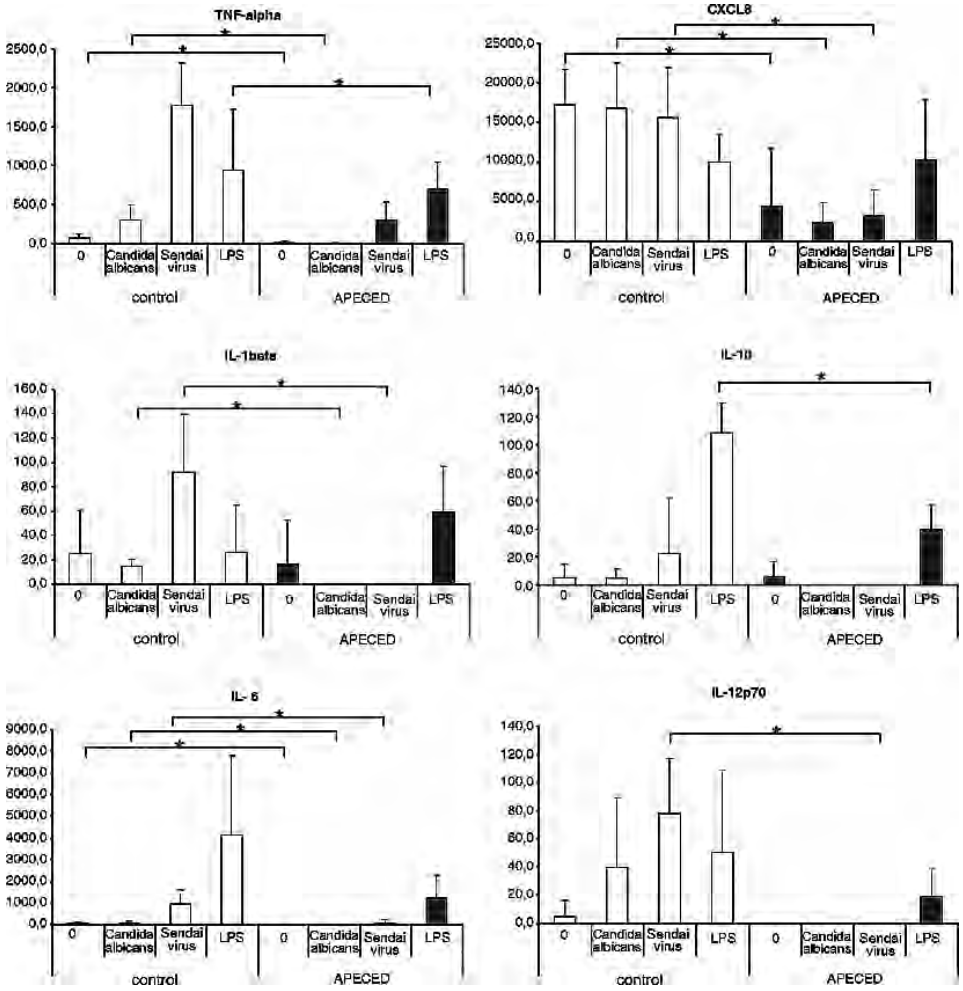



**Figure 11.** ***Cytokine production was reduced by patient moDCs (grey) compared to moDCs from healthy controls. MoDCs differentiated from APECED patients or control individuals had been stimulated with LPS (100 ng/ml), C. albicans (MOI 1), or infected with Sendai virus (MOI 5). Supernatants were collected at 24 h after microbial stimulation and cytokine levels were determined by flow cytometry. Statistical significances between the groups were calculated using Students t-test (*p<0.05).***

## 2.5 Expression profiles of AIRE deficient DCs show aberrations in immunologically important pathways

Differences in the genome-wide transcript patterns in APECED and control moDCs, were observed using Affymetrix expression array U133 Plus 2.0 chip analyses. Total cellular RNAs were isolated from three APECED patient-derived and three healthy donor-derived moDCs individually. Additionally, moDCs from the same three patients and healthy controls were stimulated with *C.albicans* for 24h and analyzed using individual Affymetrix chips. After data processing and quality control, data was analyzed using GeneSpring software. Lists of individual genes over two-fold up- or down-regulated ($p<0.05$) in APECED moDCs prior to and after stimulation were obtained. Out of these genes, those belonging to the most consistently and differentially expressed Gene Ontology (GO) categories are emphasized in the Volcano plots. A few genes, like CXCL8, IL-10, and TNF-α, were down-regulated at both protein and at mRNA levels as well as/or in Affymetrix array analyses in our study.

After studying individual genes, we proceeded to study changes that cannot be detected at analysis on the single gene level in order to gain more insight into the groups of genes, or pathways modestly down-regulated in the patients. For this purpose, we applied an in-house developed tool for pathway analysis. We observed changes in the functional groups of genes or their regulatory pathways using the Gene Ontology (GO) category classification in patient moDCs. Quite a few immunologically important pathways were down-regulated in APECED moDCs. Down-regulated pathways included cell-cell signalling, cytokine and chemokine activity, host defence and inflammatory response, lymphocyte activity, and cell motility in unstimulated APECED moDCs. After *C. albicans* stimulation of APECED moDCs, the pathways described above, as well as those involved in chemotaxis and G-protein regulated signalling pathway were clearly downregulated (Figure 12). Some pathways like cell-cell signalling, defence response and cytokine activity were down-regulated in both experimental settings.

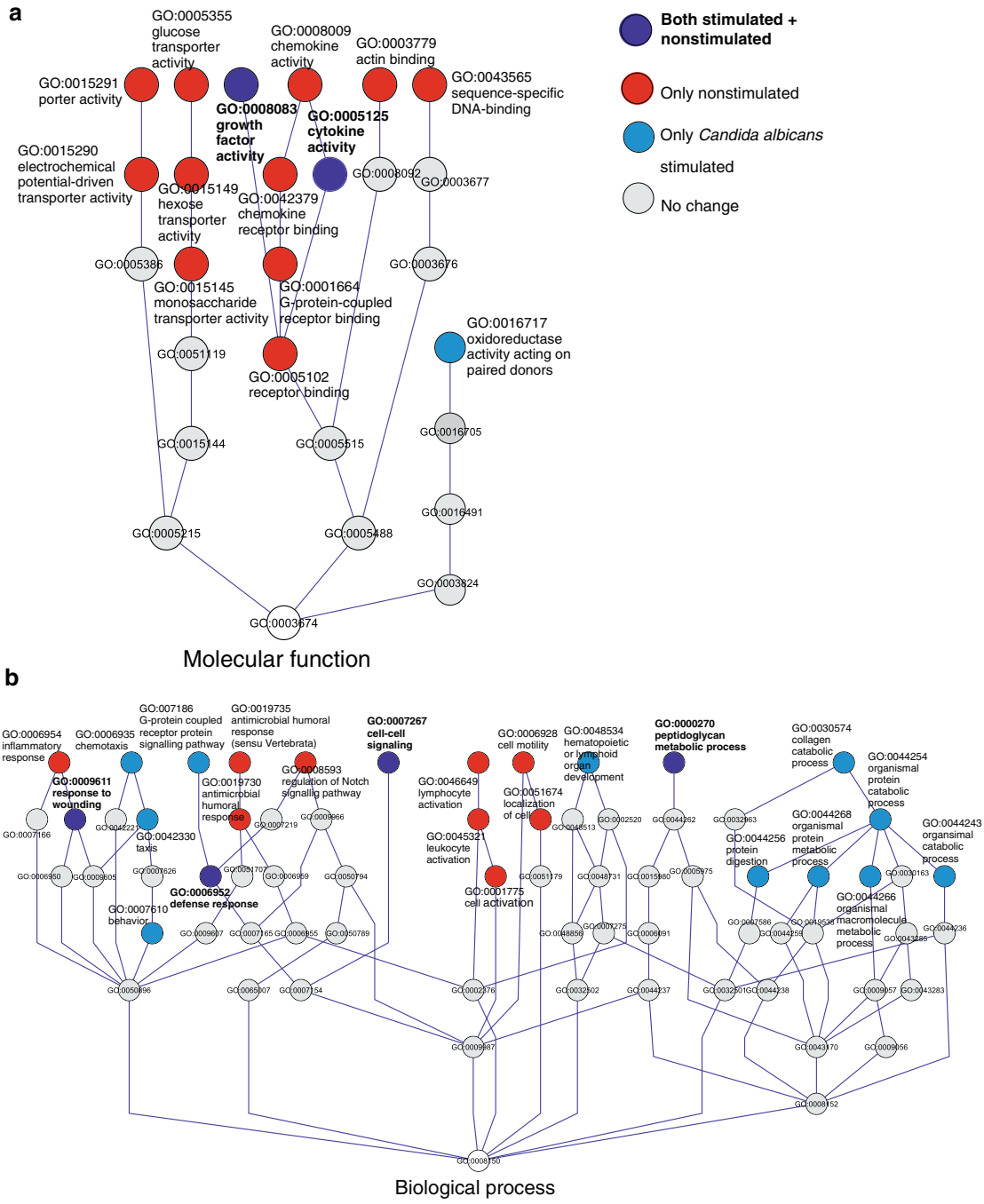

a
GO:0005355 glucose transporter activity
GO:0008009 chemokine activity
GO:0003779 actin binding
GO:0015291 porter activity
GO:0043565 sequence-specific DNA-binding
GO:0008083 growth factor activity
GO:0005125 cytokine activity
GO:0015290 electrochemical potential-driven transporter activity
GO:0008092
GO:0003677
GO:0015149 hexose transporter activity
GO:0042379 chemokine receptor binding
GO:0005386
GO:0003676
GO:0015145 monosaccharide transporter activity
GO:0001664 G-protein-coupled receptor binding
GO:0016717 oxidoreductase activity acting on paired donors
GO:0051119
GO:0005102 receptor binding
GO:0016705
GO:0015144
GO:0005515
GO:0016491
GO:0005215
GO:0005488
GO:0003824
GO:0003674
Both stimulated + nonstimulated
Only nonstimulated
Only Candida albicans stimulated
No change
Molecular function
b
GO:0006954 inflammatory response
GO:0006935 chemotaxis
GO:007186 G-protein coupled receptor protein signalling pathway
GO:0019735 antimicrobial humoral response (sensu Vertebrata)
GO:0007267 cell-cell signaling
GO:0006928 cell motility
GO:0048534 hematopoietic or lymphoid organ development
GO:0000270 peptidoglycan metabolic process
GO:0030574 collagen catabolic process
GO:0044254 organismal protein catabolic process
GO:0009611 response to wounding
GO:0019730 antimicrobial humoral response
GO:0008593 regulation of Notch signalling pathway
GO:0046649 lymphocyte activation
GO:0051674 localization of cell
GO:0044243 organismal catabolic process
GO:0042330 taxis
GO:0045321 leukocyte activation
GO:0044256 protein digestion
GO:0044268 organismal protein metabolic process
GO:0006952 defense response
GO:0001775 cell activation
GO:0044266 organismal macromolecule metabolic process
GO:0007610 behavior
GO:0050896
GO:0065007
GO:0007154
GO:0002376
GO:0032502
GO:0044237
GO:0009987
GO:0043170
GO:0009056
GO:0008152
GO:0008150
Biological process

**Figure 12.** ***Gene Ontology classes of the (a) molecular function and (b) biological process are shown schematically. The most significant gene sets differentially expressed in nonstimulated and C. albicans-stimulated APECED patient moDCs are enriched according to the pathway analysis. Colored circles show regulated pathways and gray circles represent nonregulated pathways (cut-off p≤0.0001). Several immunologically important molecular functions and biological processes were downregulated in both unstimulated and stimulated patient cells. Thus, important differences in patient moDCs were found compared to control moDCs. More pathways were down-regulated in nonstimulated patient moDCs than C. albicans stimulated moDCs (red vs. light blue circles). This indicates differences in patient moDCs already at the unstimulated state***

## 3 Gamma delta T cells in APECED (III)

Absence of AIRE/Aire has been shown to disrupt αβ T cell selection and to have a profound effect on αβ T cell development, allowing autoreactive T cells to mature. However it is not known whether Aire is needed for normal γδ T cell development. How γδ T cells develop is not well understood, but it differs in many ways from that of αβ T cells. In this study, we have analyzed the γδ T cell compartment in $Aire^{-/-}$ mice and in APECED patients.

### 3.1 γδ T cells are present at normal frequency in Aire deficient mice

We began by testing if the absence of Aire in MECs in the thymus would affect the frequency of T cells committed to the γδ T cell lineage. We used the "Ramsey Aire knock-out mouse" (Ramsey et al. 2002), which mimics the $Finn_{Major}$ mutation and syngeneic parental mice as controls. FACS analysis revealed that the mean amount of γδ TCR-positive T cells was not significantly different from that of wt mice. In the spleen, the number of γδ T cells was also similar between knock-outs and controls, as was the fraction of γδ T cells out of all $CD3^+$ splenocytes.

An important location of γδ T cells is the intestinal epithelium. Flow cytometry could not be used for quantitation in these solid tissues, so we homogenized pieces of intestine, followed by Q-RT-PCR in order to measure the relative abundancy of γδ T cells within the intestine. We selected the TCR Cδ gene for measurement, as it is deleted during TCR α locus rearrangement, whereas TCR γ mRNA can also be expressed by αβ T cells. No differences were noted between the amounts of TCR Cδ in the intestines of knock-out and wt mice (see Figure 13).

We also analyzed the subset of γδ T cells in the skin. However, since their thymic development at embryonic days 12-13 precedes the expression of Aire, which is first

detected at embryonic day 14.5, it is not likely that they would be affected by Aire-deficiency. Indeed, the amount of Cδ mRNA was similar in Aire$^{-/-}$ and Aire$^{+/+}$ mice.

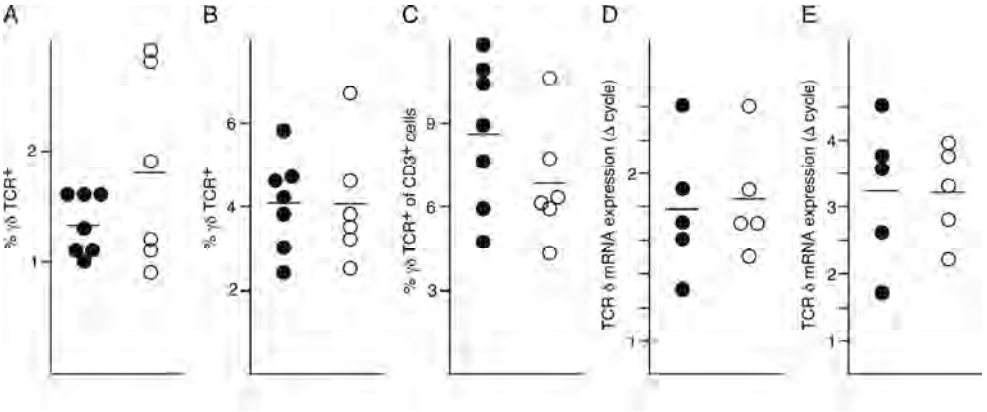



**Figure 13.** ***The number of γδ T cells is similar in Aire$^{-/-}$ and Aire$^{+/+}$ mice. (A) Thymus, (B) and (C) spleen, (D) ileum, and (E) skin. In A, B and C flow cytometry was used, while in D and E the amount of TCR Cδ mRNA was measured using Q-PCR, normalized against HPRT expression levels. Black circles symbolize Aire$^{-/-}$ mice and open circles WT mice. Mean values are indicated by horizontal lines. None of the differences are statistically significant.***

## 3.2 The TCR γ repertoire of γδ T cells is normal in Aire$^{-/-}$ mice

For a more detailed view of γδ T cell development we next analyzed the TCR γ chain repertoire in Aire knock-out and wt mice. An earlier analysis of this mouse model had shown abnormalities in certain TCR Vβ genes, while others were normal. A set of primers specific to all expressed Vγ genes was designed, with one primer amplifying the conserved Vγ5.1 – 5.3 area. The 3' primers were designed to an area conserved in the 3 functional Cγ genes. This allowed amplification of the full γ repertoire. Then, the CDR3 length patterns were determined by electrophoresis.

In the thymus, polyclonality was observed. The CDR3 pattern in all five Vγ amplifications produced a generally Gaussian distribution and the patterns were virtually identical in Aire$^{-/-}$ and Aire$^{+/+}$ mice. Additionally, the same CDR3 lengths dominated in each Vγ gene. In the spleen, no significant differences were observed either and the repertoire was very similar to that of thymic γδ T cells. In the ileum, oligoclonality was observed. This finding was consistent with previous observations, and the repertoires were visibly different from those of the thymus or spleen. Yet, the range and the average rearrangement patterns were quite alike in knock-outs and controls. We also carried out a limited analysis of the Vδ5 repertoire, which is

associated with polyclonal γδ T cells in lymphoid organs and the ileum and obtained similar results. To summarize, our results indicated that despite the absence of Aire, γδ T cells develop in normal numbers and contain a normal TCR repertoire.

## 3.3 γδ T cells of Aire$^{-/-}$ mice do not show signs of increased activation

The disruption of thymic negative selection of αβ T cells in Aire deficient mice leads to hyperreactivity and augmented expression of activation markers. Since CD25 is an activation marker on γδ T cells, we measured the expression of CD25 on γδ T cells of the spleen. No significant differences were found between Aire deficient and wt mice, nor were there differences in the frequencies of large γδ blasts in the spleen.

Next, we stimulated splenic γδ T cells with a mAb specific to γδ TCR, immobilized to the culture well bottom, in order to test the reactivity of the cells. The mAb did not induce significant proliferation on its own. After that, we added cytokines and growth factors found in the culture medium of Concanavalin A-stimulated spleen cells. This resulted in an obvious proliferative response in most knock-outs and wt mice. Surprisingly, the response of knock-outs was significantly higher that that of the wt mice, although individual variation was considerable. Therefore, the γδ T cells in Aire deficient mice were not found to be hyperreactive.

## 3.4 Target tissue infiltrates are not composed of γδ T cells

One important feature of the immunopathology of Aire deficient mice is infiltrating lymphocytes in target tissues. Consequently, the amount of TCR Cα mRNA was significantly higher in the salivary glands of Aire$^{-/-}$ mice compared to Aire$^{+/+}$ mice. However, no difference in the amount of TCR Cδ mRNA was observed, confirming that there were no increases in numbers of γδ T cells. Next, we analyzed the γ chain repertoire of the cells located in the salivary glands. The patterns were more oligoclonal than in the spleen, and not all the Vγ genes were detected in every animal, but no differences could be observed between wt mice and Aire knock-outs.

An oligoclonal repertoire may point to either an ongoing autoimmune response, or it could be due to the scarcity of T cells in the tissue. We wanted to distinguish between these possibilities and thus, we compared the local TCR γ repertoire in salivary glands to the systemic repertoire in spleen of the same mouse. Our hypothesis was that given the similar numbers of γδ T cells in the salivary glands of both Aire$^{+/+}$ and Aire$^{-/-}$ mice, an active process in the Aire$^{-/-}$ mice would be characterized by a more intensive skewing of the local repertoire, due to clonal expansions.

In order to study this hypothesis and to obtain a quantitative view of the difference between a systemic and local repertoire, we adapted a previously described method (Gorochov et al. 1998). In this method, areas of individual in-frame peaks in the CDR3 length profiles are expressed as a percentage of the combined areas of all the in-frame peaks in the profile. Two profiles are then compared by extracting the relative peak areas of one profile from the other, and the total difference is expressed as a range of 0-100%. Therefore, a value of 100% means totally different profiles and 0% completely overlapping profiles.

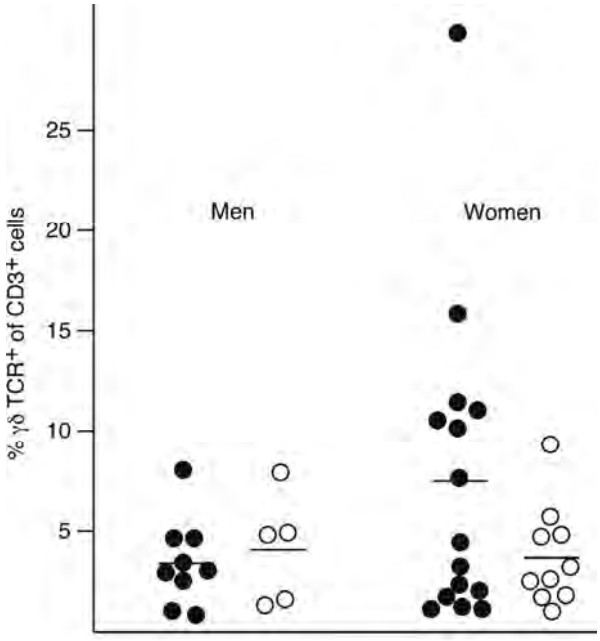
Quantitative comparison of the TCR γ repertoire in salivary glands and spleen confirmed oligoclonality. The average difference was roughly similar in both (39 ± 25% and 34 ± 17%, respectively). Detailed analyses of each Vγ gene alone did not show significant differences between the $Aire^{-/-}$ and $Aire^{+/+}$ mice either. When the same comparison was done between salivary glands and thymic repertoire, again a similar result was obtained. To summarize, our data are not consistent with significant pathological γδ T cell infiltrates in the salivary glands of the $Aire^{-/-}$ mice.

## 3.5 APECED patients have normal frequencies of γδ T cells

Finally, we measured the frequency of circulating γδ T cells in APECED patients. The mean frequency of γδ T cells within the $CD3^{+}$ population was a bit higher in the APECED patients than in healthy controls. However, the difference was not statistically significant. Interestingly, a subset of six patients had values outside the range of healthy controls and all of these patients were women. Yet, when we analyzed the whole patient cohort divided by sex, we did not find that female APECED patients would have an increased frequency of γδ T cells.

APECED is a very variable disease, with some components found in most patients and others affecting only a subset of them. Why this heterogeneity exists is not known. When we checked the clinical records of the subgroup of six female patients mentioned above, we saw no clear associations with any given disease component. Nor did we see correlation in the total number of different components. Therefore, although the findings on γδ T cells in this patient group are interesting, we have no evidence yet that they would represent anything else than random variation.

**Figure 14.** ***The frequency of peripheral blood γδ T cells in APECED patients is similar to that of controls. Black circles represent patients and open circles healthy controls. Mean values are indicated by horizontal lines. The differences are not statistically significant.***



# 4 DISCUSSION

## 1 Of mice and men: differences in autoantibodies and autoimmune diseases in the absence of AIRE/Aire

We analyzed Aire deficient and wt mouse sera for autoantibodies against eleven of the known APECED autoantigen mouse homologues, but did not find any, or higher levels of autoantibodies in Aire knock-out mice than in controls. Since most of the APECED autoantibodies are quite common in patients, present at frequencies over 20%, our analysis of 20 Aire deficient mouse sera should have been large enough to detect even low frequency autoantibodies, if they would have been present in the mice. Therefore we consider our negative results significant. Furthermore, we tested both human and mouse antigens. We also carried out several controls in order to make sure our tests were sensitive enough. However, there were a few antigens we did not test for (eg. SOX10, TPO, TG, IA-2, and $H^+K^+$-ATPase) and we cannot say whether mice have autoantibodies against them or not. Yet, our assays did include most of the known major targets in APECED and since those were absent in knock-out mice, our results showed that generally, the mice we tested do not show the same autoantibody pattern as human patients.

MHC, which is the single biggest genetic determinant in organ specific autoimmunity in man, may have an effect also in mice, and more particularly, in Aire deficient mice. It is known that the phenotype varies in different strains of Aire deficient mice (Jiang et al. 2005). Therefore, if the H2 haplotypes of the Aire deficient mice were studied after crossing the mice into different autoimmune susceptible backgrounds, such as those predisposing to T1D (H2g7), thyroiditis (H2k), or encephalitis (H2s), one would expect to see different phenotypes in these mice. One could expect for example higher levels of autoantibodies against the thyroid gland in the H2k mouse as well as a thyroid phenotype, whereas the H2g7 mice would probably have autoantibodies against the pancreas in addition to a more severe diabetic phenotype. However, whether additional phenotypes would arise and what role H2 plays in Aire deficient mice has not yet been revealed. What exactly accounts for the dramatic differences in organ specific autoimmunity in Aire deficient mice of the different strains is not known. Nevertheless, the differences between the phenotypes and the range of organs targeted by autoantibodies in the Aire deficient mice in the B6, Balb/c and NOD strains points at the involvement of additional factors than just Aire involved in molding the autoimmune phenotype. Just as the phenotype of APECED patients differs from one “outbred” individual, or

population of patients to another, so does the phenotype of inbred mice from one strain to the other.

Anderson et al. (Anderson et al. 2002) showed that Aire controls the expression of insulin in the thymus, but we did not detect insulin autoantibodies in our Aire deficient mice. Aire deficient mice do not develop autoimmune diabetes either, unlike APECED patients. This is in agreement with our results showing lack of autoantibodies against insulin, GAD and IA-2 in Aire deficient mice. None of the other disease components typical for APECED have been described in the Aire deficient mice either. We assume that the tissue reactivity reported by others (Anderson et al. 2002; Ramsey et al. 2002; Jiang et al. 2005) in Aire knock-out mice is targeted against some unknown organ specific or common antigens.

We also tested for common, or systemic, autoantigens. Elevated levels of anti-nuclear antibodies in the Aire knock-out mice were not found, thus these are unlikely candidates for the targets of the reported autoantibodies. Weak anti-nuclear antibodies had been reported in some Italian patients with APECED (Perniola et al. 2000). Nine out of the 10 patients tested positive for low titre ANA (Perniola et al. 2000). However, other studies show that ANA are not typical for APECED (personal communications, Aaro Miettinen). Therefore, the role of ANAs in APECED remains to be clarified.

Other studies supporting our findings were that α-fodrin antibodies were found in Aire deficient mice (Kuroda et al. 2005). Anti-α-fodrin antibodies have not been described in APECED patients and interestingly, expression of α-fodrin is not under the control of Aire in the thymus. This may be because α-fodrin is a ubiquitous protein, unlike other known APECED antigens. Also this difference between Aire knock-out mice and APECED patients is in agreement with our results and further supports the view that the targets of autoimmunity are different in these species. Another such Aire-independent antigen is pancreas specific protein disulphide isomerase (PDIp) (Niki et al. 2006), though this protein is more specific and therefore, reasons as to why this is a target for autoantibodies in mice and not in humans is more difficult to speculate.

Observations concerning the autoantibodies against different tissues in the Aire deficient mice showed that the genetic background had a strong effect on the spectrum of autoantibodies (Anderson et al. 2002; Jiang et al. 2005; Kuroda et al. 2005). In humans, genetic background may have an effect as well, as for example Iranians rarely have candidiasis. However, they also have a different disease mutation than Finns, who have nearly 100% candidiasis. Therefore it is not easy to say whether the differences in the phenotypes of patients are due to genetic background or mutation. In mice, it seems that differences in phenotype between the

different models are related to strain rather than the construct used (*i.e.* where the "mutation is introduced" in Aire).

Our study indicates that even though Aire knock-out mice have a well characterized defect in central tolerance, they do not develop autoimmunity against organs typically affected in the patients with APECED. Differing autoantigens in patients with APECED and in Aire deficient mice suggest that Aire may control a different set of genes in mice and in men. This may result from a number of reasons. For example, differences in chromosomal organization of target genes, in ubiquitinylation/methylation patterns, in promoter structures and in the mechanism of ectopic antigen presentation to T cells (Gotter et al. 2004) can be envisaged. The hierarchy of gene regulation may also differ between species. To find out the reason for these species differences, understanding of the molecular mechanism of AIRE dependent regulation of transcription (Gotter and Kyewski 2004) will be essential. Furthermore, a different panel of autoantigens suggests there are differences in the immunopathogenic mechanisms between man and mice, requiring further investigations. Finally, since the Aire deficient mice do not mimic the human APECED disease, caution should be applied when interpreting the data from analyses using the Aire mouse models.

After completing our study concerning APECED autoantibodies in Aire deficient mice, we went on to screen for new autoantibodies in sera of the Ramsey Aire deficient mice (Ramsey et al. 2002) using cDNA library screening of a few different libraries (liver and hypoparathyroid gland). We found some new candidate target antigens in addition to NALP5 (discussed below). However, these studies are still ongoing. Importantly, our collaborative efforts have preliminarily shown that NALP5 is also found in some, individual Aire knock-out mice (unpublished data and Mohammad et al. personal communications), although not as frequently, or in as high titres as it is found in patients. If this is the case, NALP would be the first autoantibody reported to be common for Aire deficient mice and APECED patients and could help to elucidate molecular mechanisms leading to the loss of tolerance to NALP5 in the absence of Aire. It would be very interesting to study further why this autoantigen is shared between species, if most of the other APECED autoantibodies are different in mice and men.

Our collaborative effort resulted in the discovery of NALP5 as an APECED autoantigen, associated with hypoparathyroidism (Alimohammadi et al. 2008). Hypoparathyroidism is a hallmark of APS-1 and affects more than 80% of the patients (Perheentupa 2002; Liston et al. 2004). Earlier, the calcium-sensing receptor (CaSR) had been suggested as an autoantibody for APECED (Gavalas et al. 2007), but other groups were not able to detect antibodies in patient sera against CaSR (Gylling et al. 2003; Soderbergh et al. 2004). It is quite exciting that a pattern

recognizing protein is the target of immune attack, although as with the other APECED autoantigens, why loss of tolerance specifically against this protein occurs is a mystery. Identification, finally, of a parathyroid specific autoantigen is important for improved serological diagnosis of APECED, and could help to provide better understanding of the molecular pathology of APECED.

Recently, a group of neutralizing, IgG autoantibodies against type 1 interferons (IFN) were discovered as "new" autoantibodies in APECED patients. This was a very exciting finding, as these antibodies are so APECED specific and were present in 100% of sera of the APECED patients tested. IFNs can act to halt viral replication and serve as danger signals by activating APCs, thus linking between innate and adaptive immunity. Autoantibodies against IFN-α and IFN-ω were the most common subtypes, and some Finnish patients also reacted with IFN-β and IFNλ1 (=IL29). IFN autoantibodies preceded other APECED autoantibodies and in some cases, the IFN antibodies were predictive of development of clinical symptoms (Meager et al. 2006; Wolff et al. 2007). Studying these antibodies further will certainly answer some of the many questions which arise from these results, such as why APECED patients with such "general" autoantibodies only get candidiasis and not other infections as well? Furthermore, it will be interesting to find out whether Aire knock-out mice express autoantibodies against interferons as well. Anti-IFNs may also be evoked by changes in APCs, such as DCs (Meager et al. 2006), studied in the II publication of this thesis discussed below. IFN autoantibody production in APECED patients is suggested to be caused by increased production of IFNs in the thymus. This would somehow result in anti-IFN antibodies, which cause down-regulation of IFN stimulated genes in patient blood cells (Kisand et al. 2008).

Novel pituitary autoantibodies in APECED were also reported recently (Bensing et al. 2007; O'Dwyer et al. 2007). TDRD6 was reported as a major autoantigen in APECED, in 49% of APECED patients studied, although it is expressed mainly in testis. This study also indicates the presence of further target autoantigens in the anterior pituitary than TDRD6 and AADC, which have already been found (Bensing et al. 2007). Indeed, a study on the prevalence of pituitary antibodies in Finnish APECED patients showed that the pituitary can be "part of the multi-organ involvement of the disease". This study identified enolase, a ubiquitous glycolytic enzyme, as a novel autoantigen. This antigen is not organ specific and as patients rarely develop hypopituaritism, why autoantigens against enolase would develop is not clear. The most interesting finding concerning enolase as autoantigen was that candidal enolase could also be targeted by these enolase autoantibodies, indicating cross-reactivity between it and pituitary enolase. This suggests that a mechanism in APECED autoimmunity could be molecular mimicry (O'Dwyer et al. 2007).

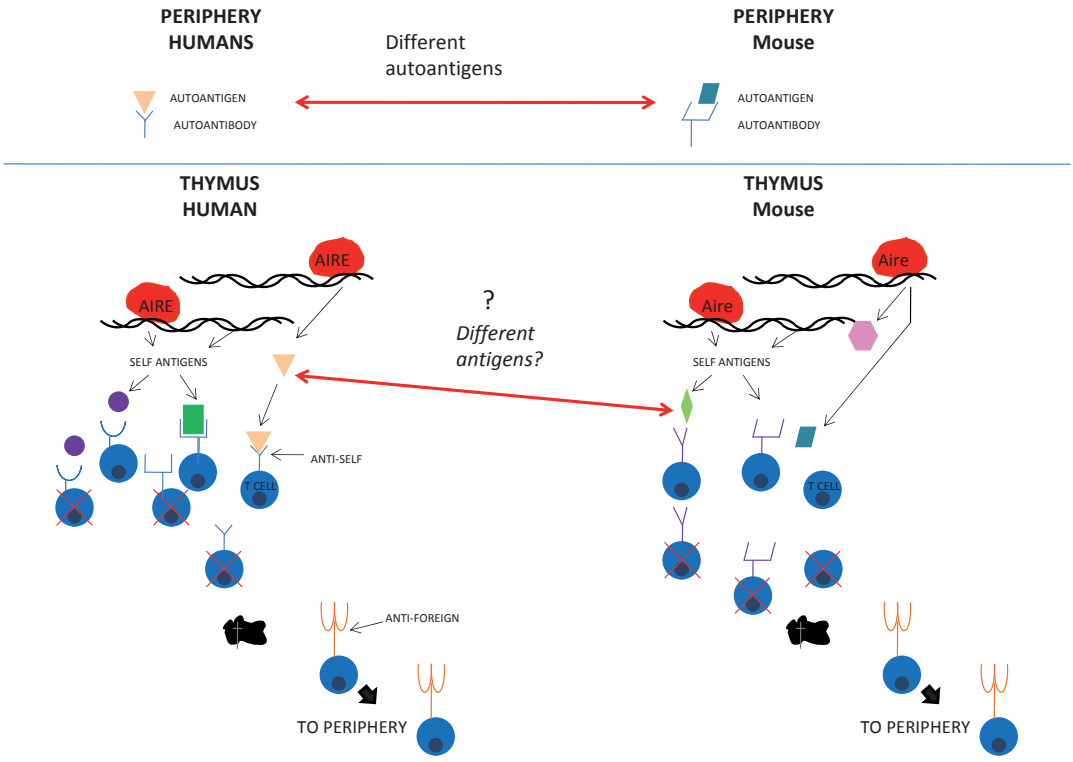



**Figure 15.** ***Conclusions from our study of APECED autoantibodies in Aire deficient mice. Autoantibodies against different autoantigens are expressed in Aire$^{-/-}$ mice compared to APECED patients. Aire may therefore control the expression of a different set of TSAs in mice than AIRE does in humans.***

None of these newly reported autoantibodies, except NALP5, have been reported to be found in Aire deficient mice yet. However, in a study of induced diabetes in Aire deficient mice, the mice did develop ICA antibodies (Hassler et al. 2008). Still, we await news of whether also these new autoimmune targets are human specific or are also found in mice. Furthermore, it will be interesting to find out whether these autoantibodies are found in different mouse strains and at which age the antibodies develop, in order to try to answer the question of which comes first, autoimmunity or autoantibodies.

## 2 What is the role of AIRE in peripheral DCs?

Our studies (II) revealed that APECED patient moDCs are functionally impaired: the transcriptional response of cytokine genes to pathogens was drastically reduced. Patient moDCs showed typical DC morphology and expressed DC marker proteins CD86 and HLA class II DR, as analyzed by FACS and Q-RT-PCR. When analyzing other typical markers of DCs by Q-RT-PCR, an interesting difference was noted in the expression of DC-SIGN (DC-specific ICAM-grabbing non-integrin) which was weakly, yet not significantly up-regulated in AIRE-deficient moDCs. This pattern recognition, adhesion and pathogen uptake receptor (Geijtenbeek et al. 2000; Geijtenbeek et al. 2000), functions as one of the major receptors for *C. albicans* enhancing its internalization (Cambi et al. 2003). However, expression of several other genes involved in cellular responses to *C. albicans* (Donini et al. 2007; Goodridge et al. 2007) was similar in APECED and control cells, and their expression was not altered either during *C. albicans* stimulation. Therefore, our studies were unable to shed light on why APECED patients are prone to candidiasis.

An important goal we had in our study was the analysis of the transcript profiles of APECED patients (carrying the same homozygote $Finn_{Major}$ mutation) versus healthy controls. Our results showed that patients differ in many respects from controls. The expression of a number of genes important for DCs was reduced in APECED moDCs. Several genes were up- or down-regulated in both unstimulated and *C. albicans* stimulated patients moDCs. Significantly more genes were repressed in patients: 5.9% in non-stimulated and 2.5% *C. albicans*-stimulated moDCs, than were activated: under 0.5% both before and after stimulation.

Our genome-wide expression analysis showed that a number of interesting genes were down-regulated in APECED moDCs, for example NFκB1, important for DC activation and maturation (Kieran et al. 1990; Chaussabel et al. 2003), CD58 (LFA-3) an MHC antigen mediating cell adhesion and T cell activation (Sanchez-Madrid et al. 1982; Suciu-Foca et al. 2005) and ITGB2 (also called CD18 or LFA-1) also important in the interactions of DCs with target cells (Sanchez-Madrid et al. 1982; Nagafuchi et al. 2007). These genes could at least partly contribute to the observed aberrations in the response to stimuli by APECED versus control cells. Expression levels of typical APECED autoantigen genes were not significantly different compared to controls, whereas those of most cytokine genes tested were down-regulated in APECED DCs. AIRE has been shown to regulate cytokine expression in the thymus (Ruan et al. 2007)

Importantly, the cytokine production was decreased in APECED moDCs, especially in response to microbial stimulus. We used three stimuli: live *C. albicans*, LPS, and Sendai virus, and all of them resulted in significantly reduced levels of DC cytokine

production. This would imply that patient moDCs respond weakly to microbial stimuli. Further, since cytokine synthesis is connected with the maturation of DCs (Veckman et al. 2004) the results could indicate that APECED patients have some impairment in their DC maturation processes as well. An important observation in support of this is that some differences in the gene expression profiles of moDCs were seen already at the immature stage in moDCs of APECED patients. Reduced expression of several genes and gene sets, such as those involved in cytokine activity, host defence response and cell-cell signalling were seen already in unstimulated patient moDCs. This could suggest that immature patient moDCs have a lowered tolerogenic capacity.

The ability for antigen uptake of APECED moDCs is likely to be normal since DEC-205 and other uptake receptors were expressed at the levels comparable to controls. The antigen presenting capacity of APECED moDCs appeared normal, since the expression of HLA class II genes was normal, or even weakly up-regulated in APECED moDCs both at mRNA and protein level. Perniola et al. (Perniola et al. 2008) studied patient monocyte and leucocyte effector mechanisms and found nothing abnormal in those in response to *e.g. C. albicans*. This is in line with our finding of the DCs being the cell type defective in APECED. Brännström et al. (Brannstrom et al. 2006) studied the role of AIRE in defence against candidiasis. They saw a proliferation in patient T cells in response to *Candida* antigens probably due to clonal expansion of *Candida* recognizing T cell clones. They also investigated the capacity of patient DCs to internalize yeast cell wall zymosan and found a defect in this process. Many receptors and other molecules are involved in binding and internalization of *C. albicans*. Thus, the defect in the internalization and activation of Aire deficient DCs may be due to multiple pathways or one common effect (Brannstrom et al. 2006). Since we did not see altered levels in either mannose or lectin receptors, nor of *e.g.* dectin-1 either, identification of the pathway dysregulated in the absence of Aire needs to be studied further, in a more systematic fashion. Interestingly, Brännström et al. also noted a possible defect in monocyte maturation in APECED patients and hypothesized that this may affect the communication between APCs and effector T cells, just as our studies proposed.

Using pathway analysis in order to observe changes in groups of genes too small on the individual gene level to be noted, we found a decrease in several immunologically relevant pathways in patient moDCs. Our results fit in well with previous findings on the role of AIRE in the maturation of DCs (Sillanpaa et al. 2004) and the role of DCs in peripheral activation of T cells (Ramsey et al. 2006). The cytokine pathway was down-regulated, in accordance with our cytokine analyses. Cell-cell signalling was also down-regulated. A decrease in DC function, leading to deteriorate DC – target cell communication could, in turn, lead to

diminished lymphocyte activation and an impaired T cell response. On the other hand, since APECED patients are not more prone to infections other than those caused by *C. albicans*, these findings are slightly puzzling.

We compared our human moDC array results with those of Johnnidis et al. (Johnnidis et al. 2005) arrays performed on thymic medullary epithelial cells (MECs) from Aire deficient and *wt* mice. Individual gene comparison did not show the same genes to be up-or down-regulated in both data sets. However, when the data sets were compared using our in-house pathway analysis, we found some shared differentially regulated functional categories. Aire knockout mouse MECs and human AIRE-deficient DCs both had down regulated cytokine activity, inflammatory response, G-protein-coupled receptor binding, chemotaxis, taxis, chemokine activity and chemokine receptor binding. Interestingly, in Aire-deficient mice, pathways involving T cell activation and proliferation as well as MHC class II biosynthesis were up-regulated, whereas only few pathways were found to be up-regulated in APECED moDCs (mRNA processing, antigen processing and presentation, MHC protein complex). These differences in gene expression profiles likely result – at least to some extent – from differences in species and cell types compared. Yet, they could also be partly due to an apparently different role of AIRE in central *vs.* peripheral tolerance and in the mechanisms in humans vs. mice.

A few other studies have addressed gene expression patterns in AIRE/Aire deficient and control cells. One study (Sillanpaa et al. 2004) compared the expression profiles of AIRE-positive and -negative monocytic U937 cell lines. Expression of the tissue-specific antigens under control of AIRE in the thymus was not detected in the U937 cells, again suggesting that the role of AIRE in the periphery may be different from that in the thymus. Array data presented by another group (Anderson et al. 2005) on mTECs from Aire deficient mice showed alterations in the expression of several genes involved in antigen processing and presentation. Aire deficient mice showed less effective antigen presenting capacity and chemokine expression was found to be up-regulated by Aire. We also observed a possible decrease in antigen presentation and in chemokine expression in the absence of AIRE. However, since our observation was in a different cell type and species, these two findings are not necessarily linked. However, it was not clear from the study by Anderson et al. whether the antigen presentation of splenic DCs from Aire-deficient mice was less effective as compared to wild type mice. On the contrary, Ramsey et al. (Ramsey et al. 2006) showed that peripheral DCs from Aire deficient mice were more effective activators of naïve T cells. Additionally, they reported that the number of DCs in the spleen, lymph nodes and blood of these mice was increased. They propose that the inappropriate T cell response in APECED could be due to overexpression of

costimulatory molecules on DCs, which is quite contrary to our findings, which suggest lack of sufficient target cell activation by DCs.

DC maturation and DC – Treg interactions are important for immune response and tolerance (Hubert et al. 2007). We found some genes (ITGB2 and CD58 or LFA3) involved in DC-Treg interplay (Grossman et al. 2004; Suciu-Foca et al. 2005; Wethmar et al. 2006; Nagafuchi et al. 2007; Varga et al. 2007) to be down-regulated in patient moDCs. These findings of impaired DC function are in concordance with the findings of Kekäläinen et al. (Kekalainen et al. 2007). They suggested that the defect in AIRE-deficient regulatory T cells (Tregs) in humans could be due to impaired DCs stimulated Treg development. Defects found in both DCs and Tregs could implicate that there is a defect in the interplay between these two cell types. A study exploring the possibility that routing of antigens from mTECS may decide whether the cells become Tregs or not, showed that interactions of Tregs with mTECs do affect their development (Aschenbrenner et al. 2007). Therefore, one possibility is that the role of Aire in the thymus may additionally influence peripheral tolerance by affecting the development of Tregs.

IFN over-production by peripheral DCs has not been observed and DCs were shown to produce normal levels of IFN stimulated genes in a recent study (Kisand et al. 2008). However, increased serum levels of CXCL10, TNF-α and IL-6 were noted. As our patient DCs produced very low levels of CXCL10, the source of this chemokine in the patient sera is most likely other cell types than DCs (Kisand et al. 2008).

So far, it seems that more genes are positively regulated than negatively regulated by Aire. According to one study, the genes IL-1R1 and MHC class II were suppressed by Aire, among other genes not reported yet, as they are still being studied. These findings are only partly in agreement with our studies, as we saw suppression of cytokines in the absence of AIRE, but up-regulation of MHC class II (Sato et al. 2004). Clearly, further studies on larger study samples are needed in order to reach a consensus on these, and several other issues concerning AIRE function.

A study in line with our findings concerning a role for Aire in the periphery was that Aire deficient mice showed induced susceptibility to induced diabetes (Hassler et al. 2008), although, strangely, highest levels were found in mice heterozygous for Aire ($Aire^{+/-}$). We have worked on a project concerning injection of insulin into $Aire^{+/+}$ and $Aire^{-/-}$ mice, in order to monitor differences in tolerance to insulin in the mice, but these studies have not yet been completed (Vaarala et al. unpublished data).

Finally, functional defects in DCs, especially defects in DC maturation, have been shown to be involved in other, more common autoimmune diseases, like T1D (Angelini et al. 2005), SLE (Monrad et al. 2007), and arthritis (Laborde et al. 2007).

Findings concerning the monogenic disease APECED and its causative gene AIRE have indicated that well-functioning peripheral and central tolerance are important in preventing autoimmunity in humans.

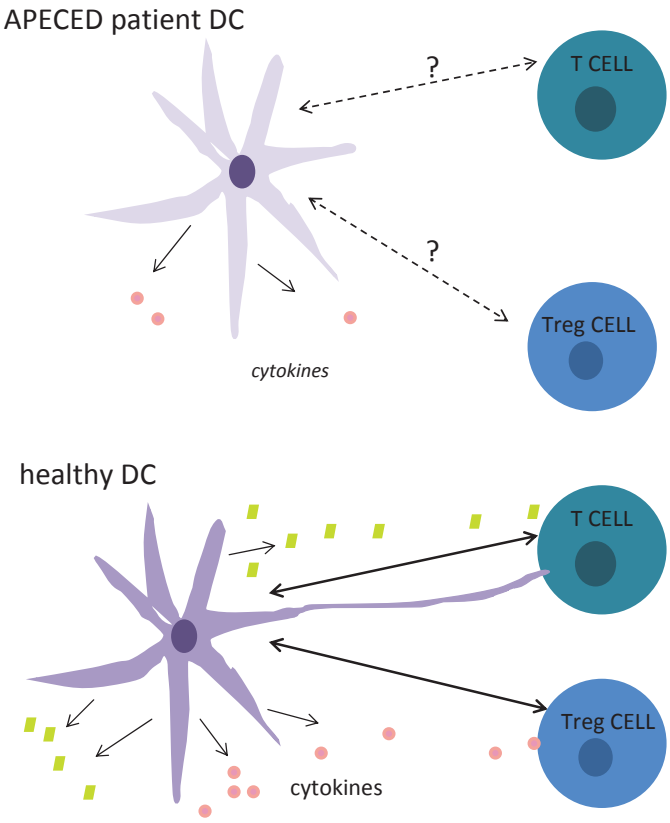



**Figure 16.** ***Our comparison of DCs from healthy donors with DCs from APECED patients showed that patient DCs have defects in cytokine production and other immunologically relevant pathways. This may result in a reduced function of patient DCs.***

Our observations support a role for AIRE in the maintenance of peripheral tolerance regulated by dendritic cells. Ours are the first results to show that AIRE plays a role in DC responses to microbial stimuli in humans. Additionally, our findings emphasize the need for further functional studies related to peripheral tolerance and how it is regulated in APECED patients. Revealing the functions of AIRE will help us understand the fundamental molecular mechanisms controlling the development of immune tolerance.

# 3 AIRE/Aire does not affect the development of γδ T cells and γδ T cells do not participate in the autoimmunity in APECED in mice or in men

Our results show that in the absence of Aire γδ T cells develop in normal numbers and display a normal TCR repertoire in all of the tissues studied. γδ T cells do not resemble activated cells in $Aire^{-/-}$ mice and did not seem to participate in the pathological tissue infiltrates and thus, the immunopathology associated with Aire-deficiency. We conclude that the development of γδ T cells is not dependent on Aire function

However, there are many ways in which loss of Aire function could affect γδ T cell development and why we set out to look at γδ T cells. Some of the molecules participating in γδ T cell selection might be controlled by Aire, like the thymic stromal element which has been shown to be needed for the positive selection of skin-associated γδ T cells (Xiong et al. 2004). Aire may be involved in the expression or function of nonclassical antigen-presenting molecules linked to antigen recognition by some γδ T cell subsets, or it may regulate the expression of cofactors, such as cytokines, important for γδ T cell maturation. Expression of Aire in the periphery and extrathymic selection, or maturation of γδ T cells could be linked, too, even though these mechanisms are not well-understood. Yet another interesting option is that the disruption of orderly αβ T cell maturation might be indirectly reflected on γδ T cell commitment. Recently, it was reported that $CD4^{+}CD8^{+}$ αβ thymocytes may be able to regulate the maturation of precursors into γδ T cell lineages by an unknown mechanism (Silva-Santos et al. 2005). For example, in mice lacking TCR β, the development of γδ T cells is also disturbed, even though γδ T cells have no built-in need for TCR β chain expression. Interactions between thymocytes like these at different developmental stages may transfer abnormalities of one lineage onto others.

The thymic TCR γ repertoire was remarkably similar in both $Aire^{-/-}$ and wt mice and the same CDR3 lengths were dominant in almost all mice. This suggests that despite the disruption of Aire-regulated ectopic transcription, whatever the antigens important to γδ T cell selection are, they were present in normal amounts.

There was no sign of γδ $TCR^{+}$ clonal expansions in the salivary glands, which would be expected in an antigen-driven local immune response. This last finding of ours makes it unlikely that γδ T cells would play a pathogenic role in APECED. Further, it suggests that the presumed immunoregulatory function of γδ T cells does not play a protective role in $Aire^{-/-}$ mice. The function of γδ T cells has been shown to take place in peripheral tissues, not in the secondary lymphoid tissues. Also other regulatory subsets suppress autoreactive T cells within the affected tissue, and such

findings have also been reported in Aire$^{-/-}$ mice (Kekalainen et al. 2007). We did not find significant numbers of γδ T cells in the target tissues and this is inconsistent with such local suppression.

γδ T cells could be recruited to participate in the immunopathology of Aire deficient mice or the autoimmune disease of APECED patients. Although the functional significance of γδ T cells is still poorly known, in most cases the early γδ T cell response is proinflammatory, whereas at later stages, immunoregulatory functions dominate (Hayday and Tigelaar 2003). Therefore, γδ T cells could have either a pathogenetic or a protective role in APECED. Earlier studies have not shown defects of immunoregulation in Aire deficient mice, and experiments in which normal regulatory T cells were transferred to Aire deficient mice were not able to reverse the immunopathology. Yet, γδ T cells have not been studied separately, and defects in them could have been hidden by the normal function of other regulatory subsets.

Since γδ T cells interact with DCs, it would have been possible that defects in DCs cause a defect in γδ T cells, or vice versa, thus leading to autoimmunity. Insufficient activation of γδ T cells could also have explained differences between species. It still may explain some species differences, if different γδ T cell subsets or functions are affected in mice and men. We already showed that there are aberrations in APECED patients DCs (II). If, γδ T cells would also have been defective, this would probably have lead to severe autoimmunity, since two cell types are affected. However, as γδ T cells function normally despite absence of Aire, γδ T cells may "take over" some of the tasks of the incapacitated DCs. How well γδ T cells can "cover" antigen presentation in mice and men may differ, thus leading to a difference in the severity of autoimmunity.

The data on human γδ T cells showed substantial variation. This is not uncommon in human studies. Six female APECED patients in our study had a high frequency of circulating γδ T cells. However, our analysis of all the patients together was not able to confirm a link between sex and γδ T cell frequency. Moreover, we could not link this finding to disease severity or any specific disease component. One possible explanation could be that, given the association of γδ T cells with epithelial tissues, the increased frequency of γδ T cells in some patients could be associated with a local process such as the candidiasis, which affects the patients with variable severity.

It would be interesting to study further the different subsets of γδ T cells in APECED patients and Aire deficient mice, but as so little is still known about γδ T cells in general, such analyses may not bring much new knowledge before the basic characteristics of γδ T cell are more clearly defined.

To summarize, our data underlines the difference in the developmental pathway and the function of αβ and γδ T cells. In the absence of Aire, the αβ T cell population shows several signs of dysregulation, but no such aberrations were observed in the γδ T cell population. The autoimmune pathology in Aire$^{-/-}$ mice does not seem to be driven by γδ T cells. Therefore, our results not only shed light on the role of γδ in APECED and the role of Aire on γδ T cells, but more generally, support the view that the thymic selection process and the molecules and antigens involved in it are different for αβ and γδ T cells.

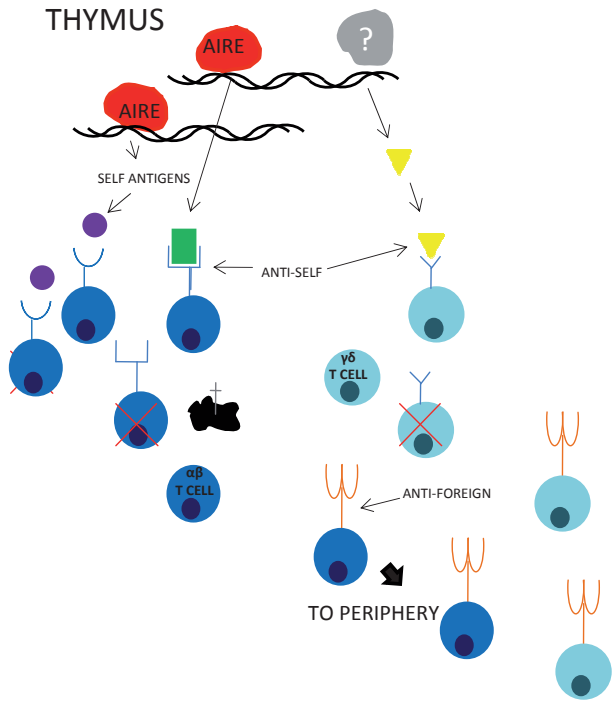



**Figure 17.** ***Our study on the γδ T cells in Aire deficient mice and in APECED patients showed that Aire does not affect the development of γδ T cells. Expression of self-antigens to γδ Tcells during their development may therefore be under the control of some other gene/s than Aire.***

# 5 CONCLUSIONS

The three studies included in this thesis have addressed different aspects of the function of Aire and tolerance. This approach was chosen because different strategies are needed in trying to solve an issue as complicated as the breakdown of tolerance – otherwise this key question would not have remained unanswered for so long. It is quite difficult, if not impossible, to study the function of a gene without access to the cells or organs in which the gene in expressed. Therefore, cell and animal models provide vital tools for revealing functions which cannot be, and would not be ethical to be studied in patients.

In the first part of this thesis, we wanted to characterize the Aire deficient mice further and focused on a typical characteristic of APECED: autoantibodies. Autoantibodies typical for human APECED were not found in Aire deficient mice, a model constructed to mimic the human APECED disease. Thus, since autoantibodies still are present in the knock-out mice, they must be targeted against other than the human APECED antibody homologues. Even though the Aire deficient mouse is a very valuable tool in studying the role of AIRE/Aire, differences between the phenotype of the mice and the APECED patients are quite large and this must be kept in mind when interpreting the results from the mice. The species difference is also very interesting in itself; why are the mechanisms of immunopathology in the mouse different from those in humans?

Findings concerning new autoantibodies expressed in both species, such as NALP5, may help shed light on both the reasons for species differences concerning autoantibodies as well as the mechanisms for why they are generated in the first place. Why autoantibodies are generated and the question “which is the hen and which is the egg – immune destruction or autoantibodies?” also remains unanswered.

Next, we focused on human DCs. Since so many mouse models had been published and many groups were working on them, and because we have access to these rare patients, we wanted to address the unanswered question of the role of Aire in the periphery. Our main findings were that DCs of APECED patients show defects in response to microbial stimulus, and a decrease in cytokine production. Gene expression profiles of cells isolated from patients were different from those in cells isolated from healthy controls. In the patients the pathways involved in immunological functions were down-regulated, also indicating aberration in DC function. Still, which DC subsets are involved, how exactly DC functions are defected, how badly defective are their communication skills with target cells, with

which target cells and what about the situation in mice, all remain to be specified. Also the mystery of why solely CMC is a plague for APECED patients and other infections are not more common as well remains unsolved.

Finally, we studied γδ T cells in both Aire deficient mice and in APECED patients, in order to find out whether Aire in involved in their development, as it is so important in the development of αβ T cells. We found that γδ T cells develop independently of Aire, as the numbers and characteristics of the γδ T cells were not altered in mice. Although we saw some variation in patient cell numbers, the cells themselves did not appear to be defected either. Therefore, γδ T cells do not seem to be involved in the peripheral damage or regulation of autoimmunity in APECED. However, since so much is still unknown concerning γδ T cells on the whole, there will be a whole lot to be found out about γδ T cell biology yet.

Taken together, our studies show that there are substantial differences in the breakdown of tolerance between mice and men, and that Aire does play a role in peripheral tolerance, effecting DCs but not γδ T cells.

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