THE AUTOIMMUNE REGULATOR

STUDIES OF IMMUNOLOGICAL TOLERANCE

IN MOUSE AND MAN

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"They look like idiots to their cousins, they look like idiots to their peers, they need courage to continue. No confirmation comes to them, no validation, no fawning students, no Nobel, no Shnobel. 'How was your year?' brings them a small but containable spasm of pain deep inside, since almost all of their years will seem wasted to someone looking at their life from the outside. Then bang, the lumpy event comes that brings the grand vindication. Or it may never come."

Nassim Nicholas Taleb

Till Jonas, min man.

ABSTRACT

The aim of the work presented in this thesis was to investigate how failure in the mechanisms that regulate self-tolerance can lead to autoimmune disease. In particular, I have studied a key player in immunological tolerance, the autoimmune regulator gene (*AIRE*). Mutations in this gene lead to a severe autoimmune disorder called autoimmune polyendocrine syndrome type I (APS I). APS I patients suffer from a combination of diseases caused by the immunological destruction of various tissues and organs, mainly the endocrine organs. The most common disease components are hypoparathyroidism, adrenocortical insufficiency and chronic mucocutaneous candidiasis (CMC). It has been clearly shown that AIRE is involved in the negative selection of autoreactive thymocytes. The suggested mechanism is that AIRE induce expression of tissue-specific antigens (TSAs) in the thymus that are needed for the deletion of autoreactive thymocytes, but the exact molecular events and relative importance of this are controversial.

The first publication on which this thesis is based, reports that AIRE is involved in the regulation of T cell-independent B cell-responses, and that B cells in *Aire*$^{-/-}$ mice have an increased activation status. This finding was thought to be connected to the increased serum levels of B cell activating factor of the TNF family (BAFF) found in both *Aire*$^{-/-}$ mice and APS I patients. The excessive BAFF was produced by AIRE-deficient dendritic cells upon IFN-γ stimulation, which was independent of the presence of autoreactive T cells. It was suggested that AIRE regulates peripheral tolerance by inhibiting STAT1 signaling downstream of the IFN-γ receptor. The second paper describes how AIRE deficiency results in impaired development of *i*NKT cells, which may contribute to the pathogenesis of APS I. This finding suggests that AIRE has other functions apart from inducing TSAs in the thymus, given that *i*NKT cells recognize lipid antigens and are not dependent on ectopic peptide expression in the thymus for their development. In the third paper, the mechanisms behind CMC in APS I are described. It is shown that APS I patients have defects in innate immune mechanisms, i.e. the anti-fungal activity of the saliva. The patients exhibit IgA autoantibodies recognizing salivary glands as an indication of ongoing immunological destruction. Furthermore, they lack expression of salivary cystatin SA1, a protein with potent anti-fungal activity. The final paper describes investigations into possible mechanisms governing thymocyte development in the absence of AIRE. This was performed in a system independent of TSA expression where endogenous superantigens mediate selection and activation of transgenic T cells specific for an ovalbumin peptide presented by H2-IA^b (OT-II T cells). It was found that the OT-II T cells were reduced in *Aire*$^{-/-}$ mice compared to wild type littermates, and showed an immature phenotype. It was also found that the OT-II *Aire*$^{-/-}$ mice had increased TCR revision after activation in peripheral organs.

The findings in *Aire*$^{-/-}$ mice and APS I patients presented in this thesis are not explained by reduced TSA expression in the thymus. They show that AIRE has additional functions in both central and peripheral tolerance.

LIST OF PUBLICATIONS

I. Lindh, E., Lind, S.M., Lindmark, E., Hässler, S., Perheentupa, J., Peltonen, L., Winqvist, O., and Karlsson, M.C.I. (2008).
    AIRE regulates T cell independent B cell responses through BAFF
    Proc Natl Acad Sci U S A 105, 18466-18471.

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II. Lindh, E., Rosmaraki, E., Berg, L., Brauner, H., Karlsson, M.C.I., Peltonen, L., Höglund, P., and Winqvist, O. (2009).
    AIRE deficiency leads to impaired *i*NKT cell development
    J Autoimmunity. In print, doi:10.1016/j.jaut.2009.07.002

III. Lindh, E., Brännström, J., Jones, P., Wermeling, F., Karlsson, M.CI., Hässler, S., Stridsberg, M., Herrmann, B., Winqvist, O.
    Autoimmunity causes altered oral microenvironment and candidiasis in APS I
    In manuscript

IV. Lindh. E., Hässler, S., Janson, P., Eberhardson, M., Lindmark, E., Karlsson, M.C.I., Peltonen, L., Winqvist, O.
    AIRE deficiency alters T cell selection and activation mediated by endogenous superantigens
    In manuscript

LIST OF ABBREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
AChR	Acetylcholine receptor
AD	Addison's disease
AIRE	Human autoimmune regulator gene
Aire	Mouse autoimmune regulator gene
AIRE	Autoimmune regulator protein
ALPS	Autoimmune lymphoproliferative syndrome
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS I	Autoimmune polyendocrine syndrome type I
BAFF	B cell activating factor of the TNF family
BCR	B cell receptor
CBP	CREB-binding protein
CD	Cluster of differentiation
CMC	Chronic mucocutaneous candidiasis
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
CYP450	Cytochrome P450
B6	C57BL/6
DC	Dendritic cell
DN	Double negative
DP	Double positive
FOB	Follicular B cell
GAD	Glutamate decarboxylase
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
HSR	Homogeneously staining region
IA2	Insulinoma associated tyrosine phosphatase like protein
IFN	Interferon
IL	Interleukin
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy, X-linked
iNKT	Invariant natural killer T cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
MZB	Marginal zone B cell
NALP5	NACHT leucine-rich-repeat protein 5
NOD	Non-obese diabetes
PAMP	Pathogen associated molecular patterns
PIAS/*Pias*	Protein inhibitor of activated STAT
PML	Promyelocytic leukemia bodies
RAG	Recombination activating gene
RIP	Rat insulin promoter

SCC	Side chain cleavage enzyme
SLE	Systemic lupus erythematosus
SP	Single positive
STAT	Signal transducers and activator of transcription
T1D	Type 1 diabetes
TCR	T cell receptor
TSA	Tissue-specific antigen
tDC	Thymic dendritic cell
Treg	T regulatory cell
21-OH	21-hydroxylase
17-OH	17α-hydroxylase

CONTENTS

1 INTRODUCTION

The immune system has evolved to protect the host from potentially harmful pathogens; viruses, bacteria, fungi and parasites. However, immune responses may also cause disease, so-called hypersensitivity diseases. In the early 20th century, the German scientist Paul Ehrlich described how the immune system destroyed the body's own organs and tissues, a condition he referred to as "horror autotoxicus". This condition, later termed autoimmunity has, during the past century, been the subject of extensive investigation, yet the underlying mechanisms are still poorly understood. The prevalence of autoimmune disorders is estimated to be 3-5% in the general population and is higher in women than men (Jacobson et al., 1997). These diseases often affect young and middle-aged adults, and may cause severe disability and morbidity.

To prevent autoimmunity, lymphocytes are subjected to mechanisms that mediate tolerance, i.e. the ability of the immune system to distinguish self-structures from non-self. This thesis will focus on a key player in immunological tolerance, the autoimmune regulator gene (*AIRE*).

1.1 IMMUNE TOLERANCE

Tolerance mechanisms include the selection processes of T and B cells during the development in thymus and bone marrow, referred to as central tolerance. Central tolerance results in the maturation of only those lymphocytes that are functional in the defense against pathogens, but are non-responsive towards self-structures. Central tolerance is not absolute, and autoreactive cells escape and enter the circulation and secondary lymphoid organs where they are regulated by peripheral tolerance mechanisms. The following section describes how the immune system generates cells that are harmless to tissues and organs, but are able to respond to potentially harmful pathogens.

1.1.1 T cell development

Early in fetal development, the thymic stroma is organized into epithelium and mesenchyme and starts to become colonized by T cell progenitors (Owen and Jenkinson, 1984). The stroma can be defined as the non-hematopoietic component of the thymus, which provides the matrix on which thymocytes develop. T cell progenitors enter the thymus as CD4CD8 double negative (DN) cells. They then differentiate through four DN stages with distinct features: $CD25^-CD44^+$, $CD25^+CD44^+$, $CD25^+CD44^-$ and $CD25^-CD44^-$ (Godfrey et al., 1993). During the $CD25^+CD44^-$ stage, recombination activating gene (RAG) expression is induced, and the T cells start to rearrange their T cell receptor (TCR) β loci. Conventional T cells primarily express a rearranged TCRβ- chain together with a surrogate pre-TCRα- chain. Successful signaling via this pre-TCR, so-called beta-selection, leads to proliferation, expression of the co-receptor molecules CD4 and CD8, and rearrangement of the TCRα locus (Hoffman et al., 1996). Once a functional TCR is expressed on the surface, the fate of the developing T cell is dependent on the avidity and affinity of the TCR inter-action with major histocompatibility complex (MHC) molecules on the thymic stroma. The critical parameters for this are TCR specificity, the type of co-stimulatory signals provided, and the number of TCRs engaged with MHC molecules at any given time (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Low affinity and avidity interactions between the TCR and ligand mediate positive selection. This process

occurs in the thymic cortex and ensures that the T cell repertoire has self-specificity. Thus, failure to bind MHC on cortical thymic epithelial cells (cTECs) leads to elimination by apoptosis, while engagement of the TCR mediates survival and differentiation (Surh and Sprent, 1994). This phenomenon has been illustrated in bone marrow chimera experiments, where the MHC-restricted specificity of the donor T cells was determined by the chimeric host. Furthermore, thymus transplantation has shown that the T cell restriction is dependent on the MHC type of the thymus epithelial cells (Bevan, 1977; Zinkernagel et al., 1978). Positive selection leads to down-regulation of either CD4 or CD8 molecules, depending on whether the T cell has specificity for MHC class I or II, respectively. RAG transcription is turned off, preventing further rearrangement of the TCR genes (Brandle et al., 1992).

The next stage of development is negative selection which occurs mainly in the thymic medulla where the TCR expression is fully up-regulated (Surh and Sprent, 1994). Thymocytes that bind with high affinity to MHC-antigen complexes presented by medullary thymic epithelial cells (mTECs) or bone-marrow-derived thymic dendritic cells (tDCs) are deleted by apoptosis (Kappler et al., 1987). Negative selection leads to the deletion of potentially autoreactive cells and is one of the major mechanisms of self-tolerance. The ability of the thymic medulla to present a diverse range of tissue-specific antigens (TSAs), termed promiscuous antigen expression, is crucial in mediating negative selection (Derbinski et al., 2001; Klein et al., 1998). This phenomenon has been demonstrated in a diabetes tolerance model where the authors found intrathymic expression of a transgenic antigen under the rat insulin promoter. They suggested intrathymic expression to be a physiological property of the insulin locus (Jolicoeur et al., 1994). Since then, a wide variety of tissue antigens, representing nearly all organs, have been found to be expressed by mTECs. The mTECs can be divided into two subsets with regard to their expression of MHC class II and CD80. It has been suggested that the subset with high expression of CD80 and MHC class II represents the most mature stage of mTEC development. The complexity of TSA expression is highest in this population (Gotter et al., 2004). However, this so called "terminal differentiation model" has been challenged, as the CD80^{high} MHC class II^{high} cells have been shown to have a higher turnover and proliferation rate than the CD80^{low} MHC class II^{low} mTECs (Gray et al., 2006). A given TSA is expressed by 1-3% of the mTECs and each mTEC expresses only a few antigens (Derbinski et al., 2001). Nevertheless, the dynamics of the DC-T cell interaction; a single DC can scan approximately 5000 T cells per hour (Miller et al., 2004), makes it is possible that the time a T cell spends in the thymic medulla is enough to encounter all CD80^{high} mTECs. However, the ability of mTECs to mediate negative selection is poor, especially for CD4^{+} thymocytes. Instead, this is mediated by professional bone-marrow-derived DCs that have a superior expression of co-stimulatory molecules (Brocker et al., 1997). The mTECs can act as TSA reservoirs for DCs, i.e. DCs pick up antigen from mTECs and delete autoreactive cells via cross-presentation (Gallegos and Bevan, 2004). A schematic illustration of thymic development is shown in Figure 1.

Less than 5% of the T cell precursors that enter the thymus will survive positive and negative selection and leave as mature naïve T cells. Most of them succumb in the positive selection process by failing to express a functional TCR. An important notion is that the TCRs appear to have an intrinsic affinity for MHC, probably as a result of co-evolution, otherwise even fewer T cells would survive (Blackman et al., 1986). In addition, the DP T cell has the ability to recombine its TCR-

α locus in several rounds, thus increasing the chance of expressing a functional TCR.

The T cells are selected for their ability to interact with self-peptides presented by MHC, yet in the periphery their function is to react to foreign antigens. This is achieved by the ability of the TCR to cross-react with several different peptides, a property necessary for the immune system to adapt to a changing environment (Mason, 1998). However, this ability, together with an incomplete negative selection processes, lead to the risk of potentially autoreactive T cells in the periphery. Therefore, further mechanisms of tolerance are needed in peripheral organs in order to prevent autoimmunity, referred to as peripheral tolerance.
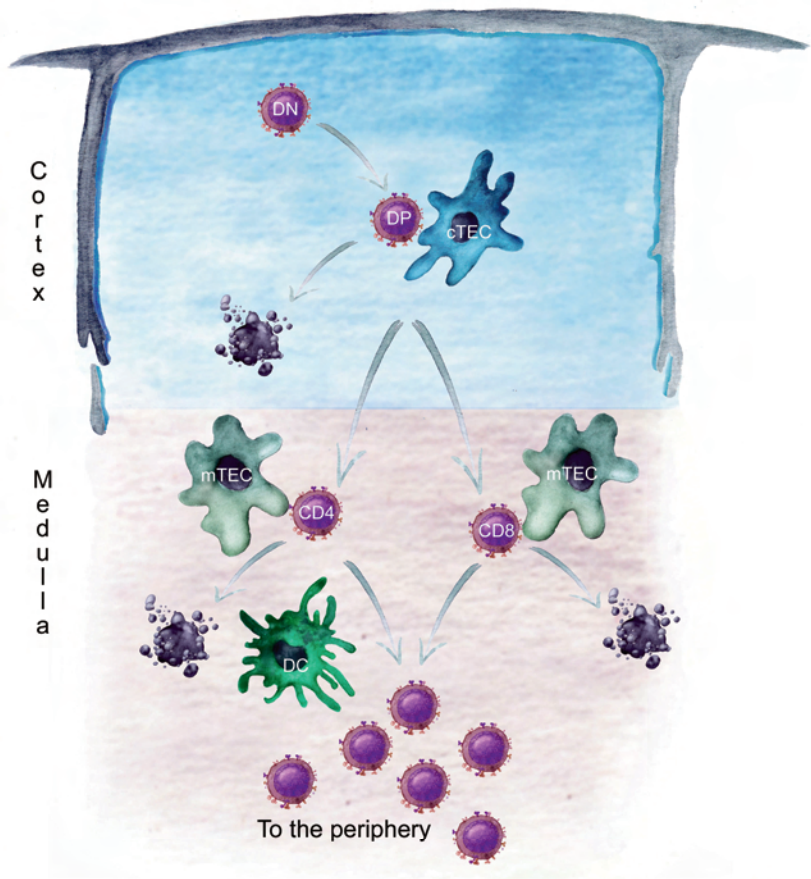

Figure 1. Schematic illustration of thymocyte development.
Illustration courtesy of Dr M. Winerdal.

1.1.1.1 Unconventional T cells

Some of the T cells will diverge from the conventional αβ T cell lineage during development in the thymus and develop into γδ T cells, natural killer T cells (NKTs), and natural T regulatory cells (Tregs). Although the affinity-avidity model for thymic selection applies to αβ T cells, the unconventional T cells seem to be selected under different conditions. NKT cells express a highly restricted set of TCRs that recognize glycolipids bound to the MHC class I-like molecule CD1d (Gumperz et al., 2000). Most NKT cells also express markers that were previously thought to be exclusively expressed on natural killer (NK) cells, for example CD161 (NK1.1 in mouse) (Arase et al., 1992). There is also a population of NKT cells that expresses NK1.1, but that is not restricted to CD1d, so-called NKT-like cells (Cardell, 2006). Two types of CD1d-restricted NKT cells have been characterized. Type I, also called invariant NKT cells (*i*NKT), express a semi-invariant TCR that consists of Vα24-Jα18 combined with Vβ11 in humans and Vα14-Jα18 combined with Vβ8.2/7/2 in mice (Lantz and Bendelac, 1994). The *i*NKT cells can be specifically identified by binding to CD1d dimers loaded with the glycosphingolipid α-galactosylceramide (αGalCer) (Kawano et al., 1997). The type II NKT cells express a more diverse TCR repertoire. Much less is known about this population due to a lack of specific reagents to identify them. However, the glycosphingolipid sulfatide was recently identified as a ligand for type II NKT cells (Blomqvist et al., 2009; Jahng et al., 2004). The *i*NKT cells diverge from the conventional T cell line at the DP stage of thymocyte development in the cortex (Egawa et al., 2005). By restricting CD1d expression to hematopoietic or stroma cells in the thymus, it was shown that *i*NKT cells are positively selected only when CD1d/ligand is expressed by hematopoietic DP thymocytes. Thus, their selection appears to be independent of thymic epithelial cells (Coles and Raulet, 2000; Wei et al., 2005). The DP thymocytes probably provide certain signals or are capable of presenting specific glycolipids that are necessary for *i*NKT cell development. A search is ongoing for natural ligands in the selection process of *i*NKT cells, as αGalCer is not a mammalian product. A key candidate is isoglobotrihexosylceramide (iGb3), which is a relatively weak activating ligand compared to αGalCer. This was suggested by findings of developmental arrest of *i*NKT cells in the absence of the enzyme β-hexosaminidase B, which produces iGb3 (Zhou et al., 2004). However, this has been challenged by the finding that mice deficient in another enzyme in the iGb3 biosynthesis, iGb3 synthase, show no defect in *i*NKT cell development (Porubsky et al., 2007). *i*NKT cells also appear to be subjected to negative selection, as the presence of αGalCer or overexpression of CD1d on DCs during development leads to the abrogation of the *i*NKT cells (Chun et al., 2003).

The γδ T cells develop without MHC restriction, although the exact pathway for their development is unclear. They make up 1-5% of the circulating T cells, while in epithelial tissues, such as the skin and mucosa, they are more frequent (Brenner et al., 1986). The natural Tregs originate in the thymus, and are defined by high expression of CD25, CD4, and the transcription factor FOXP3 (Schubert et al., 2001). For these cells, high-affinity interaction with the MHC/self-peptide complex presented by the thymic stroma, leads to differentiation rather than deletion. Their development seems to be mediated by cTECs rather than mTECs (Bensinger et al., 2001; Jordan et al., 2001)

The Tregs, NKT cells and γδ T cells all show regulatory functions and as such have the capacity to be involved in maintaining peripheral tolerance. The NKT cells can produce high amounts of immunomodulatory cytokines, including IL-4, IFN-γ and

IL-17, thereby regulating a wide variety of immune responses. They have been reported to be involved in microbial immunity, tumor rejection, and in suppressing autoimmune disease, as illustrated by a decreased number of circulating *i*NKT cells in for example systemic lupus erythematosus (SLE) and multiple sclerosis (Ronchi and Falcone, 2008). Tregs are able to suppress the activation and proliferation of T cells, either via direct cell-cell contact or by secreting cytokines. This is described further in the section on peripheral tolerance. The γδ T cells are considered "innate-like", and appear to act as a bridge between the innate and adaptive immune system. For example, γδ T cells have been shown to function as antigen presenting cells to αβ T cells (Brandes et al., 2005), and can also regulate the activation of NK cells and B cells (Brandes et al., 2003; Leslie et al., 2002).

1.1.2 B cell development

In adults, B cell generation occurs in the bone marrow and, as for T cells, it requires stroma cells. B cell development has been best characterized in mice, but the developmental pathway in humans seems to be similar (Carsetti et al., 2004). Early B cell precursors express the RAG genes and begin to rearrange the Ig heavy chain gene of the B cell receptor (BCR). The heavy chain is expressed with a surrogate light chain that together constitutes the pre-BCR. In the absence of signaling from a functional pre-BCR, the B cell will die by apoptosis. Otherwise, the B cell starts to proliferate and rearrange the light-chain gene. In humans, 60% of the B cells express Igκ light chains, while 40% express Igλ (Ghia et al., 1996). The rearrangement of gene segments of the heavy and light chains, together with the addition of random nucleotides to the DNA-ends created during recombination, creates a highly diverse antibody repertoire. The B cell precursors enter transitional stage 1 (T1), where the majority of the B cells leave the bone marrow via the blood stream and enter the spleen, although some remain and develop in the bone marrow (Cariappa et al., 2007; Loder et al., 1999). The immature T1 B cells continue to differentiate through the T2 stage into mature long-lived B cells.

During development, B cells are subjected to selection mechanisms that ensure the survival of those B cells expressing BCRs with certain specificities. This selection process not only serves to promote the development of B cells with functional antigen receptors (positive selection), but also provides a mechanism for the elimination of autoreactive clones (negative selection). Negative selection is mediated by self-antigen cross-linking of the BCR which leads to subsequent apoptotic death of the B cell or rearrangement of a new light chain (receptor editing) (Cyster et al., 1994; Gay et al., 1993). Between 20 and 50% of all developing B cells appear to undergo receptor editing (Casellas et al., 2001). B cells that fail to undergo receptor editing are deleted by apoptosis. Clonal deletion and receptor editing are the most important mechanisms in ensuring B cell tolerance. A third mechanism of B cell tolerance is anergy, but it is unclear to what extent this contributes to overall tolerance (Goodnow et al., 1988). Only about 5-10% of the B cells produced in the bone marrow will enter the mature peripheral B cell pool, the rest will be deleted during development (Wardemann and Nussenzweig, 2007).

1.1.2.1 *Peripheral B cell subsets*

Naïve, mature B cells are divided into three subsets: follicular B cells (FOBs), marginal zone B cells (MZBs) and B-1 B cells. FOBs migrate through the blood, lymph and B cell areas of the spleen, where they may present T cell-dependent antigens to activated

T cells and receive signals that lead to germinal center formation and plasma cell differentiation. In the germinal centers, the B cells undergo somatic mutations of the Ig genes and Ig class switch, which further diversifies the antibody repertoire (MacLennan, 1994). This is induced by the expression of the enzyme activation-induced cytidine deaminase (AID), which is essential for the class switch recombination process and somatic hypermutations. MZBs are more innate-like B cells that can be activated to differentiate into plasma cells in a T cell-independent manner. T cell-independent activation is also believed to mediate somatic mutation and class switch (Crouch et al., 2007). In mice, the MZBs reside in the border between the red and white pulp of the spleen, where they mediate responses to blood-borne pathogens (Martin et al., 2001). MZBs can also be activated in a T cell-dependent manner, and furthermore, they can contribute to T cell-dependent B cell responses by capturing antigens and delivering them to the FOBs (Cinamon et al., 2008). MZBs have a high expression of CD1d and have been suggested to present lipid antigens to NKT cells and to be activated to produce anti-lipid antibodies (Leadbetter et al., 2008). The human equivalent to MZBs are the extra-follicular lymph node B cells that exhibit an IgM$^+$ memory phenotype and have a function similar to the murine MZBs. The B1-B cells have mainly been described in the murine setting, and are not clearly defined in humans. These cells are innate-like B cells that reside primarily in the peritoneal cavity, but they are also found to a small extent in the spleen. Peritoneal B1 B cells are believed to be the main IgA producing plasma cell type in the lamina propria of the gut (Macpherson et al., 2000). Like the MZBs they can be activated in a T cell-independent manner (Tung and Herzenberg, 2007). The pool of natural antibodies has been attributed to MZBs and B-1 B cells (Ochsenbein et al., 1999).

Continuous BCR signaling is required for the survival of peripheral B cells (Kraus et al., 2004). It has also been suggested that BCR signaling determines B cell lineage commitment. For example, experiments with BCR transgenic mice recognizing a self-antigen show that weakly self-reactive immature B cells preferentially develop into MZBs or B-1 B cells (Wen et al., 2005).

1.1.3 Peripheral tolerance

Despite the selection processes during development, numerous low-affinity autoreactive B and T cells mature and reside in peripheral organs (Bouneaud et al., 2000). This is illustrated by the fact that immunization with a particular self-antigen in animal models can induce experimental autoimmune disease. Peripheral mechanisms that regulate an immune response are clonal anergy, i.e. unresponsiveness to antigens, and clonal suppression. For T cells, the anergic state arises when they are activated by antigen-presenting cells (APCs) via their TCR in the absence of co-stimulation, such as that of the B7-CD28/CTLA-4 pathway (Perez et al., 1997). APCs have the ability to discriminate between self and non-self, and are activated primarily upon the recognition of microbial structures, so called pathogen-associated molecular patterns (Barton and Medzhitov, 2003). This induces the up-regulation of co-stimulatory molecules, and leads to increased antigen presentation and secretion of inflammatory cytokines. The most potent APCs are denditic cells (DCs), which are important regulators of immune responses in the periphery. DCs are a heterogeneous group of cells; several different DC subsets are found in different tissues. They originate in the bone marrow and differentiate in peripheral organs into myeloid or plasmacytoid DCs, depending on the type of growth factors and cytokines they encounter. Myeloid DCs are the main

subtype in the periphery and are responsible for antigen uptake and presentation. Altered DC homeostasis or faulty regulation of for example cytokine production and expression of co-stimulatory molecules, may lead to the development of autoimmunity (Tzeng et al., 2006).

Clonal suppression is mediated by different subpopulations of T regulatory cells that can suppress the activation of T cells, either by direct cell-to-cell contact or by secreting cytokines. The best described T regulatory cells are the $CD4^+CD25^+$ $FOXP3^+$ cells. These mediate suppression by the secretion of inhibitory cytokines such as IL-10 and TGFβ, and can also perform cytolysis by the secretion of granzymes. In addition, they are believed to be able to modulate the function of DCs, for example by down-regulating their co-stimulatory molecules. The transfer of Tregs can prevent autoimmunity in mouse models, while the absence of these cells, i.e. by mutations in the *FOXP3* gene, leads to autoimmune disease in both mice and humans (Vignali 2008). Another mechanism for the elimination of autoreactive T cells is activation-induced cell death, which is triggered by the engagement of pro-apoptotic receptors on the T cell (Nagata, 1997).

The B cells that survive selection in the bone marrow and enter the spleen undergo further selection processes and need additional signals to mature fully. One of the most important is the B cell activating factor of the TNF family (BAFF), a cytokine secreted by macrophages, DCs and stroma cells (Schneider et al., 1999). Mice deficient in BAFF exhibit B cell developmental arrest and will not develop mature B cells (Batten et al., 2000). On the other hand, excessive BAFF expression may alter the fate of the developing B cell and lead to increased survival of autoreactive B cells (Mackay et al., 1999; Thien et al., 2004). In fact, self-reactive anergic B cells show decreased expression of the BAFF receptor, leading to a developmental disadvantage (Lesley et al., 2004). Increased BAFF levels are associated with several autoimmune disorders such as SLE and Sjögren's syndrome. Human studies have shown that the majority of early immature B cells are autoreactive, while the number decreases as the B cells mature, showing that selection of B cells continues in the periphery (Wardemann et al., 2003). B cell tolerance is partly regulated by T cells, given the need of T cell help for activation. These secondary signals, apart from BCR or TCR engagement, provide an extra check-point to prevent autoreactivity.

Apart from the ability of the immune system to distinguish self from non-self, it has been suggested that, in the periphery, it rather discriminates between damaged and healthy tissue. This so-called "Danger model" suggests that the immune system is activated by alarm signals from damaged tissue and not primarily by non-self structures (Matzinger, 1994). This would explain why the immune system does not attack "altered self", for example during puberty, pregnancy or aging. This model would also explain why the highest risk of tolerance failure is during infections accompanied by tissue damage.

1.2   AUTOIMMUNE DISEASES

In spite of rigorous control during development and activation, the immune system may sometimes be activated against self-structures, leading to organ destruction and autoimmune disease. As target organs are destroyed by the immune system, antigens that are normally sequestered from immune recognition are released and may elicit a response. This will lead to progression of the disease, referred to as epitope spreading. The classical definition of autoimmunity is that it can be transmitted from a sick

individual to a healthy one by the transfer of autoantigen-specific T cells or autoantibodies (Rose and Bona, 1993). The following sections discuss the classification and etiology of autoimmune diseases.

1.2.1 Classification

Autoimmune diseases can be divided into systemic and organ-specific diseases. Systemic autoimmune diseases are characterized by reactivity against ubiquitously expressed antigens, leading to pathology of a wide range of tissues and organs. An example is SLE, which is characterized by autoantibodies directed against nuclear antigens. In SLE, tissue damage is mainly caused by antibody-antigen complexes that affect the kidneys, skin and brain. Organ-specific autoimmunity can be divided into destructive and non-destructive disease. Destructive autoimmune diseases lead to the immunological destruction of the target organ, usually the endocrine organs. In non-destructive diseases, the phenotype is caused by autoantibodies that affect the target organ without causing organ destruction, for example Grave's disease where autoantibodies stimulate the TSH receptor, or myasthenia gravis where they block the acetylcholine receptor.

1.2.2 Etiology

1.2.2.1 Genetic factors

There is a general belief that autoimmunity is a result of interactive effects between genes and the environment. The genetic contribution is often very complex; however, a few autoimmune disorders are regarded as monogenic. Some of the best characterized monogenic immunodeficiency disorders are immuno-dysregulation polyendocrinopathy enteropathy, X-linked (IPEX), autoimmune lymphoproliferative syndrome (ALPS), and autoimmune polyendocrine syndrome type 1 (APS I). IPEX patients lack expression of *FOXP3*, a transcription factor that is crucial for Treg development. These patients suffer from dermatitis, enteropathy and chronic inflammation, and usually die before two years of age without treatment (Bennett et al., 2001; Powell et al., 1982). ALPS is caused by mutations of the genes involved in maintaining lymphocyte homeostasis, such as the death receptor Fas, and its ligand, or downstream signaling caspases 8 and 10 (Fisher et al., 1995). This leads to hyperproliferation of lymphocytes and severe autoimmune disease. APS I will be further described in the following sections.

In non-monogenic disorders, the most common genetic contribution is from the HLA locus (Caillat-Zucman, 2009). Some HLA alleles can lead to a higher risk of developing certain autoimmune disorders, whereas others can have a protective effect. It has been suggested that different HLA alleles are involved in autoimmunity by determining which peptides are presented to T cells, and how efficient they are bound to the MHC molecules, as this will influence the outcome of both negative selection in the thymus and T cell activation in secondary lymphoid organs. In the same manner, HLA may determine the outcome of the selection of regulatory T cells, thereby affecting development of autoimmunity. Genes that are involved in lowering the activation set point of the immune system are also associated with autoimmunity, for example, certain alleles of *CTLA-4* correlate with type 1 diabetes (T1D) (Douroudis et al., 2009). CTLA-4 is essential for the downregulation of T cell activation.

Apart from mutations, protein alterations such as posttranslational modifications, can lead to loss of tolerance and elicit autoimmune responses. For

example, protein citrullination has been implicated in rheumatoid arthritis; i.e. the patients develop antibodies recognizing citrullin, a posttranslationally modified arginin residue (Schellekens et al., 1998). Other factors behind autoimmunity are the exposure of sequestered proteins that are normally protected from immune recognition. Defects in the clearing of apoptotic cells can trigger autoimmunity; as illustrated by the development of an SLE-like syndrome in individuals with a deficiency of the complement subcomponents of C1. These complement factors are involved in the clearance of apoptotic cells by taking part in the classical pathway of the complement system, which mediates opsonization and phagocytosis (Manderson et al., 2004). At the same time, apoptosis is necessary to prevent autoimmunity, as failure in the down-regulation of an immune response and failure to maintain immune homeostasis leads to ALPS.

Women are more susceptible to autoimmune diseases; the female to male ratio being 1:3. This is thought to be due to estrogenic hormones, or possibly to immunoregulatory genes on the X chromosome, such as *IRAK1*, a susceptibility gene in SLE (Ahmed et al., 1999; Jacob et al., 2009).

1.2.2.2 *Non-genetic factors*

The concordance for most autoimmune diseases between monozygotic twins is relatively low, indicating that non-genetic factors influence the etiology. The most important triggering factor for autoimmunity is probably infections that activate the immune system and create an inflammatory milieu with up-regulation of co-stimulatory molecules and pro-inflammatory cytokines. Examples of suspected underlying infections in autoimmune disease are the Epstain-Barr virus in rheumatoid arthritis, SLE, Sjögren's syndrome, and multiple sclerosis, the Coxsackie B4 virus in T1D, and the hepatitis C virus in autoimmune hepatitis. The effect of infections is explained by molecular mimicry; the microbes have antigenic epitopes that resemble those of the host, leading to cross-reactivity. For example, scanning of protein sequence databases revealed numerous microbial regions that showed similarity to the human acetylcholine receptor (AChR), an autoantigen in myasthenia gravis (Deitiker et al., 2000). Other triggering factors are pregnancy; postpartum thyroiditis develops in one third of patients with T1D (Alvarez-Marfany et al., 1994), or pharmaceuticals; administration of IFN-alpha in the treatment of chronic hepatitis C is associated with the development of anti-21-hydroxylase (21-OH) and anti-thyroid antibodies (Wesche et al., 2001). Additional factors that may influence the development of autoimmunity are diseases or stress that leads to lymphopenia. In order to regain lymphocyte homeostasis, rapid proliferation will occur, leading to a skewed lymphocyte repertoire, which may favor autoreactive cells. For example, the depletion of B cells leads to less competition for survival factors such as BAFF, and subsequently to the increased survival of autoreactive cells that under physiological circumstances would not survive (Lesley et al., 2004).

There is an increasing conviction that stochastic elements, i.e. chance, determine whether an individual develops autoimmunity. External stochastic factors include the exposure to infections and chemicals. There are also intrinsic stochastic factors that will differ even between monozygotic twins, such as the random recombination process that generates the T and B cell repertoire, somatic mutations of the BCR, degree of cell death, availability of autoantigens, and the encounter of an autoreactive T cell with the specific antigen-loaded APC (Mackay, 2005).

1.2.3 Endocrine autoimmunity

The autoimmune destruction of endocrine organs can be manifested as isolated endocrinopathy, but it frequently results in polyendocrine disorders with two or more disease components. The autoimmune polyendocrine endocrine syndromes (APS) were originally classified by Neufeld, Maclaren and Blizzard in 1980 into 4 different groups based on clinical criteria (Neufeld et al., 1980). APS I is typically characterized by the presence of hypoparathyroidism, primary adrenocortical insufficiency (Addison's disease) and chronic mucocutaneous candidiasis (CMC), and has an onset early in childhood. APS I differs from the other polyendocrine syndromes in its monogenic origin.

APS II, also called Schmidt's syndrome after M. Schmidt who first described it, is much more common than APS I, and has a more complex genetic background. It is associated with HLA, in particular HLA-DR3 (Maclaren and Riley, 1986). APS II is characterized by the presence of Addison's disease in combination with thyroid autoimmunity and/or T1D. In a study of 107 APS II patients, 50% suffered from Addison's disease and thyroiditis, 21% from Addison's disease and Grave's disease, 18% from Addison's disease and T1D, and 11% exhibited the complete triad of Addison's disease, T1D, and thyroid disease (Betterle and Zanchetta, 2003). Other minor autoimmune diseases can also develop in APS II, such as hypogonadism, vitiligo, and alopecia. APS II affects mainly adult women and is very rarely seen in children.

APS III is classified as thyroid autoimmunity in combination with at least one other autoimmune component apart from Addison's disease. APS IV is a rare syndrome that includes all combinations that are not found in syndromes I-III.

1.3 AUTOIMMUNE POLYENDOCRINE SYNDROME TYPE I
1.3.1 Clinical phenotype

Autoimmune polyendocrine syndrome type I (APS I) also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Ahonen et al., 1990; Neufeld et al., 1981), is a monogenic, recessively inherited disease, caused by mutations in the autoimmune regulator gene (AIRE) (The Finnish-German consortium., 1997). According to its monogenic origin, APS I has a higher prevalence in genetically isolated populations, i.e. Iranian Jews, Sardinians and Finns, where the incidences are 1:9000, 1:14000 and 1:25000, respectively (Rosatelli et al., 1998; Zlotogora and Shapiro, 1992). Sporadic cases have been reported in other countries; for example the incidence in Norway is 1:90000 (Myhre et al., 2001). APS I is typically characterized by the presence of at least two features of the diagnostic triad; CMC, hypoparathyroidism, and Addison's disease (Ahonen et al., 1990). The initial manifestations include CMC, and hypoparathyroidism, but several other components such as chronic diarrhea, keratitis, periodic rash with fever, and autoimmune hepatitis, can be part of the initial picture (Husebye et al., 2009). In a study of 91 Finnish APS I patients, the onset for the first disease component was reported to range from 0.2 years to 18 years with a median of 3.3 years (Perheentupa, 2006). As the disease progresses, the patients develop additional components, and the prevalence of most components increases with age. In a study of 20 Norwegian patients, adult patients suffered from a mean of four disease components, ranging from one to seven (Myhre et al., 2001). In addition to the already mentioned, the endocrinopathies include T1D, hypothyroidism, and hypogonadism. Common non-endocrine features of APS I are alopecia, vitiligo,

enamel hypoplasia, and keratoconjunctivitis (Perheentupa, 2006). The frequency of disease components, based on studies of Finnish, Norwegian and Italian APS I patients, is summarized in Table 1.

Table 1. Frequency of disease components in APS 1 (%).

Disease component	Finland (Perheentupa et al. 2006)	Norway (Myhre et al. 2001)	Italy (Betterle et al. 1998)
Triad			
CMC	100	85	83
Hypoparathyroidism	87	85	93
Addison's disease	81	80	73
Other manifestations			
Diabetes mellitus	23	0	2
Hypogonadism (female/male)	69/28	31	43
Malabsorption	18*	10	15
Chronic hepatitis	18	5	20
Alopecia	39	40	37
Vitiligo	31	25	15
Keratoconjunctivitis	22	10	12
Hypothyroidism	21	10	10
Enamel hypoplasia	77*	40	nr

* Reported in a subgroup of the Finnish patients (Ahonen et al., 1990).
 nr, not reported.

Despite its monogenic mode of inheritance, there is a striking clinical variation in APS I, even between siblings with identical mutations, suggesting the influence of additional genes and/or environmental factors (Wolff et al., 2007). Unlike most other autoimmune diseases, no major gender difference is reported, except for a higher prevalence of hypoparathyroidism and hypogonadism in female patients (Gylling et al., 2003; Perheentupa, 2006). Diagnosis is based on the presence of at least two features of the diagnostic triad, but if a sibling has the disease, the presence of only one disease component is sufficient for diagnosis. APS I is verified by genetic testing, or serological measurement of organ-specific autoantibodies. The recently reported autoantibodies against IFN-α2 and ω at an early stage of disease are viewed as promising diagnostic markers (Meloni et al., 2008). To date, the only treatment for APS I is hormonal replacement for the endocrine deficiencies and immunosuppressive therapy for autoimmune hepatitis, keratoconjunctivitis, and intestinal dysfunction. For the other disease components, the benefits of immunosuppressive treatment seem to be overshadowed by the adverse side-effects.

Although the course of disease varies, a common trait affecting nearly all APS I patients is recurrent or persistent infection with the yeast *Candida albicans (Candida)*. An exception is the patient group carrying the Y85C mutation, most common in the Iranian Jewish population, where the prevalence of CMC is reported to be only four out

of 24 (Zlotogora and Shapiro, 1992). CMC affects the oral, esophageal and vaginal mucosa, and the infection may also spread to the skin and nails. In the APS I patients, CMC does not usually develop into a deep or systemic infection. However, a complication is the development of oral or esophageal squamous cell carcinoma (Rautemaa et al., 2007; Richman et al., 1975). Controlling *Candida* infections, and good oral hygiene are crucial for preventing the development of squamous cell carcinoma (Husebye et al., 2009).

Given the severity of APS I and the fact that new disease components may arise throughout life, regular monitoring of the patients is very important. Chronic active hepatitis, tubulointerstitial nephritis, and Addison's disease are especially dangerous; Addison's disease may result in the life-threatening condition called addisonian crisis. Factors that severely impair the quality of life for APS I patients are metabolic fluctuations due to the endocrinopathies, sterility as a consequence of hypogonadism, and painful oral ulcers caused by CMC. APS I also places a considerable psychological burden on the patient, who must live with the constant risk of developing new life-threatening disease components (Perheentupa, 2006).

1.3.2 Autoantibodies

APS I is characterized by circulating tissue-specific autoantibodies, which are often predictive of organ destruction. The dominating subclass is IgG1, indicating that Th1-mediated responses cause organ destruction. Several autoantigens in APS I have been identified; most often they are key enzymes in hormone or neurotransmitter synthesis. Examples of the former group are the P450 cytochrome steroidogenic enzymes 21-hydroxylase (21-OH), side chain cleavage enzyme (SCC) and 17α-hydroxylase (17-OH) in the adrenal cortex, and P4501A2 in the liver (Gebre-Medhin et al., 1997; Krohn et al., 1992; Uibo et al., 1994; Winqvist et al., 1992., Winqvist et al., 1993). SCC and 17-OH are also gonadal autoantigens (Winqvist et al., 1995). Enzymes associated with neurotransmitter synthesis include glutamic acid decarboxylase (GAD), the major autoantigen in T1D, and tryptophan hydroxylase expressed by enterochromaffin cells in duodenal mucosa (Ekwall et al., 1998; Velloso et al., 1994). Other islet autoantigens are aromatic L-amino acid decarboxylase (AADC), insulinoma-associated tyrosine-phosphatase-like protein (IA2) and insulin (Rorsman et al., 1995). Recently, the first parathyroid antigen was identified as NACHT leucine-rich repeat protein (NALP5) (Alimohammadi et al., 2008). Table 2 gives an overview of the autoantigens found in the different organs.

Several of the autoantigens in APS I are also autoantigens in the isolated autoimmune disorder, for example, 21-OH in Addison's disease and GAD65 in T1D, whereas others are specific to APS I, such as the SCC enzyme (Winqvist et al., 1993; Winqvist et al., 1992). In APS I, only antibodies against IA2 are predictive of islet destruction (Soderbergh et al., 2004), whereas in isolated diabetes, the anti-GAD65 antibodies are generally predictive of disease. APS I patients may exhibit organ-specific autoantibodies without any sign of disease; the reason for which is not known.

Table 2. Autoantigens in APS I.

Organ	Autoantigen
Adrenal cortex	21-OH, SCC, 17-OH
Parathyroid gland	NALP5
Gonads	SCC, 17-OH
Pancreas	GAD65, AADC, IA2, insulin
Liver	P450c1A2, P4502A6, AADC
Thyroid gland	Thyroid peroxidase, thyroglobulin
Intestine	Tryptophan hydroxylase, histidin decarboxylase
Hair follicle	Tyrosine hydroxylase
Melanocyte	SOX9, SOX10

1.4   THE AUTOIMMUNE REGULATOR
1.4.1   Molecular biology

The gene behind APS I, *AIRE*, has been identified by positional cloning and located to chromosome 21q22.3. *AIRE* contains 14 exons that encode a 545 amino acid protein with the predicted molecular mass of 58 kDa (The Finnish-German APECED consortium., 1997; Aaltonen et al., 1994; Nagamine et al., 1997).

The AIRE protein contains several domains indicating its function as a transcriptional regulator, including two plant homeodomain (PHD) zinc-fingers, a nuclear localization signal (NLS), four nuclear receptor binding LLXXL motifs, a SAND domain, and a highly conserved N-terminal homogeneously staining region (HSR) (1997; Nagamine et al., 1997; Sternsdorf et al., 1999). The SAND and PHD domains of AIRE have DNA-binding properties, and two nucleotide binding sequences have been identified in a pull-down assay; TTATTA, and a tandem repeat of ATTGGTTAA (Kumar et al., 2001). The first PHD zinc-finger of AIRE has been suggested to mediate E3 ubiquitin ligase activity (Uchida et al., 2004), although this finding has been challenged by another group (Bottomley et al., 2005). The HSR region is found in transcription factors of the Sp100 families and mediates homodimerization, while the NLS in the N-terminus directs AIRE to the nucleus. AIRE has been shown to induce transcription when fused to a heterologous DNA binding domain in *in vitro* reporter assays (Pitkanen et al., 2000).

At the subcellular level, AIRE is expressed in nuclear dots resembling promyelocytic leukemia bodies (PML). AIRE has also been shown to localize in a fibrillar cytoplasmic pattern, but this pattern is only seen in transfected cell lines and not in tissue sections or blood cells (Rinderle et al., 1999). Co-staining of AIRE and the PML-body-associated proteins Sp100 or PML protein, shows no co-localization (Bjorses et al., 1999). Instead, AIRE binds directly to another PML-associated protein, the CREB-binding protein (CBP) (Pitkanen et al., 2000). CBP is a co-activator to several transcription factors, including NFkB and the STAT proteins. A recent publication reported the functional interaction of AIRE with PIAS1 (protein inhibitor of activated STAT1) in transcriptional regulation (Ilmarinen et al., 2008). The PIAS proteins function as transcriptional co-regulators by exerting their functions

as E3 SUMO ligases, by changing the subcellular localization of their targets or by interfering with DNA binding (Rytinki et al., 2009).

Over 60 mutations in the *AIRE* gene leading to APS I have so far been reported (Wang et al., 1998; Zaidi et al., 2009). They are found throughout the coding region; most are stop mutations leading to a truncated protein lacking a PHD finger, or missense mutations in the HSR region. The most common is the R257X mutation, a C to T transition found in 83% of the Finnish patients. This mutation results in truncation of the protein before the first PHD zinc-finger, leading to loss of the nuclear dot localization (Bjorses et al., 2000). In the Norwegian population, the dominating mutation is a 13 bp deletion in exon 8 (1085-1097$_{del}$), found in 55% of the alleles (Myhre et al., 2001). The functional domains of AIRE and some disease-causing mutations are shown in Figure 2.

So far, no clear genotype-phenotype correlation has been found in APS I. Some mutations seem to correlate with a lower incidence of CMC, for example the Y85C mutation seen in Iranian Jewish patients (Zlotogora and Shapiro, 1992). Heterozygote mutations do not usually lead to APS I. The only known exception is the G228W mutation, which acts in a dominant manner; the mutated protein dimerizes with the functional protein and disrupts its functional capacity (Cetani et al., 2001).

Figure 2. Schematic of the functional domains of AIRE and common mutations.
Black shading indicates LXXLL or NR box regions.

The high variability in phenotype between patients carrying the same mutation suggests that other genes and/or environmental factors determine disease outcome, but these factors remain unknown. For example, no association with HLA has been found in APS I, except in a few disease components such as Addison's disease and alopecia.

1.4.2 AIRE-deficient mice

The mouse orthologue *Aire* has been mapped to the murine chromosome 10 and shares 71% homology with human *AIRE* (Shi et al., 1999; Wang et al., 1999). The expression sites of *AIRE/Aire* have been investigated in several studies, with varying results. However, it is concluded that at the cellular level, the AIRE protein is mainly expressed in mTECs, in particular in the MHC class II^{high} subset, and in monocyte-derived DCs and macrophages. Human AIRE expression has also been detected in peripheral CD14^{+} blood monocytes (Halonen et al., 2001; Heino et al., 1999; Kogawa et al., 2002; Nagamine et al., 1997). Mouse AIRE expression has been reported in radio-resistant stroma cells of the lymph nodes (Gardner et al., 2008). In addition, low levels of AIRE expression have been detected in a number of other tissues and organs, but it is difficult to rule out the possibility that this is due to the presence of tissue macrophages and DCs.

In 2002, the first mouse models of APS I were described by two independent groups. The first was engineered to mimic the most common Finnish *AIRE* mutation

by the insertion of a termination codon in exon 6 (Ramsey et al., 2002). These *Aire*^{-/-} mice were of mixed 129/Sv (129) and C57BL/6 (B6) background. The phenotype was relatively mild, and the only signs of abnormality were infertility, organ infiltrates of lymphocytes and circulating organ-specific autoantibodies. The development and distribution of T and B cells in blood and lymphoid organs was found to be normal. The most striking finding was increased T cell proliferation in response to immunization with hen egg lysozyme (HEL) antigen and *in vitro* restimulation of draining lymph nodes post-immunization. The authors also observed a change in the distribution of the TCR repertoire, i.e. over-representation of three Vβ-families. This mouse was later back-crossed onto a congenic B6 background (Hassler et al., 2006). These mice showed normal endocrinology, but developed MZB lymphoma with age.

The second mouse model was created by Anderson and colleagues, and was a conditional knock-out mouse generated by a deletion in exon 2 and portions of the upstream and downstream introns (Anderson et al., 2002). These mice were also of mixed 129 and B6 background. The authors found lymphocytic infiltrates in several organs such as the salivary glands, ovaries, stomach, and eye, and autoantibodies with the same kind of target distribution. Bone marrow transfer experiments and thymic grafting showed that the phenotype was correlated with AIRE deficiency in thymic radio-resistant stroma cells. These cells showed reduced expression of several genes, some of which were TSAs. Thus, the authors hypothesized that the function of AIRE was to induce the expression of TSAs, thereby promoting negative selection of autoreactive T cells. Two of the TSAs that were found to be down-regulated in the thymus of *Aire*^{-/-} mice are autoantigens in APS I, i.e. insulin and IA2.

The Ramsey mouse model was crossed onto a double transgenic system, where the mice expressed HEL as an organ-specific antigen under the control of the rat-insulin promoter (RIP) and the T cells exressed a TCR specific for HEL. Studies if this model revealed that AIRE is fundamental to the deletion of autoreactive cells in the thymus (Liston et al., 2003). Furthermore, loss of AIRE affected thymic deletion induced by an antigen under control of the thyroglobulin promoter (Liston et al., 2004). AIRE deficiency also led to decreased thymic expression of endogenous insulin and to an increased population of islet-specific T cells in the periphery. Anderson and colleagues used a similar system to the HEL transgene but with ovalbumin. However, in this system, AIRE deficiency led to a striking defect in the negative selection of OT-II T cells, even though the ovalbumin transcript was present in the thymus at the same level as in the wild type mice (Anderson et al., 2005).

In 2005, Kuroda and colleagues described a third mouse model of APS I in which the targeting vector was constructed to replace exons 5-12 of the *Aire* gene (Kuroda et al., 2005). The mice were back-crossed to B6 and BALB/c backgrounds. The authors observed Sjögren's syndrome-like pathological changes, such as lymphocytic infiltration of the salivary and lacrimal glands, and a decrease in the secretion of tear fluid. Furthermore, the mice exhibited autoreactivity against a Sjögren's syndrome antigen, alpha-fodrin. Alpha-fodrin was expressed in the thymus of *Aire*^{-/-} mice to the same extent as in wild type mice, and thus the authors demonstrated that *Aire*^{-/-} mice have reactivity against normally expressed thymic antigens. Furthermore, they reported a normal expression of FOXP3 in the thymus and spleen, and normal numbers of CD4^{+}CD25^{+} Tregs. The authors also showed that the genetic background seemed to influence which targets were affected. For example

the BALB/c *Aire*^{-/-} mice developed autoantibodies against gastric mucosa, a feature that is rarely observed in B6 mice.

The influence of background genes on AIRE-deficient phenotype has been further evaluated. The mouse model described by Anderson and colleagues was back-crossed onto B6, BALB/c, non-obese diabetes (NOD) and SJL backgrounds (Jiang et al., 2005). The range of organs affected was found to differ; for example thyroiditis and pancreatitis were exclusive to the NOD and SJL backgrounds, while B6 and BALB/c *Aire*^{-/-} mice were protected against these manifestations. Interestingly, autoreactivity against the pancreas in *Aire*^{-/-} NOD and SLJ mice was directed against exocrine cells and not against the insulin-producing beta-cells. The mice of NOD and SLJ backgrounds also exhibited more severe lymphocytic infiltrates in general.

A double knock-out mouse was developed by crossing *FoxP3*^{-/-} mice with *Aire*^{-/-} mice carrying the exon 2 deletion (Chen et al., 2005). These mice suffered from extensive lymphocytic infiltration of the lungs and liver, which resulted in death at about 28 days of age. However, they did not exhibit any autoantibodies against these organs, excluding a pathogenic role of autoantibodies in this model.

Another report on AIRE deficiency on the NOD background has been published by Niki and colleagues (Niki et al., 2006). This model used the Kuroda *Aire*^{-/-} mouse back-crossed onto the NOD background. These mice exhibited body weight loss starting in week 8, and lymphocyte infiltration of various organs. In the pancreas, the reactivity was directed against acinar cells surrounding the beta-cell islets, rather than the islets themselves. Thus, also in this study, AIRE deficiency on a NOD background altered the pancreatic specificity from endocrine to exocrine cells. This subsequently resulted in a resistance to the development of diabetes in the absence of AIRE.

A forth *Aire*^{-/-} mouse has recently been reported (Hubert et al., 2009). This model was constructed to mimic the 13bp deletion in exon 8 found in patients. A *LacZ* reporter gene was brought under control of the *Aire* promoter, creating an *Aire-LacZ* fusion gene. In this model, the thymic epithelial cell compartment was found to be disturbed, i.e. *Aire*^{-/-} mice showed an increased frequency of MCH class II^{high} mTECs, while the immature mTECs were reduced. The authors found a normal CD4^{+} TCR repertoire; the length of the different Vβ-CDR3s showed a Gaussian distribution and thus no oligoclonal expansion dominated the repertoire of *Aire*^{-/-} mice. Furthermore, the *Aire*^{-/-} mice did not show increased susceptibility to oral or systemic infection with *Candida albicans*.

In summary, *Aire*^{-/-} mice provide a useful tool in studying the mechanism behind APS I, and have greatly contributed to our knowledge of AIRE. However, it is important to bear in mind that the mouse phenotype differs from that of the human. While humans suffer from very severe autoimmune disease and multiple organ failure that is lethal if not treated, the murine phenotype is relatively mild. Apart from infertility, which is observed in most *Aire*^{-/-} mouse models, the only mice that actually develop clinical manifestations are those on the NOD background. Even in this case, the mice develop exocrine pancreatitis and not diabetes, in contrast to APS I patients (Kekalainen et al., 2007a). Furthermore, the autoantigen profiles of mouse and man has been found to be completely different (Pontynen et al., 2006). The mouse models of APS I are summarized in Table 3.

Table 3. *Aire*$^{-/-}$ mouse strains.

Aire mutation	Background	Major findings
Exon 6 (Ramsey et al., 2002)	Mixed B6/129	Liver infiltrates of lymphocytes; autoantibodies; increased proliferative response against HEL; altered TCRβ repertoire.
Exon 6 (Hassler et al., 2006)	B6	MZB lymphoma; liver infiltrates of B cells; normal endocrinology.
Exon 2 (Anderson et al., 2002)	Mixed B6/129	Lymphocytic infiltrates in salivary glands, ovaries, stomach, and eye; reduced expression of several genes in mTECs.
Exon 5-12 (Kuroda et al., 2005)	B6 and BALB/c	Sjögren's syndrome-like manifestations in the BALB/c mice; normal numbers of Tregs.
Exon 2 (Jiang et al., 2005)	B6, BALB/c, NOD, SJL	Organs affected dependent on background strain; exocrine pancreatitis in NOD and SJL strains.
Exon 2 (Chen et al., 2005)	*FoxP3*$^{-/-}$ B6	Extensive lymphocytic infiltrates in lungs and liver; lethal before 28 days of age.
Exon 5-12 (Niki et al., 2006)	NOD	Exocrine pancreatitis; diabetes protection.
Exon 8 (Hubert et al., 2009)	B6	Disturbed thymic epithelium; no increased susceptibility to *Candida* infections.

1.4.3 Central vs. peripheral tolerance defect in the absence of AIRE

AIRE is clearly involved in the negative selection of T cells. The suggested mechanism is by inducing TSA expression in mTECs, thereby mediating negative selection of autoreactive T cells (Anderson et al., 2002). The impaired negative selection in the absence of AIRE was first shown in transgenic systems where an antigen was expressed under the control of the rat insulin promoter, and the T cells carried a transgenic TCR recognizing the same antigen. In absence of AIRE, the antigen-specific T cells were not deleted in the thymus (Anderson et al., 2005; Liston et al., 2003). Thymic expression of insulin was shown to be dependent on AIRE, and the absence of AIRE led to the escape of islet-specific T cells. Furthermore, AIRE deficiency affected thymic deletion induced by an antigen under control of the thyroglobulin promoter in a dose-dependent manner, i.e. thymic deletion was less efficient in homozygotes than heterozygotes (Liston et al., 2004). Unexpectedly, later studies showed that AIRE deficiency led to impaired negative selection of the antigen-specific T cells, even when the antigen was normally expressed in the thymus (Anderson et al., 2005). Furthermore, *Aire*$^{-/-}$ mice do not develop anti-insulin antibodies, or antibodies against any of the known APS I autoantigens (Pontynen et al., 2006). In fact, introducing an *Aire* mutation in the diabetes-prone NOD strain led to the development of exocrine

pancreas insufficiency and not diabetes (Jiang et al., 2005). In contrast to insulin, *Aire*^{-/-} mice have been shown to have reduced thymic expression of two antigens that also appear as autoantigens in the periphery: mucin 6 and inerphotoreceptor retinoid-binding protein (DeVoss et al., 2006; Gavanescu et al., 2007). However, *Aire*^{-/-} mice also show autorecativity against autoantigens that are normally expressed in AIRE-deficient thymus, i.e. alpha-fodrin and pancreas-specific protein disulfide isomerase (Kuroda et al., 2005; Niki et al., 2006).

AIRE expression seems to be dependent on a normal thymic architecture, given that mice with an abnormally developed thymus lack AIRE expression. For example, AIRE expression is not detected in *RelB*^{-/-} mice that lack thymic medulla and epithelial cells; RelB is responsible for the differentiation of mTECs and thymic DCs (Heino et al., 2000). New Zealand White (NZW) mice, a model for systemic autoimmunity, also have reduced numbers of AIRE expressing mTECs and reduced expression of AIRE per cell (Fletcher et al., 2009). A recent publication showed that *Aire*^{-/-} mice have a disrupted thymic ultrastructure, and signs of increased activation of mTECs. Whether this is caused by AIRE deficiency, or if AIRE deficiency is a consequence of this has not been established (Milicevic et al., 2009). Furthermore, AIRE deficiency leads to a blockage in the development of CD4SP thymocytes; Li and colleagues showed that the SP3 stage of development was increased by 70% whereas the SP4 cells were reduced by 80% (Li et al., 2007). Thus, it is possible that AIRE is important for the final maturation of thymocytes. For example, AIRE regulates the expression of co-stimulatory molecules and/or chemokines involved in thymocyte migration and maturation. An example of the former is the increased expression of VCAM-1 seen in AIRE-deficient DCs (Ramsey et al., 2006). Expression of chemokine CCL22 is reduced in AIRE deficient thymus; CCL22 functions by attracting DP thymocytes to the medulla (Anderson et al., 2005). In contrast, CCL19 and CXCL10 are up-regulated in AIRE-deficient mTECs. These chemokines are expressed in the cortico-medullary junction and attract SP T cells to the blood vessels through which they leave the thymus (Annunziato et al., 2001). This shows that AIRE may influence central tolerance through several mechanisms.

In addition to defects in central tolerance mechanisms, AIRE deficiency also affects peripheral tolerance. One of the most striking findings was the increased proliferation in response to the foreign HEL antigen after immunization of *Aire*^{-/-} mice (Ramsey et al., 2002). AIRE-deficient peripheral APCs are increased in number and have an altered phenotype, both in *Aire*^{-/-} mice and APS I patients. The APCs have an increased ability to activate naïve T cells, in part due to the increased expression of VCAM-1 (Ramsey et al., 2006). In addition, aged *Aire*^{-/-} mice have a phenotype caused by overly activated B cells, i.e. autoantibodies, MZB lymphoma and liver infiltrates of B cells (Hassler et al., 2006). In conclusion, AIRE deficiency seems to lead to breakdowns in a number of both central and peripheral tolerance mechanisms. The question that remains to be answered is to what extent each of these actually contributes to APS I.

2 PRESENT STUDY

Aim

The aim of this work was to investigate how AIRE is involved in immune tolerance in order to understand the mechanisms behind APS I.

2.1 METHODOLOGY

The materials and methods used in this work are described in detail in the original publications, Papers I-IV.

Mice (Papers I-IV)
The generation of *Aire*$^{-/-}$ mice (B6.129s4-Aire^{tm1P1tn}) has been described previously (Ramsey et al., 2002). All *Aire*$^{-/-}$ mice used were C57BL/6 congenic, and age- and sex matched with wild type littermates from heterozygotic breeding as controls. In two of the studies (Papers I and III), congenic *Aire*$^{+/-}$ C57BL/6 mice crossed with OT-II TCR transgenic C57BL/6 mice were used with the permission of Dr F. Carbone. The animals were kept at the Department of Microbiology, Tumor and Cell Biology animal facility at Karolinska Institutet.

Patients (Papers I-III)
Blood and saliva were collected from APS I patients and used with their informed consent.

Bone marrow transfer experiments (Papers I, II, IV)
Bone marrow cells were obtained by flushing mouse femur. Bone marrow transfer was performed by i.v. injection of donor-derived bone marrow cells into recipient mice irradiated with 900 rad at least 4 h prior to reconstitution.

Immunization (Paper I)
To measure specific Ig response, mice were immunized with antigen and serum was collected post-immunization.

Flow cytometry (Papers I, II. IV)
Single cell suspensions were prepared from murine organs and human PBMCs were purified from blood. Cells were stained with fluorochrome-conjugated antibodies and analyzed on flow cytometers: a FACSAria using FACSDiva software or a FACSCalibur using CellQuestPro software (Becton Dickinson).

ELISA and ELISPOT (Papers I-III)
Cytokine levels in cell culture supernatants and in serum from mice and humans were measured using specific ELISA kits according to manufacturer's instructions. In one of the studies (Paper I), an ELISPOT assay was performed to identify IgM-secreting B cells.

Immunofluorescent staining (Paper III)
Immunohistochemistry was used to analyze protein expression in frozen tissue cryosections.

PCR amplification (Papers I, II, IV)
Total RNA was extracted with Trizol® reagent (Invitrogen) and cDNA was synthesized by reverse transcription. Transcript levels or genomic DNA was amplified using specific primers.

Electrophoresis and immunoblotting (Papers I, III)
Proteomic analysis was performed with 2-D electrophoresis, SDS page and immuno-blotting.

MALDI (Papers III)
In gel digestion (trypsin) and matrix-associated-desorption/ionization mass spectrometry (MALDI) was performed by the proteomics core facility at Karolinska Institutet (PAC).

Candida growth inhibition assay (III)
Candida albicans (ATCC 90028) was cultured in the presence of saliva or anti-fungal substances. Inhibition was analyzed by measuring the incorporation of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

^{51}Cr release assay (Paper II)
A standard 4-h ^{51}Cr release assay was used to measure natural killer cell activity *in vitro*.

Statistical analysis (I-IV)
The data were analyzed with student's T-test or Wilcoxon's rank sum test in order to test for differences between 2 groups. Mean values are given together with SEM in the figures. $P < 0.05$ was considered significant.

Ethical considerations
The studies were evaluated and approved by the local ethics committees and have been conducted according to the regulations for handling laboratory animals and patient data.

2.2 RESULTS AND COMMENTS

2.2.1 Increased B cell activation in the absence of AIRE

The majority of the characteristics of *Aire*^{-/-} mice, i.e. autoantibodies, liver infiltrates of B cells, and the development of MZB cell lymphoma are caused by overly activated B cells (Hassler et al., 2006). In this study (Paper I), B cell activation in the absence of AIRE was studied. *Aire*^{-/-} mice were immunized with T cell independent antigen type I, TNP-Ficoll, and a significant increase in the production of specific antibodies was observed compared to wild type mice. The response to TNP-Ficoll is largely dependent on MZBs, as illustrated by a marked suppression of specific antibodies in mice lacking MZBs (Guinamard et al., 2000). The distribution of peripheral B cell subsets appeared normal, but the B cell activation status was increased in *Aire*^{-/-} mice, in particular in the MZB cell compartment. As the phenotype of *Aire*^{-/-} mice is very similar to that of mice overexpressing the cytokine BAFF (Mackay et al., 1999), serum levels of this cytokine were measured. Both *Aire*^{-/-} mice and APS I patients exhibited significantly higher levels of circulating BAFF than wild type mice and healthy control subjects (Figure 3). BAFF is secreted by DCs and radio-resistant stroma cells upon stimulation with IFN-γ, and is crucial for B cell development and activation (Schneider et al., 1999).
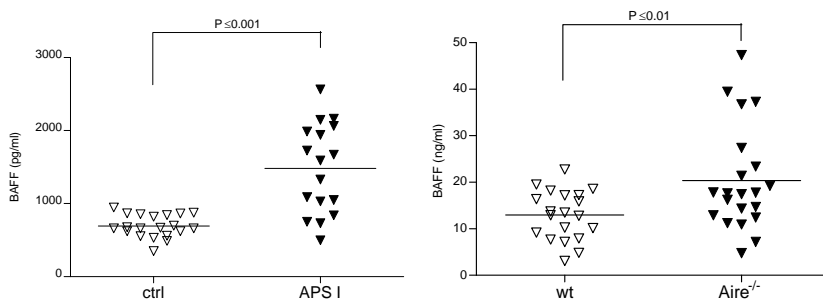
Figure 3. Increased serum BAFF in APS I patients (left lane) and *Aire*^{-/-} mice (right lane), analyzed with ELISA. Each dot represents one individual, and the mean values are indicated by horizontal lines.

To determine whether the excessive BAFF production was dependent on activated T cells, bone marrow reconstitution experiments using T-cell-deficient nude mice as recipients were performed. These experiments revealed that the excessive BAFF levels were produced by AIRE-deficient DCs independent of the presence of autoreactive T cells. Furthermore, when bone marrow-derived DCs were cultured *in vitro* from *Aire*^{-/-} and wild type mice, AIRE-deficient cells, correlated for numerical comparison, were found to produce excessive BAFF upon IFN-γ stimulation.

The IFN-γ receptor signals via the STAT1 pathway, leading to the differential expression of several genes. It has recently been shown that AIRE interacts functionally with PIAS1 (protein inhibitor of activated STAT1) (Ilmarinen et al., 2008). PIAS1 is involved in negatively regulating STAT1 signaling. We therefore suggest that AIRE is involved in the inhibition of STAT1 signaling downstream of the IFN-γ receptor, and that IFN-γ stimulation leads to exacerbated STAT1 signaling in AIRE-deficient cells. This subsequently leads to increased expression of BAFF (Figure 4). In accordance,

another STAT1 target gene, *Gbp1*, was found to be up-regulated in splenocytes from *Aire*^{-/-} mice. It has previously been shown that *Gbp1* is up-regulated in *Pias1*^{-/-} mice, further supporting the present findings (Liu et al., 2004).

In summary, AIRE has been shown to be involved in regulating the activation of peripheral DCs, leading to increased production of BAFF and subsequent activation of B cells in the absence of AIRE. Hence, AIRE is involved in maintaining tolerance and immune homeostasis in the periphery.
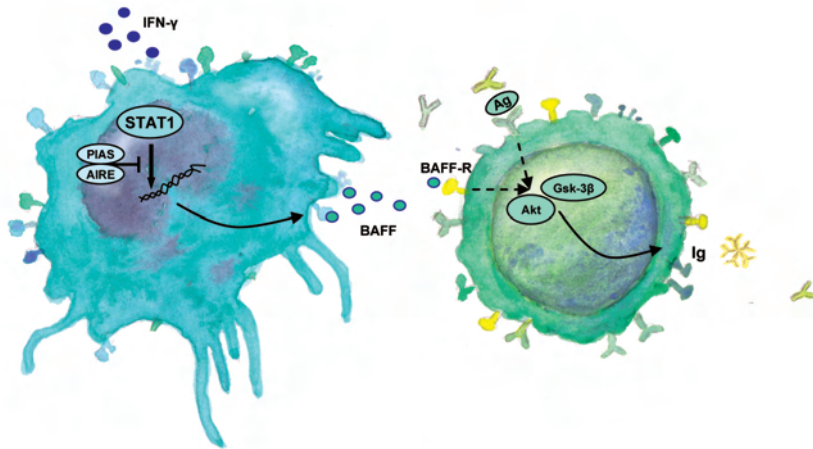
Figure 4. Hypothesized schematic of the role of AIRE in STAT1 signaling.
In the absence of AIRE, STAT1 signaling is not inhibited, leading to excess
BAFF production and subsequent activation of B cells. Illustration courtesy
of Dr M. Winerdal.

Comments

Since this paper was published, further evidence of the role of AIRE in STAT1 signaling has been found. The expression of STAT1-regulated genes was investigated and found to be increased in *Aire*^{-/-} mice (Figure 5). Furthermore, Levi and Polychronakos have reported that insulin expression in mTECs is regulated by IFN-γ, i.e. insulin expression is reduced upon IFN-γ stimulation (Levi and Polychronakos, 2009). The discovery that the expression of insulin is reduced in AIRE-deficient mTECs has led to the hypothesis that the role of AIRE is to induce expression of TSAs in the thymus, thereby promoting negative selection of autoreactive T cells (Anderson et al., 2002). The molecular mechanisms behind this have not yet been elucidated. These new data suggest that AIRE is involved in STAT1 signaling downstream of the IFN-γ receptor and regulates IFN-γ activating genes. I believe that this can explain many of the characteristics seen in APS I patients and *Aire*^{-/-} mice.
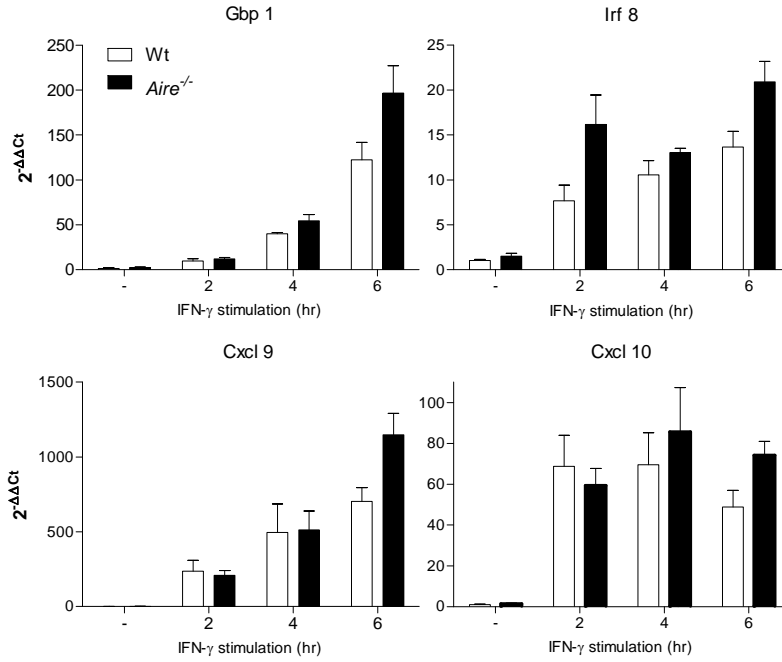
Figure 5. STAT1-target genes *Gbp1*, *Irf8*, *Cxcl9*, and *Cxcl10* are up-regulated in AIRE-deficient DCs upon IFN-γ stimulation. The same genes are upregulated in *Pias*^{-/-} mice, supporting the role of AIRE in PIAS1-mediated inhibition of STAT1 signaling.

2.2.2 AIRE deficiency affects *i*NKT cell development

In the present work (Paper II) an investigation was made on the effects of AIRE deficiency on the development of *i*NKT and NK cells. Both *i*NKT cells and NK cells have immunoregulatory functions and have been implicated in autoimmune disease. It was found that the frequency of circulating *i*NKT cells was reduced in APS I patients, as well as in the thymus and peripheral organs of *Aire*^{-/-} mice (Figure 6). Furthermore, murine splenic *i*NKT cells produced less of the cytokines IFN-γ, IL-17, and IL-4 upon stimulation with α-galactosylceramide, measured with ELISA.
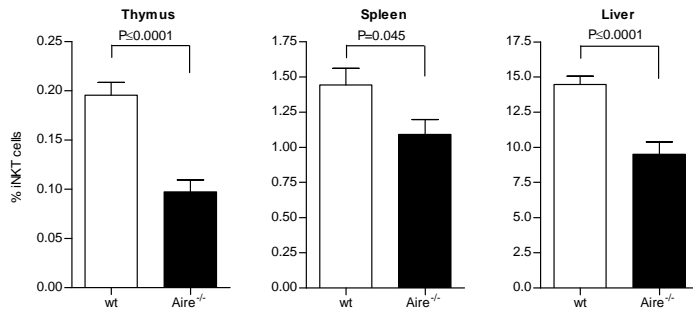
Figure 6. Flow cytometric analysis of *i*NKT cell frequency in the thymus, spleen and liver of *Aire*^{-/-} mice and wild type littermates. The mean and SEM of the result from at least 8 mice in each group are shown.

The impairment of *i*NKT cell development was found to be dependent on AIRE expression in radio-resistant cells, as shown by bone marrow transfer experiments in which bone marrow from AIRE-deficient or AIRE-sufficient donor mice was transferred into lethally irradiated hosts (Figure 7).

In contrast, the NK cells developed normally in the absence of AIRE, both phenotypically and functionally. Neither *i*NKT cells nor NK cells expressed any detectable levels of *Aire* transcripts and it was thus concluded that the reduced *i*NKT cell population in the absence of AIRE was caused by the interaction with other cells, leading to defective development in the thymus and/or defective distribution and activation in peripheral organs.
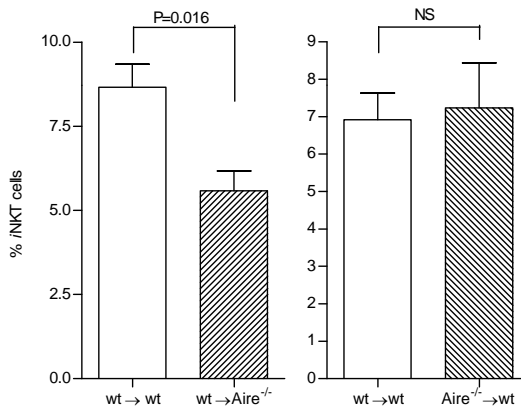
Figure 7. The development of *i*NKT cells is dependent on AIRE expression in radio-resistant stroma cells. Bone marrow from *Aire*^{-/-} or wild type donors was transferred into lethally irradiated hosts. The frequency of *i*NKT cells was analyzed 8 weeks after reconstitution using flow cytometry.

Comments

The effect of AIRE-deficiency on regulatory cell populations has been investigated in several studies. The Tregs are found to be normal in *Aire*^{-/-} mice but impaired in APS I patients, whereas the γδ T cells are phenotypically and functionally normal in both mice and patients (Anderson et al., 2005; Kekalainen et al., 2007b; Tuovinen et al., 2009). During the work on the present study, a report on the normal development and function of *i*NKT cells was published by another group (Pitt et al., 2008). However, this report was based on studies of a different *Aire*^{-/-} mouse strain than the present study, which could explain the discrepancy of results.

It is not known how exactly AIRE contributes to the development of *i*NKT cells. Since selection of *i*NKT cells seems to be mediated by hematopoietic cells and not by radio-resistant stroma cells in the thymus, it is likely that the phenotype seen here is caused by tDCs. The outcome in the thymic selection process of *i*NKT cells, as for conventional T cells, is probably dependent on a combination of factors, such as TCR-signaling strength, co-stimulatory molecules and chemokines. AIRE deficiency might alter these conditions and change the threshold for selection. It is also possible that the disrupted thymic ultrastructure, which is reported in *Aire*^{-/-} mice, influences the development of *i*NKT cells.

2.2.3 Autoimmunity behind *Candida* infections in APS I

A common trait that affects nearly all APS I patients is recurrent or persisting mucocutaneous infections with the yeast *Candida albicans (Candida)*. In this study (Paper III), innate immune responses in APS I patients were investigated to establish whether CMC is caused by changes in the oral microenvironment. The initial part of the study involved the investigation of the ability of saliva from APS I patients to inhibit the growth of *Candida in vitro*. The anti-fungal activity of the saliva is an important means of defense in healthy individuals to prevent colonization of microorganisms in the oral compartment. Saliva from APS I patients was found not to inhibit the growth of *Candida* in the same manner as saliva from healthy controls. When the protein expression pattern of saliva from APS I patients was analyzed and compared to that of healthy controls using 2-dimensional electrophoresis, it was found that one protein in particular was missing (Figure 8).
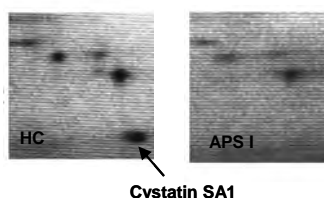
Figure 8. 2-dimensional electrophoresis of saliva
from APS I patients and healthy controls. The protein
in saliva from healthy controls was identified as
cystatin SA1.

This protein was excised from the gel containing the healthy control saliva and analyzed by matrix-associated-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and identified as cystatin SA1. Cystatin SA1 belongs to a large family of salivary proteins that function as cysteine protease inhibitors, several of which have been shown to exert anti-microbial capacity. To evaluate whether cystatin SA1 could be involved in anti-*Candida* immunity, full-length cystatin SA1 was synthesized and inhibition assays performed. Cystatin SA1 was found to inhibit the growth of *Candia* in a dose-dependent manner and at similar concentrations as commercial anti-fungal drugs. The anti-fungal capacity of cystatin SA1 was found to be dependent on the full-length protein, as the capacity was lost when using overlapping peptides of cystatin SA1 in the inhibition assay (Figure 9).
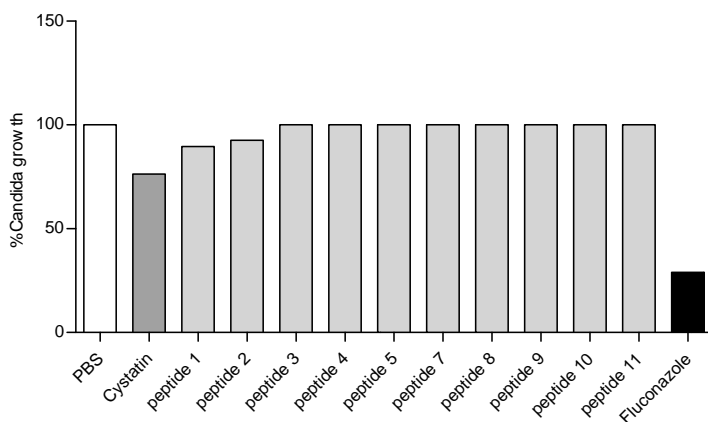
Figure 9. Anti-fungal activity of full-length cystatin SA1 and peptides, analyzed with MTT inhibition assay.

The mechanism behind the loss of cystatin SA1 in saliva from APS I patients was then investigated. As *Aire*^{-/-} mice are reported to exhibit lymphocytic infiltrates in the salivary glands, we investigated whether APS I patients showed immune recognition of salivary glands. Saliva from APS I patients showed IgA antibodies recognizing a large 250 kDa band in human submandibular gland, suggesting that APS I patients have autoimmunity against the main cystatin-producing salivary gland. The large band was identified as myosin-9, also known as non-muscle myosin heavy-chain IIa. These findings suggest that myosin-9 is an autoantigen of the oral compartment in APS I patients.

Comments

The molecular mechanisms behind CMC seen in APS I have remained elusive. The patients have an overly activated immune system in most respects, and do not show increased susceptibility to other infections. Furthermore, they appear to have normal cell-mediated responses to *Candida*-antigens *in vitro* (Brannstrom et al., 2006), and normal expression of pattern recognition receptors involved in the recognition of *Candida*-antigens (Hong et al., 2009). In this study, it was found that APS I patients showed reactivity against oral tissue, particularly salivary glands. Furthermore, they lack expression of a potent anti-fungal peptide in the saliva. I believe that the lack of cystatin SA1 could be implicated in the increased susceptibility to *Candida* infections in APS I patients. Hence, defects in the regulation of the adaptive immune system (autoimmunity) lead to impaired innate immunity (anti-fungal activity of saliva). *Candida* infections are increasing in the world, in particular due to the increased group of immunocompromised patients such as AIDS patients and cancer patients on chemotherapy. As the commercial anti-fungal drugs that are now available on the market are often associated with liver toxicity, the role of cystatin SA1 in anti-*Candida* treatment should be further evaluated.

2.2.4 Altered superantigen-mediated T cell selection and activation in AIRE-deficient mice

In order to evaluate the role of AIRE in central tolerance, a system was created in which the negative selection was mediated by superantigens. Disruption of the *Aire* gene was introduced in a TCR transgenic system where the T cells recognize an ovalbumin peptide presented by H2-IA^b molecules. In this system, negative selection is mediated by mammary tumor virus superantigens that are integrated in the mouse genome and expressed on the surface of MCH class II-bearing cells. The superantigen can bind to the Vβ5-chain of the transgenic TCR and mediate activation of the T cell.

It was found that OT-II *Aire*$^{-/-}$ mice exhibited a significant decrease in the OT-II T cells, in the thymus, blood and spleen, compared to OT-II wild type littermates (Figure 10).
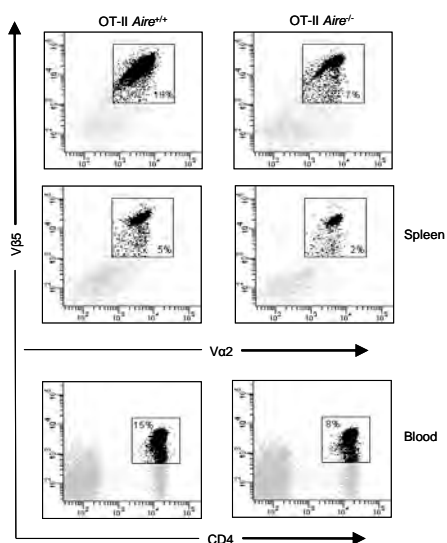
Figure 10. Decreased population of OT-II transgenic T cells in the absence of AIRE. Flow cytometric analysis of OT-II T cells in the thymus, blood and and spleen from OT-II *Aire*$^{-/-}$ and wild type littermates.

The frequency of AnnexinV-positive OT-II T cells was not increased in the thymus of OT-II *Aire*$^{-/-}$ mice, suggesting that the superantigen-mediated negative selection was not changed. However, the OT-II T cells exhibited an immature phenotype, illustrated by decreased expression of the maturation marker Qa-2. This suggests that the OT-II T cells have a developmental defect in the thymus and leave the thymus prematurely. These findings were somewhat unexpected, given the increased number of transgenic cells in the thymus of *Aire*$^{-/-}$ mice in the presence of the cognate antigen (Liston et al., 2004). The transgenic T cell will probably receive distorted signals in absence of AIRE that alter the outcome of superantigen-mediated selection and activation in a different

way from TCR-specific activation. Furthermore, an increased frequency of CD49d+ T cells and highly up-regulated expression of RAG2 were seen in the spleen of OT-II *Aire*^{-/-} mice; a sign of ongoing TCR revision (Figure 11). In addition, OT-II *Aire*^{-/-} mice exhibited an increased frequency of T cells not bearing the transgenic TCR, further indicating revision of the endogenous TCR.
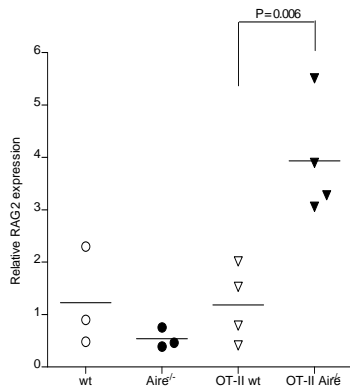

Figure 11. Increased RAG2 expression in spleen of OT-II *Aire*^{-/-} mice, measured with QT-PCR.

Comments

The belief that APS I is caused by a lack of TSA expression in the thymus has been challenged by the finding that *Aire*^{-/-} mice develop autoantibodies against unrepressed target genes in mTECs (Kuroda et al., 2005). Furthermore, in an OT-II system where ovalbumin was expressed in the thymus under control of RIP, negative selection of the OT-II T cells was severely impaired in absence of AIRE, even though the level of ovalbumin was the same as in wild type mice (Anderson et al., 2005). In contrast to these findings, the present study shows that AIRE influences superantigen-mediated T cell selection in the thymus, leading to a decreased number of OT-II T cells. (Anderson et al., 2005). It is likely that superantigen-mediated T cell activation differ from conventional TCR-specific activation with regards to signaling thresholds and the requirements of co-stimulation. Thus, the present findings of an altered development of OT-II cells could be a consequence of differential expression of co-stimulatory molecules and/or chemokines that mediate the migration of thymocytes that have been reported in *Aire*^{-/-} mice.

Little is known about the mechanism behind superantigen-mediated TCR revision in peripheral organs, but it is known to be dependent on co-stimulatory signals. As for the thymus, the present findings could be caused by a change in the splenic environment, for example by the differential expression of co-stimulatory signals mediated by AIRE-deficient DCs and/or stroma cells.

2.3 GENERAL DISCUSSION

It has been clearly shown that AIRE controls the development of thymocytes. In the absence of AIRE, negative selection is severely impaired, and the number of mature naïve T cells leaving the thymus is increased. The suggested mechanism is that AIRE functions as a transcriptional inducer of TSAs, leading to clonal deletion of autoreactive T cells. Although the function of AIRE as a TSA-inducing factor explains the increased population of autoreactive T cells escaping from the thymus in *Aire*^{-/-} mice, several other findings cannot be explained by this model.

The hypothesis that APS I is caused by reduced expression of TSAs in the thymus was first proposed by Anderson and colleagues in 2002 (Anderson et al., 2002). In elegant transfer studies in mice they showed that AIRE expression in thymic stroma cells is crucial for adequate negative selection of thymocytes, and that *Aire*^{-/-} mice had reduced levels of insulin and P450IA2 transcripts in the thymus, both of which are known autoantigens in APS I. However, the same group also performed studies using a double transgenic system where ovalbumin was expressed in the thymus under the control of the rat insulin promoter, and the T cells were specific for an ovalbumin peptide. Surprisingly, in this system, the negative selection of the ovalbumin-specific T cells was severely impaired in *Aire*^{-/-} mice, despite the fact that ovalbumin was expressed at the same level as in wild type thymus (Anderson et al., 2005). Furthermore, Kuroda and colleagues showed that *Aire*^{-/-} mice have autoreactive T cells that recognize autoantigens that are normally expressed in the thymus (Kuroda et al., 2005). Thus, there seems to be no clear correlation between the TSAs induced by AIRE in the thymus and the peripheral autoantigens that elicit organ destruction in APS I (Figure 12). In fact, introducing an *Aire* mutation into diabetes-prone NOD mice led to the development of exocrine pancreatitis, and not to reactivity against insulin and development of diabetes (Jiang et al., 2005).
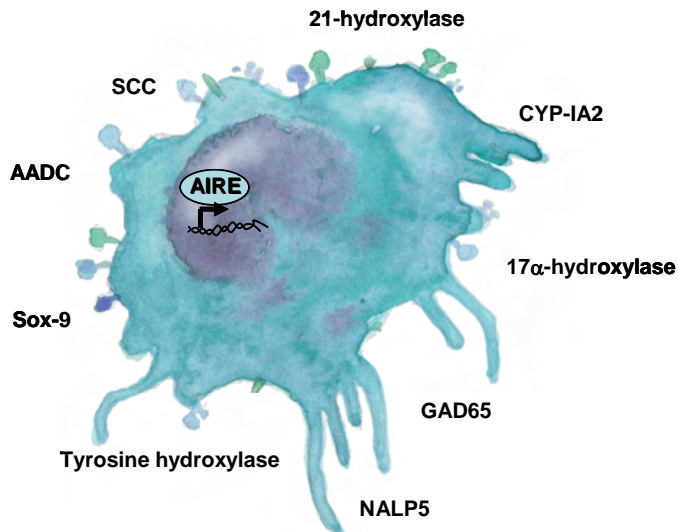

Figure 12. The ectopic expression of TSAs induced by AIRE does not correlate with the autoantigens in affected organs of APS I patients. Picture courtesy of Dr. M. Winerdal.

The above findings suggest that APS I is not mainly caused by reduced expression of TSAs in the thymus and that AIRE has other functions, in both central and peripheral tolerance. For example, AIRE deficiency leads to disruption of the thymic ultrastructure and to signs of activation and increased intracellular traffic in mTECs (Milicevic et al., 2009). Furthermore, several chemokines involved in thymocyte migration, such as CCL19 and CXCL22, are differentially regulated in the absence of AIRE. These findings are likely to influence thymocyte development and alter the outcome of negative selection. Both the reduced number of *i*NKT cells reported in Paper II and the altered development of OT-II transgenic thymocytes in Paper IV are unlikely to be a consequence of reduced TSA expression in the medulla, given that the *i*NKT cells recognize glycolipids and the OT-II T cells are specific to an ovalbumin peptide. It is tempting to speculate that these findings are a consequence of altered thymic ultrastructure and/or differential expression of co-stimulatory molecules and chemokines in the absence of AIRE.

In addition to the expression of AIRE in the medullary epithelial cells of the thymus, AIRE is expressed in peripheral dendritic cells and in a special subset of cortical lymph node stroma cells. Previous studies have shown that AIRE deficiency affects the phenotype of peripheral cells in a number of ways, in turn affecting the regulation of several immune responses. For example, AIRE-deficient peripheral DCs show differential expression of numerous genes, as demonstrated by micro-array analysis. Furthermore, AIRE-deficient DCs have an increased ability to activate naïve T cells, in part mediated by over-expression of the co-stimulatory molecule VCAM-1(Ramsey et al., 2006). Paper I describes an investigation into whether the hyper-activation status of DCs in the absence of AIRE could affect B cells in a T cell-independent manner. The motivation for this study was the observation that many characteristics of *Aire*^{-/-} mice were caused by activated B cells, i.e. autoantibodies, liver infiltrates of B cells, and MZB lymphoma (Hassler et al., 2006). Indeed, *Aire*^{-/-} mice showed increased *in vivo* responses to the T cell-independent antigen TNP-Ficoll, and increased overall activation status of, in particular, the MZBs. This is suggested to be linked to the increased circulating levels of BAFF found in both *Aire*^{-/-} mice and APS I patients. Using bone marrow transfer into T cell-deficient nude mice it was shown that the increased BAFF was independent of activated T cells, and was caused by overly activated DCs upon stimulation with IFN-γ. During the work course of this study, Ulmanen and colleagues reported a functional interaction of AIRE with PIAS1 (Ilmarinen et al., 2008). PIAS1 is a negative regulator of STAT1 signaling, which is induced upon binding of IFN-γ to its receptor. Their findings is in accordance with our observation of increased signaling downstream of the IFN-γ receptor in AIRE-deficient DCs. These two findings suggest that AIRE and PIAS1 function together by inhibiting the STAT1 pathway, leading to excessive signaling in the absence of AIRE. In accordance with this, AIRE-deficient DCs was found to express increased levels of *Gbp1* and several other STAT1-target genes when stimulated with IFN-γ. Other groups have reported increased thymic expression of STAT1-regulated chemokines CXCL10 and CCL19 in *Aire*^{-/-} mice, and increased serum levels of CXCL10 in sera from APS I patients (Anderson et al., 2005, Kisand et al., 2008). The gene expression profile correlates with that seen in *Pias1*^{-/-} mice, further supporting the role of AIRE in STAT1 signaling. Interestingly, insulin expression is also reported to be regulated by STAT1 signaling, i.e. the stimulation of mTECs with IFN-γ leads to the down-regulation of

Insulin transcripts (Levi and Polychronakos, 2009). Thus, altered STAT1 signaling might affect tolerance through several mechanisms.

In conclusion, AIRE is involved in both central and peripheral tolerance mechanisms, possibly by taking part in STAT1 signaling. Loss of the expression of AIRE leads to several differentially expressed genes in both mTECs and DCs, leading to changes in the performance of these cells. In the thymus, this results in impaired negative selection of autoreactive T cells, whereas in the secondary lymphoid organs it causes hyperactivation of B and T cells and breakdown in peripheral immunological tolerance mechanisms. Unraveling the function of AIRE in these events will provide new insight into the regulation of the immune system and improve our understanding of how autoimmune diseases arise and, hopefully, can be treated.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar har utvecklats för att skydda oss mot patogener såsom bakterier, virus, svamp och encelliga mikroorganismer. Ibland går regleringen av immunförsvaret fel och det angriper kroppens egna organ och vävnader, vilket kallas autoimmunitet. Autoimmuna sjukdomar såsom diabetes och multipel skleros blir allt vanligare och är en vanlig orsak till ohälsa hos framför allt unga människor. Idag finns ingen bot mot autoimmuna sjukdomar, endast symptomen kan behandlas.

Under sin utveckling genomgår immuncellerna en rad selektionsprocesser för att se till att de 1) är funktionella och kan angripa sjukdomsorsakande organismer samt 2) inte kan angripa kroppsegna strukturer. Under dessa processer kommer celler som är reaktiva mot självstrukturer att elimineras medan celler som är reaktiva mot patogener men icke-reaktiva mot organ och vävnader att få leva vidare och cirkulera i kroppen. Immunförsvarets förmåga att skilja mellan kroppsegna och främmande strukturer kallas tolerans och är det främsta skyddet mot autoimmunitet. I min avhandling har jag studerat en viktig faktor i toleransmekanismer, ett protein som kallas AIRE (autoimmune regulator). Mutationer i genen som kodar för AIRE-proteinet leder till en mycket allvarlig autoimmun sjukdom kallad autoimmunt polyendokrint syndrom typ 1 (APS I). APS I är en recessiv monogen sjukdom. Det innebär att om man ärver anlaget från båda sina föräldrar kommer man med största sannolikhet att utveckla sjukdomen. Patienterna blir svårt sjuka i en rad manifestationer. Vanligast är att immunförsvaret angriper och förstör hormonproducerande organ såsom bisköldkörteln, bukspottkörteln, binjurebarken samt fortplantningsorganen, vilket leder till hormonbrist och komplikationer med dödlig utgång om de inte behandlas. Patienterna drabbas också av svåra kroniska svampinfektioner i munhålan.

AIREs roll i tolerans är fortfarande inte helt utredd. För att få en ökad förståelse för hur APS I uppstår har vi modifierat möss så att de saknar AIRE-proteinet, så kallade knock-out möss. Dessa möss fungerar som en djurmodell av APS I. AIRE-proteinet uttrycks framför allt i två celltyper, dels i en specialiserad cell i tymus samt i dendritceller. Dendritcellens funktion är att äta upp invaderande patogener, migrera till en lymfkörtel och där visa upp patogena strukturer för T-celler. T-cellerna blir då aktiverad och kan ge signaler till B-celler att börja producera antikroppar, samt tar sig till infektionsplatsen där de kan angripa och döda infekterade celler. T-cellens betydelse illustreras i AIDS som leder till att T-cellerna dör och att patienterna inte kan bekämpa olika infektioner.

AIREs uppgift har visat sig vara att mediera att kroppsreaktiva T-celler dör under utvecklingen i tymus så att endast de celler som kan skilja på kroppsegna strukturer och främmande cirkulerar i kroppen. I avsaknad av AIRE verkar det som om T-cellerna inte utvecklas rätt, vilket leder till att de kan bli självreaktiva och angripa t.ex. bisköldkörteln. Den första artikeln i avhandlingen handlar om att AIRE är inblandad i aktiveringen av B-celler. I avsaknad av AIRE blir dendritcellerna överaktiverade och producerar överdrivna mängder av en faktor som i sin tur aktiverar B-celler. Denna faktor kallas BAFF och hittas i höga nivåer i blod från APS I-patienter. I den andra artikeln visas att APS I-patienterna och AIRE knock-out mössen har en reducerad population av en speciell typ av immunceller som kallas NKT-celler. NKT-celler fungerar som regulatorer av immunförsvaret och det har visat sig att de även är färre i en rad andra autoimmuna sjukdomar. Den reducerade populationen av NKT-celler skulle kunna medverka i autoimmunitetsutvecklingen i APS I-patienterna. Denna

studie inkluderar även NK-celler. NK-celler fungerar i försvaret mot intracellulära patogener och kan döda t.ex. virusinfekterade celler. NK-cellerna visade sig vara helt normala i både APS I-patienter och AIRE knock-out möss.

I den tredje artikel studeras orsakerna bakom de kroniska svampinfektionerna som APS I-patienterna drabbas av. Dessa svampinfektioner är paradoxala eftersom de vanligtvis bara drabbar immunosupprimerade patienter såsom AIDS-patienter eller cancerpatienter som behandlas med kemoterapi. Då APS I-patienterna snarare har ett överaktivt immunförsvar och inte är överkänsliga för några andra typer av infektioner är det märkligt att de inte kan bekämpa svampinfektionerna. Dessutom sprider sig sällan dessa svampinfektioner till blodet utan håller sig lokaliserade till munhålan, vilket tyder på ett lokalt problem och inte en systemisk defekt. Vi fann att saliv från APS I-patienter inte kunde hämma tillväxt av svamp på samma sätt som saliv från friska personer, vilket tyder på att APS I-patienterna sakar faktorer i saliven som kan motverka svampväxt. Proteininnehållet i saliven från APS I-patienterna analyserades och jämfördes med friska kontrollpersoner. Patienterna saknade ett speciellt protein i saliven som identifierades som cystatin SA1. Detta protein syntetiserades och visade sig ha svampdödande egenskaper. Vidare så undersöktes bakgrunden till att cystatin fattades i saliven hos APS I-patienterna. Det visade sig att patienterna hade tecken som tydde på att spottkörtlarna, som producerar cystatin, var angripna av immunförsvaret. I den sista artikeln undersöktes mekanismerna bakom T-cellsutvecklingen i avsaknad av AIRE. Detta gjordes genom att skapa möss som endast har T-celler som känner igen ett speciellt protein. I dessa möss påverkas T-cellsutvecklingen av så kallade superantigen. Våra resultat tyder på att AIRE är inblandat i T-cellsutvecklingen på andra sätt än vad som tidigare rapporterats.

Sammantaget pekar våra data på att AIRE är inblandat i tolerans på flera olika sätt. Vi hoppas att en ökad förståelse för hur AIRE fungerar kommer att ge mer kunskap, inte bara om APS I, utan även om andra autoimmuna sjukdomar. Detta är viktigt för att i framtiden kunna förhindra dessa tillstånd och hitta botemedel.

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REFERENCES

The Finnish-German APECED consortium (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet *17*, 399-403.

Aaltonen, J., Bjorses, P., Sandkuijl, L., Perheentupa, J., and Peltonen, L. (1994). An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. Nat Genet *8*, 83-87.

Ahmed, S.A., Hissong, B.D., Verthelyi, D., Donner, K., Becker, K., and Karpuzoglu-Sahin, E. (1999). Gender and risk of autoimmune diseases: possible role of estrogenic compounds. Environ Health Perspect *107 Suppl 5*, 681-686.

Ahonen, P., Myllarniemi, S., Sipila, I., and Perheentupa, J. (1990). Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med *322*, 1829-1836.

Alimohammadi, M., Bjorklund, P., Hallgren, A., Pontynen, N., Szinnai, G., Shikama, N., Keller, M.P., Ekwall, O., Kinkel, S.A., Husebye, E.S.*, et al.* (2008). Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. N Engl J Med *358*, 1018-1028.

Alvarez-Marfany, M., Roman, S.H., Drexler, A.J., Robertson, C., and Stagnaro-Green, A. (1994). Long-term prospective study of postpartum thyroid dysfunction in women with insulin dependent diabetes mellitus. J Clin Endocrinol Metab *79*, 10-16.

Anderson, M.S., Venanzi, E.S., Chen, Z., Berzins, S.P., Benoist, C., and Mathis, D. (2005). The cellular mechanism of Aire control of T cell tolerance. Immunity *23*, 227-239.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Annunziato, F., Romagnani, P., Cosmi, L., Lazzeri, E., and Romagnani, S. (2001). Chemokines and lymphopoiesis in human thymus. Trends Immunol *22*, 277-281.

Arase, H., Arase, N., Ogasawara, K., Good, R.A., and Onoe, K. (1992). An NK1.1+ CD4+8- single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor V beta family. Proc Natl Acad Sci U S A *89*, 6506-6510.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell *76*, 651-663.

Barton, G.M., and Medzhitov, R. (2003). Toll-like receptor signaling pathways. Science *300*, 1524-1525.

Batten, M., Groom, J., Cachero, T.G., Qian, F., Schneider, P., Tschopp, J., Browning, J.L., and Mackay, F. (2000). BAFF mediates survival of peripheral immature B lymphocytes. J Exp Med *192*, 1453-1466.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet *27*, 20-21.

Bensinger, S.J., Bandeira, A., Jordan, M.S., Caton, A.J., and Laufer, T.M. (2001). Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. J Exp Med *194*, 427-438.

Betterle, C., and Zanchetta, R. (2003). Update on autoimmune polyendocrine syndromes (APS). Acta Biomed *74*, 9-33.

Bevan, M.J. (1977). In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature *269*, 417-418.

Bjorses, P., Halonen, M., Palvimo, J.J., Kolmer, M., Aaltonen, J., Ellonen, P., Perheentupa, J., Ulmanen, I., and Peltonen, L. (2000). Mutations in the AIRE gene: effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy protein. Am J Hum Genet *66*, 378-392.

Bjorses, P., Pelto-Huikko, M., Kaukonen, J., Aaltonen, J., Peltonen, L., and Ulmanen, I. (1999). Localization of the APECED protein in distinct nuclear structures. Hum Mol Genet *8*, 259-266.

Blackman, M., Yague, J., Kubo, R., Gay, D., Coleclough, C., Palmer, E., Kappler, J., and Marrack, P. (1986). The T cell repertoire may be biased in favor of MHC recognition. Cell *47*, 349-357.

Blomqvist, M., Rhost, S., Teneberg, S., Lofbom, L., Osterbye, T., Brigl, M., Mansson, J.E., and Cardell, S.L. (2009). Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. Eur J Immunol *39*, 1726-1735.

Bottomley, M.J., Stier, G., Pennacchini, D., Legube, G., Simon, B., Akhtar, A., Sattler, M., and Musco, G. (2005). NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. J Biol Chem *280*, 11505-11512.

Bouneaud, C., Kourilsky, P., and Bousso, P. (2000). Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. Immunity *13*, 829-840.

Brandes, M., Willimann, K., Lang, A.B., Nam, K.H., Jin, C., Brenner, M.B., Morita, C.T., and Moser, B. (2003). Flexible migration program regulates gamma delta T-cell involvement in humoral immunity. Blood *102*, 3693-3701.

Brandes, M., Willimann, K., and Moser, B. (2005). Professional antigen-presentation function by human gammadelta T Cells. Science *309*, 264-268.

Brandle, D., Muller, C., Rulicke, T., Hengartner, H., and Pircher, H. (1992). Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. Proc Natl Acad Sci U S A *89*, 9529-9533.

Brannstrom, J., Hassler, S., Peltonen, L., Herrmann, B., and Winqvist, O. (2006). Defect internalization and tyrosine kinase activation in Aire deficient antigen presenting cells exposed to Candida albicans antigens. Clin Immunol *121*, 265-273.

Brenner, M.B., McLean, J., Dialynas, D.P., Strominger, J.L., Smith, J.A., Owen, F.L., Seidman, J.G., Ip, S., Rosen, F., and Krangel, M.S. (1986). Identification of a putative second T-cell receptor. Nature *322*, 145-149.

Brocker, T., Riedinger, M., and Karjalainen, K. (1997). Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. J Exp Med *185*, 541-550.

Caillat-Zucman, S. (2009). Molecular mechanisms of HLA association with autoimmune diseases. Tissue Antigens *73*, 1-8.

Cardell, S.L. (2006). The natural killer T lymphocyte: a player in the complex regulation of autoimmune diabetes in non-obese diabetic mice. Clin Exp Immunol *143*, 194-202.

Cariappa, A., Chase, C., Liu, H., Russell, P., and Pillai, S. (2007). Naive recirculating B cells mature simultaneously in the spleen and bone marrow. Blood *109*, 2339-2345.
Carsetti, R., Rosado, M.M., and Wardmann, H. (2004). Peripheral development of B cells in mouse and man. Immunol Rev *197*, 179-191.

Casellas, R., Shih, T.A., Kleinewietfeld, M., Rakonjac, J., Nemazee, D., Rajewsky, K., and Nussenzweig, M.C. (2001). Contribution of receptor editing to the antibody repertoire. Science *291*, 1541-1544.

Cetani, F., Barbesino, G., Borsari, S., Pardi, E., Cianferotti, L., Pinchera, A., and Marcocci, C. (2001). A novel mutation of the autoimmune regulator gene in an Italian kindred with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, acting in a dominant fashion and strongly cosegregating with hypothyroid autoimmune thyroiditis. J Clin Endocrinol Metab *86*, 4747-4752.

Chen, Z., Benoist, C., and Mathis, D. (2005). How defects in central tolerance impinge on a deficiency in regulatory T cells. Proc Natl Acad Sci U S A *102*, 14735-14740.

Chun, T., Page, M.J., Gapin, L., Matsuda, J.L., Xu, H., Nguyen, H., Kang, H.S., Stanic, A.K., Joyce, S., Koltun, W.A.*, et al.* (2003). CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. J Exp Med *197*, 907-918.

Cinamon, G., Zachariah, M.A., Lam, O.M., Foss, F.W., Jr., and Cyster, J.G. (2008). Follicular shuttling of marginal zone B cells facilitates antigen transport. Nat Immunol *9*, 54-62.

Coles, M.C., and Raulet, D.H. (2000). NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. J Immunol *164*, 2412-2418.

Crouch, E.E., Li, Z., Takizawa, M., Fichtner-Feigl, S., Gourzi, P., Montano, C., Feigenbaum, L., Wilson, P., Janz, S., Papavasiliou, F.N., and Casellas, R. (2007). Regulation of AID expression in the immune response. J Exp Med *204*, 1145-1156.

Cyster, J.G., Hartley, S.B., and Goodnow, C.C. (1994). Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. Nature *371*, 389-395.

Deitiker, P., Ashizawa, T., and Atassi, M.Z. (2000). Antigen mimicry in autoimmune disease. Can immune responses to microbial antigens that mimic acetylcholine receptor act as initial triggers of Myasthenia gravis? Hum Immunol *61*, 255-265.

Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol *2*, 1032-1039.

DeVoss, J., Hou, Y., Johannes, K., Lu, W., Liou, G.I., Rinn, J., Chang, H., Caspi, R.R., Fong, L., and Anderson, M.S. (2006). Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. J Exp Med *203*, 2727-2735.

Douroudis, K., Laine, A.P., Heinonen, M., Hermann, R., Lipponen, K., Veijola, R., Simell, O., Knip, M., Uibo, R., Ilonen, J., and Kisand, K. (2009). Association of CTLA4 but not ICOS polymorphisms with type 1 diabetes in two populations with different disease rates. Hum Immunol *70*, 536-539.

Egawa, T., Eberl, G., Taniuchi, I., Benlagha, K., Geissmann, F., Hennighausen, L., Bendelac, A., and Littman, D.R. (2005). Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity *22*, 705-716.

Ekwall, O., Hedstrand, H., Grimelius, L., Haavik, J., Perheentupa, J., Gustafsson, J., Husebye, E., Kampe, O., and Rorsman, F. (1998). Identification of tryptophan hydroxylase as an intestinal autoantigen. Lancet *352*, 279-283.

Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middleton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935-946.

Fletcher, A.L., Seach, N., Reiseger, J.J., Lowen, T.E., Hammett, M.V., Scott, H.S., and Boyd, R.L. (2009). Reduced thymic Aire expression and abnormal NF-kappa B2 signaling in a model of systemic autoimmunity. J Immunol *182*, 2690-2699.

Gallegos, A.M., and Bevan, M.J. (2004). Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med *200*, 1039-1049.

Gardner, J.M., Devoss, J.J., Friedman, R.S., Wong, D.J., Tan, Y.X., Zhou, X., Johannes, K.P., Su, M.A., Chang, H.Y., Krummel, M.F., and Anderson, M.S. (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. Science *321*, 843-847.

Gavanescu, I., Kessler, B., Ploegh, H., Benoist, C., and Mathis, D. (2007). Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity. Proc Natl Acad Sci U S A *104*, 4583-4587.

Gay, D., Saunders, T., Camper, S., and Weigert, M. (1993). Receptor editing: an approach by autoreactive B cells to escape tolerance. J Exp Med *177*, 999-1008.

Gebre-Medhin, G., Husebye, E.S., Gustafsson, J., Winqvist, O., Goksoyr, A., Rorsman, F., and Kampe, O. (1997). Cytochrome P450IA2 and aromatic L-amino acid decarboxylase are hepatic autoantigens in autoimmune polyendocrine syndrome type I. FEBS Lett *412*, 439-445.

Ghia, P., ten Boekel, E., Sanz, E., de la Hera, A., Rolink, A., and Melchers, F. (1996). Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. J Exp Med *184*, 2217-2229.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8-triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol *150*, 4244-4252.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., and et al. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676-682.

Gotter, J., Brors, B., Hergenhahn, M., and Kyewski, B. (2004). Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med *199*, 155-166.

Gray, D.H., Seach, N., Ueno, T., Milton, M.K., Liston, A., Lew, A.M., Goodnow, C.C., and Boyd, R.L. (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood *108*, 3777-3785.

Guinamard, R., Okigaki, M., Schlessinger, J., and Ravetch, J.V. (2000). Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat Immunol *1*, 31-36.

Gumperz, J.E., Roy, C., Makowska, A., Lum, D., Sugita, M., Podrebarac, T., Koezuka, Y., Porcelli, S.A., Cardell, S., Brenner, M.B., and Behar, S.M. (2000). Murine CD1d-restricted T cell recognition of cellular lipids. Immunity *12*, 211-221.

Gylling, M., Kaariainen, E., Vaisanen, R., Kerosuo, L., Solin, M.L., Halme, L., Saari, S., Halonen, M., Kampe, O., Perheentupa, J., and Miettinen, A. (2003). The hypoparathyroidism of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy protective effect of male sex. J Clin Endocrinol Metab *88*, 4602-4608.

Halonen, M., Pelto-Huikko, M., Eskelin, P., Peltonen, L., Ulmanen, I., and Kolmer, M. (2001). Subcellular location and expression pattern of autoimmune regulator (Aire), the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). J Histochem Cytochem *49*, 197-208.

Hassler, S., Ramsey, C., Karlsson, M.C., Larsson, D., Herrmann, B., Rozell, B., Backheden, M., Peltonen, L., Kampe, O., and Winqvist, O. (2006). Aire-deficient mice develop hematopoetic irregularities and marginal zone B-cell lymphoma. Blood *108*, 1941-1948.

Heino, M., Peterson, P., Kudoh, J., Nagamine, K., Lagerstedt, A., Ovod, V., Ranki, A., Rantala, I., Nieminen, M., Tuukkanen, J.*, et al.* (1999). Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla. Biochem Biophys Res Commun *257*, 821-825.

Heino, M., Peterson, P., Sillanpaa, N., Guerin, S., Wu, L., Anderson, G., Scott, H.S., Antonarakis, S.E., Kudoh, J., Shimizu, N.*, et al.* (2000). RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse. Eur J Immunol *30*, 1884-1893.

Hoffman, E.S., Passoni, L., Crompton, T., Leu, T.M., Schatz, D.G., Koff, A., Owen, M.J., and Hayday, A.C. (1996). Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev *10*, 948-962.

Hong, M., Ryan, K.R., Arkwright, P.D., Gennery, A.R., Costigan, C., Dominguez, M., Denning, D.W., McConnell, V., Cant, A.J., Abinun, M.*, et al.* (2009). Pattern recognition receptor expression is not impaired in patients with chronic mucocutanous candidiasis with or without autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. Clin Exp Immunol *156*, 40-51.

Hubert, F.X., Kinkel, S.A., Crewther, P.E., Cannon, P.Z., Webster, K.E., Link, M., Uibo, R., O'Bryan, M.K., Meager, A., Forehan, S.P.*, et al.* (2009). Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype. J Immunol *182*, 3902-3918.

Husebye, E.S., Perheentupa, J., Rautemaa, R., and Kampe, O. (2009). Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med *265*, 514-529.

Ilmarinen, T., Kangas, H., Kytomaa, T., Eskelin, P., Saharinen, J., Seeler, J.S., Tanhuanpaa, K., Chan, F.Y., Slattery, R.M., Alakurtti, K.*, et al.* (2008). Functional interaction of AIRE with PIAS1 in transcriptional regulation. Mol Immunol *45*, 1847-1862.

Jacob, C.O., Zhu, J., Armstrong, D.L., Yan, M., Han, J., Zhou, X.J., Thomas, J.A., Reiff, A., Myones, B.L., Ojwang, J.O.*, et al.* (2009). Identification of IRAK1 as a risk gene with critical role in the pathogenesis of systemic lupus erythematosus. Proc Natl Acad Sci U S A *106*, 6256-6261.

Jacobson, D.L., Gange, S.J., Rose, N.R., and Graham, N.M. (1997). Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol *84*, 223-243.

Jahng, A., Maricic, I., Aguilera, C., Cardell, S., Halder, R.C., and Kumar, V. (2004). Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. J Exp Med *199*, 947-957.

Jiang, W., Anderson, M.S., Bronson, R., Mathis, D., and Benoist, C. (2005). Modifier loci condition autoimmunity provoked by Aire deficiency. J Exp Med *202*, 805-815.
Jolicoeur, C., Hanahan, D., and Smith, K.M. (1994). T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. Proc Natl Acad Sci U S A *91*, 6707-6711.

Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Holenbeck, A.E., Lerman, M.A., Naji, A., and Caton, A.J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol *2*, 301-306.

Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 273-280.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E.*, et al.* (1997). CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science *278*, 1626-1629.

Kekalainen, E., Miettinen, A., and Arstila, T.P. (2007a). Does the deficiency of Aire in mice really resemble human APECED? Nat Rev Immunol *7*, 1.

Kekalainen, E., Tuovinen, H., Joensuu, J., Gylling, M., Franssila, R., Pontynen, N., Talvensaari, K., Perheentupa, J., Miettinen, A., and Arstila, T.P. (2007b). A defect of regulatory T cells in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Immunol *178*, 1208-1215.

Kisand, K., Link,M., Wolff AS., Meager A., Tserel A., Org, T., Murumägi, A., Uibo, R., Willcox, N., Trebusak Podkrajsek, K., Battelino T., Lobell, A., Kämpe O., Lima, K., Meloni A., Ergun-Longmire, B., Maclaren, NK, Perheentupa, J., Krohn, KJ., Scott, HS., Husebye, ES., Peterson P. (2008). Interferon autoantibodies associated with AIRE deficiency decrease the expression of IFN-stimulated genes. Blood *112*, 2657-2666

Klein, L., Klein, T., Ruther, U., and Kyewski, B. (1998). CD4 T cell tolerance to human C-reactive protein, an inducible serum protein, is mediated by medullary thymic epithelium. J Exp Med *188*, 5-16.

Kogawa, K., Nagafuchi, S., Katsuta, H., Kudoh, J., Tamiya, S., Sakai, Y., Shimizu, N., and Harada, M. (2002). Expression of AIRE gene in peripheral monocyte/dendritic cell lineage. Immunol Lett *80*, 195-198.

Kraus, M., Alimzhanov, M.B., Rajewsky, N., and Rajewsky, K. (2004). Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. Cell *117*, 787-800.

Krohn, K., Uibo, R., Aavik, E., Peterson, P., and Savilahti, K. (1992). Identification by molecular cloning of an autoantigen associated with Addison's disease as steroid 17 alpha-hydroxylase. Lancet *339*, 770-773.

Kumar, P.G., Laloraya, M., Wang, C.Y., Ruan, Q.G., Davoodi-Semiromi, A., Kao, K.J., and She, J.X. (2001). The autoimmune regulator (AIRE) is a DNA-binding protein. J Biol Chem *276*, 41357-41364.

Kuroda, N., Mitani, T., Takeda, N., Ishimaru, N., Arakaki, R., Hayashi, Y., Bando, Y., Izumi, K., Takahashi, T., Nomura, T.*, et al.* (2005). Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. J Immunol *174*, 1862-1870.

Lantz, O., and Bendelac, A. (1994). An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. J Exp Med *180*, 1097-1106.

Leadbetter, E.A., Brigl, M., Illarionov, P., Cohen, N., Luteran, M.C., Pillai, S., Besra, G.S., and Brenner, M.B. (2008). NK T cells provide lipid antigen-specific cognate help for B cells. Proc Natl Acad Sci U S A *105*, 8339-8344.

Lesley, R., Xu, Y., Kalled, S.L., Hess, D.M., Schwab, S.R., Shu, H.B., and Cyster, J.G. (2004). Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. Immunity *20*, 441-453.

Leslie, D.S., Vincent, M.S., Spada, F.M., Das, H., Sugita, M., Morita, C.T., and Brenner, M.B. (2002). CD1-mediated gamma/delta T cell maturation of dendritic cells. J Exp Med *196*, 1575-1584.

Levi, D., and Polychronakos, C. (2009). Regulation of insulin gene expression by cytokines and cell-cell interactions in mouse medullary thymic epithelial cells. Diabetologia *52*, 2151-2158.

Li, J., Li, Y., Yao, J.Y., Jin, R., Zhu, M.Z., Qian, X.P., Zhang, J., Fu, Y.X., Wu, L., Zhang, Y., and Chen, W.F. (2007). Developmental pathway of CD4+CD8- medullary thymocytes during mouse ontogeny and its defect in Aire-/- mice. Proc Natl Acad Sci U S A *104*, 18175-18180.

Liston, A., Gray, D.H., Lesage, S., Fletcher, A.L., Wilson, J., Webster, K.E., Scott, H.S., Boyd, R.L., Peltonen, L., and Goodnow, C.C. (2004). Gene dosage--limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. J Exp Med *200*, 1015-1026.

Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C.C. (2003). Aire regulates negative selection of organ-specific T cells. Nat Immunol *4*, 350-354.

Liu, B., Mink, S., Wong, K.A., Stein, N., Getman, C., Dempsey, P.W., Wu, H., and Shuai, K. (2004). PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat Immunol *5*, 891-898.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J Exp Med *190*, 75-89.

Mackay, F., Woodcock, S.A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., Tschopp, J., and Browning, J.L. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med *190*, 1697-1710.

Mackay, I.R. (2005). The etiopathogenesis of autoimmunity. Semin Liver Dis *25*, 239-250.

Maclaren, N.K., and Riley, W.J. (1986). Inherited susceptibility to autoimmune Addison's disease is linked to human leukocyte antigens-DR3 and/or DR4, except when associated with type I autoimmune polyglandular syndrome. J Clin Endocrinol Metab *62*, 455-459.

MacLennan, I.C. (1994). Somatic mutation. From the dark zone to the light. Curr Biol *4*, 70-72.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-2226.

Manderson, A.P., Botto, M., and Walport, M.J. (2004). The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol *22*, 431-456.

Martin, F., Oliver, A.M., and Kearney, J.F. (2001). Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity *14*, 617-629.

Mason, D. (1998). A very high level of crossreactivity is an essential feature of the T-cell receptor. Immunol Today *19*, 395-404.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu Rev Immunol *12*, 991-1045.

Meloni, A., Furcas, M., Cetani, F., Marcocci, C., Falorni, A., Perniola, R., Pura, M., Boe Wolff, A.S., Husebye, E.S., Lilic, D.*, et al.* (2008). Autoantibodies against type I interferons as an additional diagnostic criterion for autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab *93*, 4389-4397.

Milicevic, Z., Milicevic, N.M., Laan, M., Peterson, P., Kisand, K., Scott, H.S., and Westermann, J. (2009). Ultrastructure of medullary thymic epithelial cells of autoimmune regulator (Aire)-deficient mice. Immunol Cell Biol.

Miller, M.J., Hejazi, A.S., Wei, S.H., Cahalan, M.D., and Parker, I. (2004). T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. Proc Natl Acad Sci U S A *101*, 998-1003.

Myhre, A.G., Halonen, M., Eskelin, P., Ekwall, O., Hedstrand, H., Rorsman, F., Kampe, O., and Husebye, E.S. (2001). Autoimmune polyendocrine syndrome type 1 (APS I) in Norway. Clin Endocrinol (Oxf) *54*, 211-217.

Nagamine, K., Peterson, P., Scott, H.S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K.J., Lalioti, M.D., Mullis, P.E., Antonarakis, S.E.*, et al.* (1997). Positional cloning of the APECED gene. Nat Genet *17*, 393-398.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.
Neufeld, M., Maclaren, N., and Blizzard, R. (1980). Autoimmune polyglandular syndromes. Pediatr Ann *9*, 154-162.

Neufeld, M., Maclaren, N.K., and Blizzard, R.M. (1981). Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. Medicine (Baltimore) *60*, 355-362.

Niki, S., Oshikawa, K., Mouri, Y., Hirota, F., Matsushima, A., Yano, M., Han, H., Bando, Y., Izumi, K., Matsumoto, M., *et al.* (2006). Alteration of intra-pancreatic target-organ specificity by abrogation of Aire in NOD mice. J Clin Invest *116*, 1292-1301.

Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., and Zinkernagel, R.M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. Science *286*, 2156-2159.

Owen, J.J., and Jenkinson, E.J. (1984). Early events in T lymphocyte genesis in the fetal thymus. Am J Anat *170*, 301-310.

Perez, V.L., Van Parijs, L., Biuckians, A., Zheng, X.X., Strom, T.B., and Abbas, A.K. (1997). Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity *6*, 411-417.

Perheentupa, J. (2006). Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab *91*, 2843-2850.

Pitkanen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vahamurto, P., Ollila, J., Vihinen, M., *et al.* (2000). The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. J Biol Chem *275*, 16802-16809.

Pitt, L.A., Hubert, F.X., Scott, H.S., Godfrey, D.I., and Berzins, S.P. (2008). NKT cell development in the absence of the autoimmune regulator gene (Aire). Eur J Immunol *38*, 2689-2696.

Pontynen, N., Miettinen, A., Arstila, T.P., Kampe, O., Alimohammadi, M., Vaarala, O., Peltonen, L., and Ulmanen, I. (2006). Aire deficient mice do not develop the same profile of tissue-specific autoantibodies as APECED patients. J Autoimmun *27*, 96-104.

Porubsky, S., Speak, A.O., Luckow, B., Cerundolo, V., Platt, F.M., and Grone, H.J. (2007). Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. Proc Natl Acad Sci U S A *104*, 5977-5982.

Powell, B.R., Buist, N.R., and Stenzel, P. (1982). An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. J Pediatr *100*, 731-737.

Ramsey, C., Hassler, S., Marits, P., Kampe, O., Surh, C.D., Peltonen, L., and Winqvist, O. (2006). Increased antigen presenting cell-mediated T cell activation in mice and patients without the autoimmune regulator. Eur J Immunol *36*, 305-317.

Ramsey, C., Winqvist, O., Puhakka, L., Halonen, M., Moro, A., Kampe, O., Eskelin, P., Pelto-Huikko, M., and Peltonen, L. (2002). Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet *11*, 397-409.

Rautemaa, R., Hietanen, J., Niissalo, S., Pirinen, S., and Perheentupa, J. (2007). Oral and oesophageal squamous cell carcinoma--a complication or component of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, APS-I). Oral Oncol *43*, 607-613.

Richman, R.A., Rosenthal, I.M., Solomon, L.M., and Karachorlu, K.V. (1975). Candidiasis and multiple endocrinopathy. With oral squamous cell carcinoma complications. Arch Dermatol *111*, 625-627.

Rinderle, C., Christensen, H.M., Schweiger, S., Lehrach, H., and Yaspo, M.L. (1999). AIRE encodes a nuclear protein co-localizing with cytoskeletal filaments: altered sub-cellular distribution of mutants lacking the PHD zinc fingers. Hum Mol Genet *8*, 277-290.

Ronchi, F., and Falcone, M. (2008). Immune regulation by invariant NKT cells in autoimmunity. Front Biosci *13*, 4827-4837.

Rorsman, F., Husebye, E.S., Winqvist, O., Bjork, E., Karlsson, F.A., and Kampe, O. (1995). Aromatic-L-amino-acid decarboxylase, a pyridoxal phosphate-dependent enzyme, is a beta-cell autoantigen. Proc Natl Acad Sci U S A *92*, 8626-8629.

Rosatelli, M.C., Meloni, A., Devoto, M., Cao, A., Scott, H.S., Peterson, P., Heino, M., Krohn, K.J., Nagamine, K., Kudoh, J.*, et al.* (1998). A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. Hum Genet *103*, 428-434.

Rose, N.R., and Bona, C. (1993). Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today *14*, 426-430.

Rytinki, M.M., Kaikkonen, S., Pehkonen, P., Jaaskelainen, T., and Palvimo, J.J. (2009). PIAS proteins: pleiotropic interactors associated with SUMO. Cell Mol Life Sci *66*, 3029-3041.

Schellekens, G.A., de Jong, B.A., van den Hoogen, F.H., van de Putte, L.B., and van Venrooij, W.J. (1998). Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest *101*, 273-281.

Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J.L., Holler, N., Ambrose, C., Lawton, P., Bixler, S., Acha-Orbea, H.*, et al.* (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med *189*, 1747-1756.

Schubert, L.A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S.F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem *276*, 37672-37679.

Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. Science *263*, 1615-1618.

Shi, J.D., Wang, C.Y., Marron, M.P., Ruan, Q.G., Huang, Y.Q., Detter, J.C., and She, J.X. (1999). Chromosomal localization and complete genomic sequence of the murine autoimmune regulator gene (Aire). Autoimmunity *31*, 47-53.

Soderbergh, A., Myhre, A.G., Ekwall, O., Gebre-Medhin, G., Hedstrand, H., Landgren, E., Miettinen, A., Eskelin, P., Halonen, M., Tuomi, T.*, et al.* (2004). Prevalence and clinical associations of 10 defined autoantibodies in autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab *89*, 557-562.

Sternsdorf, T., Jensen, K., Reich, B., and Will, H. (1999). The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. J Biol Chem *274*, 12555-12566.

Surh, C.D., and Sprent, J. (1994). T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature *372*, 100-103.

Thien, M., Phan, T.G., Gardam, S., Amesbury, M., Basten, A., Mackay, F., and Brink, R. (2004). Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. Immunity *20*, 785-798.

Tung, J.W., and Herzenberg, L.A. (2007). Unraveling B-1 progenitors. Curr Opin Immunol *19*, 150-155.

Tuovinen, H., Pontynen, N., Gylling, M., Kekalainen, E., Perheentupa, J., Miettinen, A., and Arstila, T.P. (2009). gammadelta T cells develop independently of Aire. Cell Immunol *257*, 5-12.

Tzeng, T.C., Suen, J.L., and Chiang, B.L. (2006). Dendritic cells pulsed with apoptotic cells activate self-reactive T-cells of lupus mice both in vitro and in vivo. Rheumatology (Oxford) *45*, 1230-1237.

Uchida, D., Hatakeyama, S., Matsushima, A., Han, H., Ishido, S., Hotta, H., Kudoh, J., Shimizu, N., Doucas, V., Nakayama, K.I.*, et al.* (2004). AIRE functions as an E3 ubiquitin ligase. J Exp Med *199*, 167-172.

Uibo, R., Aavik, E., Peterson, P., Perheentupa, J., Aranko, S., Pelkonen, R., and Krohn, K.J. (1994). Autoantibodies to cytochrome P450 enzymes P450scc, P450c17, and P450c21 in autoimmune polyglandular disease types I and II and in isolated Addison's disease. J Clin Endocrinol Metab *78*, 323-328.

Wang, C.Y., Davoodi-Semiromi, A., Huang, W., Connor, E., Shi, J.D., and She, J.X. (1998). Characterization of mutations in patients with autoimmune polyglandular syndrome type 1 (APS1). Hum Genet *103*, 681-685.

Wang, C.Y., Shi, J.D., Davoodi-Semiromi, A., and She, J.X. (1999). Cloning of Aire, the mouse homologue of the autoimmune regulator (AIRE) gene responsible for autoimmune polyglandular syndrome type 1 (ASP1). Genomics *55*, 322-326.

Wardemann, H., and Nussenzweig, M.C. (2007). B-cell self-tolerance in humans. Adv Immunol *95*, 83-110.

Wardemann, H., Yurasov, S., Schaefer, A., Young, J.W., Meffre, E., and Nussenzweig, M.C. (2003). Predominant autoantibody production by early human B cell precursors. Science *301*, 1374-1377.

Wei, D.G., Lee, H., Park, S.H., Beaudoin, L., Teyton, L., Lehuen, A., and Bendelac, A. (2005). Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. J Exp Med *202*, 239-248.

Velloso, L.A., Winqvist, O., Gustafsson, J., Kampe, O., and Karlsson, F.A. (1994). Autoantibodies against a novel 51 kDa islet antigen and glutamate decarboxylase isoforms in autoimmune polyendocrine syndrome type I. Diabetologia *37*, 61-69.

Wen, L., Brill-Dashoff, J., Shinton, S.A., Asano, M., Hardy, R.R., and Hayakawa, K. (2005). Evidence of marginal-zone B cell-positive selection in spleen. Immunity *23*, 297-308.

Wesche, B., Jaeckel, E., Trautwein, C., Wedemeyer, H., Falorni, A., Frank, H., von zur Muhlen, A., Manns, M.P., and Brabant, G. (2001). Induction of autoantibodies to the adrenal cortex and pancreatic islet cells by interferon alpha therapy for chronic hepatitis C. Gut *48*, 378-383.

Winqvist, O., Gebre-Medhin, G., Gustafsson, J., Ritzen, E.M., Lundkvist, O., Karlsson, F.A., and Kampe, O. (1995). Identification of the main gonadal autoantigens in patients with adrenal insufficiency and associated ovarian failure. J Clin Endocrinol Metab *80*, 1717-1723.

Winqvist, O., Gustafsson, J., Rorsman, F., Karlsson, F.A., and Kampe, O. (1993). Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. J Clin Invest *92*, 2377-2385.

Winqvist, O., Karlsson, F.A., and Kampe, O. (1992). 21-Hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet *339*, 1559-1562.

Wolff, A.S., Erichsen, M.M., Meager, A., Magitta, N.F., Myhre, A.G., Bollerslev, J., Fougner, K.J., Lima, K., Knappskog, P.M., and Husebye, E.S. (2007). Autoimmune polyendocrine syndrome type 1 in Norway: phenotypic variation, autoantibodies, and novel mutations in the autoimmune regulator gene. J Clin Endocrinol Metab *92*, 595-603.

Zaidi, G., Sahu, R., Zhang, L., George, G., Bhavani, N., Shah, N., Bhatia, V., Bhansali, A., Jevalikar, G., Jayakumar, R.*, et al.* (2009). Two novel AIRE mutations in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) among Indians. Clin Genet.

Zhou, D., Mattner, J., Cantu, C., 3rd, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y.P., Yamashita, T.*, et al.* (2004). Lysosomal glycosphingolipid recognition by NKT cells. Science *306*, 1786-1789.

Zinkernagel, R.M., Callahan, G.N., Klein, J., and Dennert, G. (1978). Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. Nature *271*, 251-253.
Zlotogora, J., and Shapiro, M.S. (1992). Polyglandular autoimmune syndrome type I among Iranian Jews. J Med Genet *29*, 824-826.