Autoimmune Regulator Deficient Mice, an Animal Model of Autoimmune Polyendocrine Syndrome Type I

SIGNE HÄSSLER
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Abstract

Autoimmune diseases develop when the immune system fails to distinguish self from non-self or when the immune system is hypersensitive to endogenous or exogenous danger signals, or when a tissue erroneously sends a danger signal to the immune system. The education of the immune system to distinguish self from non-self is mainly carried out in the thymus and gives rise to central tolerance, whereas the ability to sense a danger or a healthy tissue constitutes peripheral tolerance. In these studies we have investigated the peripheral tolerance mechanisms controlled by the autoimmune regulator (Aire) gene in Aire deficient mice, an animal model of the monogenic disease autoimmune polyendocrine syndrome type I (APS I).

Aire−/− mice displayed increased numbers of myeloid-derived antigen-presenting cells (APCs) in the spleen, lymph nodes and peritoneum as well as more blood monocytes and metallophilic macrophages in the spleen. Monocytes were also increased in the blood of APS I patients. Monocyte precursors displayed an accelerated development in the bone marrow of Aire−/− mice, and Aire−/− APCs had an altered phenotype that caused an increased immune response in several different contexts. Aire−/− splenic and lymph node dendritic cells had an increased ability to activate naive T cells, partly as a result of an upregulated expression of the costimulatory molecule VCAM-1. In Aire−/− mice increased activity of the metallophilic macrophages in the splenic marginal zone seems to be responsible both for the activated phenotype of marginal zone B cells and for the frequent development of marginal zone lymphoma with aging. In a TCR transgenic model Aire deficiency caused an increased superantigen-mediated TCR revision in the spleen, perhaps as a result of the altered phenotype of APCs in the spleen. Finally, Aire was shown to influence autoimmune disease development by a macrophage-dependent mechanism in diabetes induced with multiple low dose streptozotocin injections.

These results indicate that Aire has an important function in peripheral tolerance by controlling the phenotype of myeloid-derived APCs and thereby regulating the activation of T and B lymphocytes.

Keywords: autoimmune regulator, autoimmune polyendocrine syndrome type I, peripheral tolerance, antigen presenting cells, autoimmunity, danger signal, knockout mice

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The oak was once an acorn.
If you ever despair of achieving success because of your modest beginning,
remember that even the oak, that large and strong tree, started as a small
acorn lying on the ground.

Â maman
The cover picture “Calypso”
was painted by Giovanna Oppo Hässler
List of papers included in this thesis

This thesis is based on the following papers¹, which will be referred by their Roman numerals:

I Increased antigen presenting cell-mediated T cell activation in mice and patients without the autoimmune regulator.
   **Hässler S.,** Ramsey C., Marits P., Kämpe O., Surh C.D., Peltonen L., Winqvist O.
   *Eur J Immunol. 2006 Feb;36(2):305-17*

II Aire deficient mice develop hematopoietic irregularities and marginal zone B cell lymphoma.
   **Hässler S.,** Ramsey C., Karlsson M.C.I, Larsson D., Herrmann B., Rozell B., Backheden M., Peltonen L., Kämpe O., Winqvist O.
   *Blood 2006 Sep 15;108(6):1941-8*

III Aire deficiency causes enhanced endogenous superantigen-mediated TCR revision.
   **Hässler S.,** Lundgren E., Janson P., Eberhardson M., Kämpe O., Peltonen L., Winqvist O.
   *In manuscript*

IV Aire deficiency causes increased susceptibility to streptozotocin induced diabetes via a macrophage dependent mechanism.
   **Hässler S.,** Peltonen L., Sandler S., Winqvist O.
   *In manuscript*

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² These authors contributed equally to the work
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
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<tr>
<td>APS I</td>
<td>autoimmune polyendocrine syndrome type I</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
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<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cell</td>
</tr>
<tr>
<td>tDC</td>
<td>thymic dendritic cell</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>T reg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetes</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>PAMP</td>
<td>pattern associated molecular pattern</td>
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<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
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<tr>
<td>MZB</td>
<td>marginal zone B cell</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>LFA-1</td>
<td>leukocyte function associated antigen</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>CMC</td>
<td>chronic mucocutaneous candidiasis</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>SCC</td>
<td>side chain cleavage enzyme</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>AADC</td>
<td>aromatic acid decarboxylase</td>
</tr>
<tr>
<td>IA2</td>
<td>insulinoma associated tyrosine phosphatase like protein</td>
</tr>
<tr>
<td>HSR</td>
<td>homogeneously staining region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>LT</td>
<td>lymphotoxin</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>HEL</td>
<td>hen egg lysozyme</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promoter</td>
</tr>
<tr>
<td>Ova</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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</table>
PREFACE

Autoimmune diseases such as multiple sclerosis and type I diabetes are increasing in prevalence in western countries and are causing a considerable psychosocial burden. They usually have a complex etiology with both genetic and environmental factors involved but there are a few exceptions, where the autoimmunity is caused by mutations in single genes. This thesis deals with the animal model of a monogenic autoimmune disease, namely autoimmune polyendocrine syndrome type I.

This doctoral research was carried out at the Department of Medical Sciences, Uppsala University, and at the Department of Medicine, unit of clinical allergy research, at the Karolinska Institutet. This work was financially supported by grants from the Swedish Research Council, the Lundberg Foundation, the Ronald McDonald Foundation, the Grönwall Foundation, the Magnus Bergvall Foundation, the Sven Jerring Foundation, the Swedish Medical Society, the Agnes and Mac Rudberg Foundation, the Queen Silvia Jubilee Foundation, the Swedish Diabetes Association, and the Swedish Juvenile Diabetes Foundation.

Signe Josephine Hässler, Uppsala, 13th of October 2006
INTRODUCTION

The ability of the immune system to distinguish self from non-self is designated as self-tolerance. Despite numerous scientific efforts to understand the mechanisms of this phenomenon, it is still a mystery how the immune system can manage to recognize an almost infinite number of antigens present on pathogens and yet be innocuous to the body. The potential danger inherent in the failure of these mechanisms was envisaged as early as 1902 by Paul Ehrlich, who referred to this situation as “horror autotoxicus” (1). Later many diseases that had been previously called idiopathic were found to have an autoimmune etiology.

The prevalence of autoimmune diseases such as multiple sclerosis and type 1 diabetes is increasing. They now affect 3-5% of the population and often cause disability in young people (2), especially women since in most autoimmune diseases there is a female preponderance. Today there is no cure for autoimmunity and although many advances have been made and therapies that ameliorate some autoimmune diseases are available the treatments usually do not target the causative agent and may have side effects such as increased susceptibility to infections (3). Understanding the mechanisms that underlie autoimmune diseases is of great importance in attempts to develop therapies that specifically target the autoimmune response without affecting the normal immune response against infections. This thesis will focus on the self-tolerance mechanisms controlled by the autoimmune regulator (AIRE) gene, which when mutated causes autoimmune polyendocrine syndrome type I (APS I).

Central tolerance

Somatic rearrangement of the T cell receptor (TCR) in T cells and the B cell receptor (BCR) in B cells generates $10^{15}$-$10^{16}$ different specificities to allow the recognition of any antigen, including self-antigens. In order to avoid the dangerous consequences of an autoreactive lymphocyte repertoire, after rearrangement of their receptors B cells and T cells undergo an educational process in primary lymphoid organs where they are taught to distinguish between self and non-self. The mechanisms through which lymphocytes are
educated in the thymus (T cells) and bone marrow (B cells) are together
referred to as central tolerance.

**Negative selection and promiscuous antigen expression in the thymus**

Although it has been known for quite a long time that the thymus is responsible for T cell development (4), the mechanisms underlying this process began to be understood only in the last two decades. During embryonic development the thymic primordium is formed from the third pharyngeal pouch and the third branchial cleft and starts to be colonized by hematopoietic progenitors (5). Once in the thymic cortex, progenitors receive cytokines and signals that induce their proliferation and differentiation from CD25+CD44+ through CD44+CD25+ to CD25+CD44lo double negative (DN) (CD4+CD8-) thymocytes (6). At the same time DN thymocytes emit important signals for the differentiation of the cortical epithelial network; this communication and reciprocal influence between thymocytes and thymic epithelial cells is called thymic crosstalk (7). At the DN CD25+CD44lo stage thymocytes begin to express RAG 1 and RAG 2 (recombination activating gene) and rearrange the TCR β-chain locus and express pre-TCR, an invariant surrogate α-chain. A functional β-chain rearrangement will induce constitutive signaling from the pre-TCR, stop RAG expression and further rearrangement, and induce proliferation. At the end of this stage CD4 and CD8 expression is induced together with reexpression of the RAG genes and rearrangement of the α-chain locus (5). Once a functional TCR is produced, CD4+CD8+ double positive (DP) thymocytes go through a process of positive selection, where all self-MHC (major histocompatibility complex) binding TCRs that recognize antigens presented on cortical epithelial cells (cTECs) are induced to survive, whereas thymocytes with TCRs not able to bind to self-MHC or to presented self-antigens are programmed to die (8). During positive selection even low affinity recognition of antigens is enough to induce survival signals through the TCR, and therefore both self and non-self recognizing T cells are rescued (9). During positive selection DP thymocytes are induced to downregulate either CD4 or CD8 expression depending on whether they recognize peptides presented on MHC I or MHC II respectively.

Self-tolerance is generated in the next phase of thymocyte development, which is called negative selection and is performed by specialized epithelial cells of the medulla (mTEC) and by bone marrow derived thymic dendritic cells (tDCs) (9). CD4+ and CD8+ single positive (SP) thymocytes move to
the medulla, attracted by chemokines produced by mTECs and DCs, and they spend about 14 days there making contact with the antigen presenting cells (APCs). If they recognize a self-peptide presented by an mTEC or a tDC, they are induced to different fates depending on the affinity and avidity of the binding and on the type of costimulatory signals they receive (10-14): high-affinity autoreactive T cells are induced to die by apoptosis (clonal deletion) or to become anergic, that is, unresponsive to self-antigen. Low-affinity autoreactive T cells are thought to be induced to differentiate into CD4+CD25+ regulatory T cells (T regs), which play a pivotal role in the maintenance of peripheral tolerance during immune responses. The mechanisms and the type of APCs responsible for negative selection are still controversial. mTECs are not so efficient in uptake and processing of antigens, so the bulk of blood borne and hematopoietic self-antigens are probably taken up and presented by tDCs (9). Knockout mice with an abnormally developed medullary epithelium display defective development of T regs (15, 16), suggesting that mTECs are responsible for the selection of T regs in mice, but in humans it has been shown that mTECs have only an indirect effect through the secretion of the cytokine thymic stromal lymphopoietin (TSLP) which subsequently induces tDCs to select T regs (14). A very important issue that has emerged in the last decades is the fact that mTECs have the ability to express self-antigens that are normally considered tissue restricted, such as the pancreatic β-cell hormone insulin (17). This peculiar feature of mTECs has been termed promiscuous rather than ectopic self-antigen expression, to emphasize the fact that it is a physiological and not an aberrant property of mTECs (18). By expressing a wide range of tissue specific antigens, mTECs are thought to be the main actors in the negative selection of autoreactive T cells recognizing organ specific antigens (19, 20). It is not yet clear how this phenotype with this peculiar expression pattern is achieved; it has been observed that the number of promiscuously expressed genes increases from cTEC to mTEC and that mTECs can be subdivided into an MHC IIlo and an MHC IIhi subset, among which MHC IIhi mTECs seem to display the highest promiscuous expression in both mice and humans (21, 22). Since all the thymic epithelial cells derive from a common precursor cell (23), it has been hypothesized that MHC IIhi mTECs represent a final and most mature differentiation stage, but recently it has been shown that this subset actually proliferates more and has a higher turnover than the MHC IIlo subset, contradicting this model (24). Each promiscuous antigen seems to be expressed by 2% of the mTECs and each mTEC expresses a few antigens (17). The expression of promiscuous antigens uses different transcriptional regulatory mechanisms in mTECs compared to those in the tissue where the corresponding antigen is normally expressed; for instance the transcription factor pdx-1 is necessary for preproinsulin expression in β-cells, but pdx-1 is not expressed by insulin-expressing mTEC lines (25). In spite of their poor ability to take up antigens, mTECs are efficient antigen
presenters to T cells (24), suggesting that they are capable of direct negative selection of T cells. On the other hand this implies that they mainly perform negative selection of endogenous antigens on MHC I presented to CD8⁺ T cells, but they cannot present exogenous antigens on MHC II to CD4⁺ T cells. It has recently been reported that bone marrow derived DCs are necessary for negative selection of a neo-self-antigen presented on MHC II, but the antigen can be expressed on mTECs and cross-presented by tDCs (19); such a mechanism may explain how CD4⁺ T cells may achieve self-tolerance to organ-specific antigens (9). It is not clear how this cross-presentation by tDCs of antigens expressed by mTECs is achieved; uptake of apoptotic mTECs or delivery of exosomes might be possible mechanisms (20).

The importance of promiscuous antigen expression in central tolerance has been proven by its influence on autoimmune disease in both human and mouse models. Insulin is an important autoantigen in human type 1 diabetes (26) and in the diabetes prone NOD (non-obese diabetes) mouse strain (27). A diabetes susceptibility locus has been mapped to the VNTR (variable number of tandem repeats) allele in the insulin gene promoter, where the class III allele causes higher insulin expression in mTECs and protects from diabetes (28, 29). In a similar way, NOD mice lacking insulin expression in the thymus but with normal expression in β-cells develop diabetes earlier (30).

Although negative selection in the thymus is a very effective way of eliminating autoreactive T cell clones, it is not complete and low-affinity self-reactive clones can move out to peripheral lymphoid organs (31). To avoid autoimmunity, autoreactive T cells are kept in check by a variety of mechanisms which together constitute peripheral tolerance, which is the focus of this thesis.

Peripheral tolerance

Once a mature T lymphocyte leaves the thymus, it starts to recirculate between the blood and lymph nodes or spleen. It has been proposed that the main concern of the immune system at this point is not the distinction between self and non-self, but discrimination of what is dangerous from what is harmless (32). Danger signals may be exogenous, e.g. produced by pathogens or environmental toxins, or endogenous, e.g. resulting from transformation of a cell into a cancer or from necrosis as a result of tissue damage. The danger signals from pathogens have been well characterized whereas less is known about endogenous signals. An important issue of this theory is that the immune system cannot be separated from the rest of the body. It has to interact and communicate with the tissues to work properly and each tissue gives specific instructions that are most favourable for its own function.
(33). For example in the anterior chamber of the eye, cells constitutively express Fas ligand in order to induce apoptosis of activated lymphocytes and avoid an adaptive immune response which would easily damage such a delicate function as the sight (34).

The induction of an adaptive immune response relies on the previous activation of an innate immune response by an infectious agent. In the absence of an infection, autoreactive T cells are exposed to small amounts of self-antigens that are brought to the lymph nodes by immature dendritic cells (DCs) which have low expression of costimulatory molecules (35). In the absence of costimulation, signaling through the TCR induces anergy instead of activation. The highest risk of developing an autoimmune response is during an infection, when DCs are activated by the binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors (36). Once activated, DCs upregulate the expression of costimulatory molecules such as B7.1 and B7.2 and they increase antigen presentation on MHC I and MHC II, secrete inflammatory cytokines, migrate from the infected tissue to the draining lymph node, and effectively activate T cells (37). The type of cytokines produced is influenced not only by the type of pattern recognition receptors to which the PAMPs that are present in the pathogen bind, but also by the phenotype/subset of DCs present in the infected tissue. For instance Toll like receptor 9 (TLR9) binding on plasmacytoid DCs (pDCs) induces IFNα production, whereas TLR4 binding on myeloid DCs induces production of IL-12. These cytokines are important for the differentiation of different types of T helper cells and/or cytotoxic T lymphocytes, which in turn will influence the type of immune cells that will be recruited to the tissue to combat the infection. Infections are often associated with tissue damage, and DCs will therefore take up and present both foreign antigens from the infectious agent and self-antigens released by the injury; self-antigens activate both autoreactive effector T cells and autoreactive CD4+CD25+ T regs. Evidence for suppressor/regulatory T cells became apparent in an experimental series with day 3 neonatal thymectomy, which induced autoimmunity in different target organs in a strain-dependent manner (38, 39). T regs are able to suppress effector T cells, but because of the conflicting results between in vivo and in vitro assays the mechanisms are still controversial (40). It is clear that production of immunosuppressive cytokines is necessary, particularly TGF-β, since blocking antibodies against TGF-β prevent the suppression. In some experiments the suppression is mediated by cell contact with membrane-bound TGF-β on T regs, while in some others TGF-β acts in soluble form, but it seems that its main direct action is exerted not on effector T cells but on DCs, which are switched to a tolerogenic phenotype, which in turn can induce further development of “adaptive T regs”. Adaptive T regs are not specific for self-antigens as are CD4+CD25+ T regs and their main function is to produce TGF-β and IL-10, which suppress effector T cells (40). An immune response against a pathogen therefore always induces a
counter-response by T regs, which keeps it under control and prevents it from switching the focus from the pathogen to the self. Furthermore, T cells normally have intrinsic mechanisms to dampen the immune response and to shut it down once the pathogen has been cleared. The first of these is the expression of CTLA-4 upon activation, an inhibitory molecule that binds to B7.1 and B7.2 on APCs with higher affinity than CD28 and competes for costimulatory signals but also induces inhibitory signals that prevent proliferation and cytokine production of activated T cells (41). The second is activation-induced cell death, whereby activation makes T cells sensitive to apoptosis by upregulating the death receptor Fas (42).

Thus peripheral tolerance seems to be a function of the phenotype of the APCs in terms of expression of costimulatory molecules and proinflammatory cytokines, which in turn is influenced by the presence or absence of a danger signal and which determine the outcome of the peripheral T cell response.

**B cell tolerance**

B lymphocytes develop in the bone marrow and undergo selective steps similar to those of T cells in the thymus. Early committed B cell precursors are called pro-B cells and depend on both cell adhesion and cytokine signals from the bone marrow stroma for the expression of RAG and rearrangement of the heavy chain of the immunoglobulin gene (IgH). Once they express an IgH they are called pre-B cells, they temporarily downregulate RAG, and they proliferate and start to express a surrogate light chain that binds to the IgH to generate a pre-B cell receptor (43). If the IgH is not able to bind to the pre-light chain and build a functionally signaling pre-BCR, the pre-B cell will die by apoptosis. If the pre-BCR is functional after proliferation, the pre-B cell will re-express RAG and rearrange a light chain to become an immature B cell (43). If the light chain builds a functional BCR with the IgH, the immature B cell will undergo negative selection, which means that B cells receiving a strong BCR signal, either because they recognize multivalent self-antigens that efficiently cross-link the BCR or because they have high affinity for a monovalent self-antigen, will either die by apoptosis (clonal deletion) (44) or will rearrange a new light chain (receptor editing) (45, 46). In the case of editing, negative selection will be performed again on the new BCR for as long as lambda or kappa light chain genes are available for rearrangement; once these are finished the only possible outcome for a strongly autoreactive B cell is apoptosis. If instead a B cell has a low-affinity BCR for a soluble self-antigen, the outcome of negative selection is anergy; that is, the B cell will become unresponsive to antigen stimuli (47), but it will not die immediately and it will enter the circulation and survive in the
periphery for a few days. All the other B cells, including some with very low affinity for self-antigens, will survive this selection and go out to peripheral lymphoid organs, where they will complete their differentiation.

One important difference between the thymic and bone marrow stroma is the lack of promiscuous antigen expression in the bone marrow, which therefore only allows the negative selection of blood-borne self-proteins and hematopoietic and ubiquitous antigens present in the bone marrow. B cells recognizing organ specific antigens will survive this selection and mature in the periphery, but since antigens of this type are usually sequestered from the circulation and from peripheral lymphoid organs, autoreactive B cells presumably will not encounter organ specific antigens, and even if they do, they will not get any T cell help to be activated, since promiscuous antigen expression in the thymus will eliminate autoreactive T cell clones against organ specific antigens (48). In this way B cell tolerance is partly controlled by T cells.

**Differentiation of B cell subsets in peripheral lymphoid organs**

When they leave the bone marrow, B cells are in a transitional stage and are not yet fully mature. In peripheral lymphoid organs they will undergo negative selection for self-antigens that are not accessible to bone marrow, but they are no longer able to achieve receptor editing because at this stage they have lost expression of the RAG genes, so they can only be deleted or become anergic. Once again this process does not eliminate all the autoreactive clones, too high stringency in the selection would cause a severe restriction in the B cell repertoire and its ability to recognize foreign antigens. All the B cells that survive this further selection, including anergic B cells, need to receive other survival and differentiation signals in order to fully mature. All the transitional B cells, including anergic B cells, initially enter the T cell areas of the lymph nodes and spleen, where they are retained only if they encounter self-antigens. This retention prevents them from migrating to the areas where they can receive survival signals, so that within a few days they die (49). The most important signal that has been identified is given by the binding of the membrane-bound cytokine BAFF (B cell activating factor) to the BAFF receptor on transitional B cells (50, 51). This cytokine is expressed by macrophages and DCs in the marginal zone of the spleen and by stromal cells in the follicles (52). Depending on whether a transitional B cell migrates to the marginal zone or to the follicles, it will differentiate into a marginal zone B cell or a follicular B cell, respectively. It is not known how this decision is made; it seems that the BCR specificity and expression of certain integrins (LFA-1 and α4β1) (53) and chemokine receptors (54) which direct their migration are at least partly responsible (55). Another subset of B cells, so called B-1 cells, differentiates mainly in peritoneal and pleural cavi-
ties, is less dependent on BAFF signaling and is more dependent on positive selection through BCR signaling (56). This selection is performed by bacterial antigens from the intestinal flora and by self-antigens. B-1 cells are responsible for the secretion of the natural antibodies that constitute the greater part of the IgM in our circulating repertoire. Marginal zone B cells (MZB) are also thought to require some BCR signaling from self-antigens in order to be selected and to be able to recognize carbohydrate (T independent type 2) antigens on bacterial cell walls (55); this restricted specificity is thought to be important for the early response to pneumococcal infections in the blood, since the marginal zone is the first lymphoid site that these bacteria encounter and as marginal zone macrophages and metallophilic macrophages express innate receptors that are specialized to retain the bacteria and present them to the MZB (57). The response to these bacteria does not require T cell help, but needs “costimulation” of MZB through a receptor called TACI (58), whose ligands are APRIL and BAFF. Follicular B cells require positive selection for their survival (59), but it is not clear whether this selection is due to binding of self-antigens or to tonic antigen independent signaling through the BCR. B cells of this type require T cell help in terms of CD40L binding and cytokines for their activation, and build germinal centers where they go through somatic hypermutation and several rounds of selection in order to increase the affinity of their BCR for a foreign antigen during an immune response. BAFF seems to be necessary also for the survival of B cells in germinal centers, but not for later stages such as memory B cells (60).

Because of their role in early phases of immune responses against bacteria, both B-1 and MZB cells need to be selected by carbohydrate self-antigens and therefore they have more autoreactive potential than follicular B cells (55). On the other hand, both these subsets are more easily activated, but they do not generate any memory, so this autoreactive potential is restricted. Nevertheless, in some autoimmune diseases increased expression of BAFF seems to lead to survival of more autoreactive clones and tonic stimulation of their immunoglobulin secretion, both in mouse models (61, 62) and in human rheumatoid arthritis (63), Sjögren’s syndrome (64), and systemic lupus erythematosus (SLE) (65). Chronic activation and stimulation of autoreactive B cells through antigen and BAFF seem to contribute to the relatively frequent development of lymphoma in Sjögren’s syndrome (66).

Etiology of autoimmune diseases

Evolution has provided our immune system with many important checkpoints for prevention of autoimmunity; yet self-tolerance is not perfect and
may fail. A few monogenic autoimmune disorders have been identified and have provided unique models for studies of autoimmune mechanisms (67). Examples are: autoimmune lymphoproliferative syndrome, caused by mutations in the Fas ligand or Fas, where lymphocytes show an increased survival after activation (68); IPEX (X-linked autoimmunity allergic dysregulation syndrome), which has led to the discovery that its mutated transcription factor FoxP3 is necessary for the development of CD4\(^+\)CD25\(^+\) T regs and self-tolerance (69, 70); and APS I, an animal model of which is the main focus of this thesis.

Most of the autoimmune diseases cannot be attributed to one gene, but have a complex pathogenesis in which both genetic and environmental factors play a role; for example the concordance in the development of type 1 diabetes in monozygotic twins is approximately 30-50%. Most probably a combination of genetic factors that influence several tolerance mechanisms together with a pathogen or a toxic substance that initiates an immune response is necessary in order to circumvent self-tolerance.

A well established genetic factor that influences autoimmunity is the HLA (Human Leukocyte Antigen) haplotype. Different MHC II haplotypes, in particular, are associated with autoimmune diseases. The MHC II haplotype seems to be most relevant when a particular self-antigen is attacked in the autoimmune response, and it reflects the efficiency with which an immunodominant peptide from the self-antigen can be presented on that particular MHC II haplotype (27). In some cases MHC II protects from autoimmunity by inducing efficient deletion of self-reactive T cell clones or efficient selection of T regs in the thymus, and in some others it causes susceptibility to autoimmunity by effectively inducing presentation of the immunodominant peptide in peripheral lymphoid organs (71).

No single pathogen has yet been uniquely associated with a given autoimmune disease, suggesting that many different pathogens have the ability to start autoimmunity by inducing an immune response; it has been suggested that in certain instances pathogens might even protect from autoimmunity by deviating the cytokine profile of an immune response or by moving the “attention” of the immune system to a site other than the susceptible organ (72). There are a few exceptions. For example streptococcus infections are known to cause rheumatic heart disease by molecular mimicry; that is, antibodies produced against the bacteria cross-react with heart antigens (71). Lyme disease also seems to be caused by molecular mimicry of Borrelia burgdorferi antigens with the leukocyte function associated antigen-1 (LFA-1) molecule (71).
Endocrine autoimmunity

Autoimmune endocrine disorders such as type I diabetes, Hashimoto’s thyroiditis and Addison’s disease may present as isolated endocrinopathy of a single endocrine organ, but frequently they are associated with the destruction of two or more endocrine glands and are therefore classified as autoimmune polyendocrine syndromes (APS). These syndromes have been systematically classified into four groups by Neufeld et al. (73, 74). APS I is characterized by early onset during childhood, hypoparathyroidism and Addison’s disease as the main endocrine manifestations, and chronic mucocutaneous candidiasis; in APS II Addison’s disease is associated with either hypothyroidism or type I diabetes with a female preponderance and adult onset; in APS III hypothyroidism and diabetes are associated in the absence of Addison’s disease; and APS IV is defined as association of Addison’s disease with autoimmune diseases/endocrinopathies other than those present in APS I and II. These syndromes share an autoimmune destruction of endocrine organs and production of organ specific autoantibodies against target glands (75). It is likely that at least partly they have similar pathogenetic mechanisms, but the different ages of onset, female/male ratios and patterns of inheritance suggest that they are distinct clinical entities with different primary etiologies. In APS II an association with certain HLA haplotypes (76, 77), CII TA (MHC II transcriptional activator) (78) and CTLA-4 polymorphisms (79, 80) has been established. It has been shown that in APS II CD4⁺CD25⁺ T regs are numerically normal but defective in their suppressor function (81). T regs are therefore believed to play an important role in the maintenance of tolerance to endocrine glands, as also suggested by animal models of depletion of such cells (39).

APS I is unique among the polyendocrine syndromes for its monogenic autosomal recessive mode of inheritance, which has been linked to mutations in the AIRE gene (82, 83). This makes it a relatively simple model of endocrine autoimmune disease, and complete understanding of the pathogenetic mechanisms of APS I could lead to important insights into endocrine autoimmunity.

Autoimmune polyendocrine syndrome type I

Albeit a very rare disease APS I is prevalent in some isolated populations such as the Finnish (1:25,000) (84), Iranian Jewish (1:9,000) (85), and the Sardinian populations (1:14,000) (86). APS I has also been termed APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) to indicate the presence of non-endocrine components in the disease, such as chronic mucocutaneous candidiasis (CMC) (Figure 1), nail dystrophy and enamel hypoplasia (87).
The most common disorders are hypoparathyroidism and Addison’s disease, which together with CMC constitute the diagnostic triad: the presence of at least two of these disorders is necessary for the diagnosis, but one of them is enough if a sibling has the disease. Several other endocrine disorders may be present, but with a lower frequency, including hypothyroidism, type I diabetes, hypergonadotrophic hypogonadism and hypophysitis (88, 89) (Table 1). Autoimmune destruction of non-endocrine cells has also been described and may cause autoimmune hepatitis, keratoconjunctivitis, vitiligo, alopecia, pernicious anemia, and asplenia (88, 89) (Table 1).

Table 1. Clinical manifestations of APS I

<table>
<thead>
<tr>
<th>Disease manifestations</th>
<th>Betterle 1998</th>
<th>Perheentupa 2006</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic triad (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypoparathyroidism</td>
<td>93</td>
<td>85</td>
</tr>
<tr>
<td>CMC</td>
<td>83</td>
<td>98</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>73</td>
<td>78</td>
</tr>
<tr>
<td>Minor manifestations (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypogonadism</td>
<td>43</td>
<td>60 (F), 12 (M)</td>
</tr>
<tr>
<td>alopecia</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>chronic hepatitis</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>pernicious anemia</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>vitiligo</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>autoimmune thyroid disease</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>keratoconjunctivitis</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>type I diabetes</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>chronic atrophic gastritis</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>malabsorption</td>
<td>15</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table adapted from Betterle et al. 1998 (88) and Perheentupa 2006 (89). Data from Perheentupa 2006 were taken from patients at 30 years of age.
Malabsorption is common and may have different causes, such as exocrine pancreatic insufficiency (89) or autoimmune destruction of serotonin-producing enterochromaffin cells in the small intestine (90).

There is no major sex difference in the severity of APS I, except for a partial protection of male patients from hypoparathyroidism (91) and an increased prevalence of hypogonadism in females (89). The number and type of disease manifestations vary considerably between different patients, even among siblings (92, 93). New disease components may appear throughout life, and diagnosed patients therefore require continuous monitoring, especially for the life-threatening Addison’s disease and chronic active hepatitis, as well as prophylaxis against asplenia by pneumococcal vaccination and against CMC-induced oral carcinoma by anti-fungal therapy (89, 94). The current treatment is hormonal replacement therapy for the endocrine deficiencies (89, 94); immunosuppressive therapy is normally used only for chronic active hepatitis, but there are case reports describing its successful use for other disease complications, such as exocrine pancreatic insufficiency and keratoconjunctivitis (95), male infertility (96), and transplant rejection (97, 98).

Mutations in the \textit{AIRE} gene cause APS I with 100% penetrance, but they do not predict the phenotype of the disease (99, 100). Environmental factors and background genes probably contribute to the outcome of the disease. Although HLAs are not directly associated with APS I, associations with certain HLA haplotypes have been described for some disease components (101, 102).

Heterozygotes normally do not develop APS I, and the few cases where \textit{AIRE} mutations have been found on only one chromosome are thought to be caused by unidentified mutations in regulatory regions (103). One exception is the G228W mutation, which causes dominant transmission of the disease in heterozygotes and cosegregates with hypothyroidism (104). A molecular analysis of this mutation has shown that the mutated protein binds and multimerizes with the wild type protein and thereby disrupts its transactivating capacity (105).

**Autoantibodies**

A hallmark of APS I is the development of organ specific autoantibodies, which precede the clinical onset of disease (94, 106, 107) and are often maintained for several years after the organ destruction. Many of the antigens recognized by the autoantibodies have been identified (Table 2) (90, 101, 108-120).
Table 2. Autoantigens in APS I

<table>
<thead>
<tr>
<th>Tissue/cell-type</th>
<th>Disease component</th>
<th>Autoantigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>adrenal cortex</td>
<td>adrenal insufficiency</td>
<td>P450cSCC, P450c17, P450c21 (107-110)</td>
</tr>
<tr>
<td>testis/ovary</td>
<td>hypogonadism</td>
<td>P450cSCC, P450c17 (111-112)</td>
</tr>
<tr>
<td>pancreatic β-cells</td>
<td>type I diabetes</td>
<td>GAD65, AADC, IA2, insulin (113, 114, 101)</td>
</tr>
<tr>
<td>thyroid gland</td>
<td>hypothyroidism</td>
<td>thyroid peroxidase, thyroglobulin (115)</td>
</tr>
<tr>
<td>liver</td>
<td>chronic autoimmune hepatitis</td>
<td>P450 1A2, P450 2A6, AADC (116)</td>
</tr>
<tr>
<td>enterochromaffin cells</td>
<td>intestinal dysfunction</td>
<td>tryptophane hydroxylase (90)</td>
</tr>
<tr>
<td>parietal cells</td>
<td>chronic atrophic gastritis</td>
<td>H+/K+ ATPase (117)</td>
</tr>
<tr>
<td>gastric enterochromaffin like cells</td>
<td>intestinal dysfunction</td>
<td>histidine decarboxylase (118)</td>
</tr>
<tr>
<td>hair follicle</td>
<td>alopecia</td>
<td>tyrosine hydroxylase (119)</td>
</tr>
<tr>
<td>melanocyte</td>
<td>vitiligo</td>
<td>SOX9, SOX10 (120)</td>
</tr>
</tbody>
</table>

Typically they are key enzymes in the synthesis of hormones or neurotransmitters in the target glands, e.g. CYP450 21 in the adrenal cortex, CYP450 SCC in the adrenal cortex and gonads, and tryptophan hydroxylase in the enterochromaffin cells of the small intestine. Interestingly many autoantigens identified in APS I are also autoantigens in the corresponding isolated idiopathic/autoimmune disorders, e.g. CYP450 21 in Addison’s disease, thyroid peroxidase in autoimmune thyroiditis, and GAD 65 in type 1 diabetes. Some autoantigens have been identified by screening cDNA libraries of a target organ, but they have since proved to correlate more with disorders of another organ: for instance GAD65 is expressed in islet β-cells, but GAD65 autoantibodies in APS I correlate mainly with intestinal dysfunction and only marginally with diabetes (101, 121, 122). Other β-cell autoantigens that have been described in APS I are AADC, IA2, and insulin; only IA2 and insulin antibodies predict diabetes with high specificity but with low sensitivity, suggesting that another, unknown β-cell autoantigen remains to be discovered (101, 122). The parathyroid autoantigen is also still elusive; one group has identified the calcium sensing receptor (123), but others have not been able to reproduce this result (91, 122).

The autoantibodies often recognize the most conserved part of the enzymes among different species, i.e., the catalytic site (124-126); some of the autoantibodies have also been shown to inhibit the target enzymes in in vitro cell free assays (110, 127, 128) but not in vivo (129). It is therefore likely that these antibodies do not play a pathogenic role in APS I and that they develop after destruction of the organs has started and cause release of intracellular enzymes. The adrenal autoantibodies are mainly of IgG1 subclass, suggesting that the organ destruction is probably mediated by a Th1 response (130). An increased proportion of activated T cells has been observed in the
blood of APS I patients compared to healthy controls (131). Besides the presence of organ specific antibodies, the immunological mechanism of this disease is unknown. In particular it is not clear why the patients develop CMC in spite of a very efficient adaptive immune response against endocrine glands and a normal response against other pathogens. Previously it was assumed that CMC in APS I was caused by a T cell defect, although an early study showed normal in vitro proliferation of T cells against *Candida albicans* antigens (132), a finding which we have recently confirmed (133). Our data also suggest a specific defect in the internalization and activation against *C. albicans* in APCs from APS I patients as a possible cause of CMC.

Very recently anti-IFNα antibodies have been identified as early markers of APS I; they precede the clinical symptoms, including CMC, and are present in 100% of APS I patients and might therefore be of high diagnostic value (134). The investigators had previously studied these antibodies in patients with thymoma and myasthenia gravis (135), autoimmune disorders which in analogy with APS I may present CMC. The correlation of anti-interferon antibodies with CMC suggests that they might be involved in the etiology of this disorder, perhaps by neutralizing the local action of interferons in the skin and mucosae and thereby inhibiting a local immune response. If this proves to be the case it will imply that interferons are essential for the mucosal immune response against *C. albicans* and that CMC in APS I is not caused by an immune deficiency but may be considered as another autoimmune manifestation.

**Molecular biology of AIRE**

The autoimmune regulator was identified by positional cloning in 1997 (82, 83) and later the mouse orthologue was cloned (136, 137). The presence of several highly conserved domains has suggested a role for AIRE as a transcriptional regulator. AIRE encodes for a 545 amino acid protein and contains an HSR (homogeneously staining region) domain, three LXXLL nuclear receptor binding motives, a functional bipartite nuclear localization signal (NLS, a proline rich region, a SAND domain, and two PHD fingers (82, 83) (Figure 2).
The mouse orthologue displays 71% homology with the human protein sequence and all the domains are conserved (136, 137). To date more than 50 different mutations have been described in APS I patients and all of them are localized within one of the conserved domains, causing either a truncated polypeptide, an amino acid change, a deletion, or an insertion. The most common mutations are the R257X nonsense mutation in exon 6, and a 13bp deletion at nucleotides 1085-1097 in exon 8 that causes a frameshift (82, 83); the first is particularly frequent in the Finnish population and the second in the British one (138), where they are in linkage disequilibrium with a common haplotype, suggesting that these mutations arose in a common ancestor in these populations. The same mutations are also present in other populations but in linkage disequilibrium with several different haplotypes, indicating that they arose independently in different individuals and that these are mutation hot spots (103, 139). No genotype-phenotype correlation has been observed except for the Y85C Iranian Jewish missense mutation, which causes an amino acid change in the HSR domain, retains the normal nuclear localization and in vitro transactivating activity of the protein, and is associated with the milder phenotype seen in the Iranian Jewish population characterized by a very low frequency of CMC and of the ectodermal dystrophies (85, 103). It has been observed that this mutation causes the AIRE protein to have a shorter half-life (140), which might explain the milder phenotype as due to a partial retention of the function but lower availability of the protein. Interestingly two other mutations in the HSR domain have been correlated to the lack of CMC: W78R in a Czech patient (141) and K83E in Finnish patients (83). It has been shown that the HSR domain mediates dimerization and tetramerization of AIRE (142, 143), while the PHD fingers are necessary for transcriptional activation in vitro (103, 142, 143); although they are
thought to mainly mediate interaction with other proteins, there is evidence that they can bind to ATTAGTTA sequences (144). In the transcriptional coactivator CBP (CREB binding protein) a PHD finger has been shown to be essential for histone acetylation and also for acetylation of the basal transcription factor TFIIIE34 and for CBP autoacetylation (145), all of which are necessary for its transcriptional transactivating activity; the PHD fingers in the AIRE protein might therefore have a similar function, although this has not been tested yet. The SAND domain is a DNA-binding motif (146, 147) and gel mobility assays have suggested that it may bind to TTATTA sequences (144). It has also been found that the PHD1 but not the PHD2 domain of AIRE functions as an E3 ubiquitin ligase in cell free assays in vitro (148), but this finding has been challenged in another report (149). AIRE binds to the transcriptional coactivator CBP (142) and synergizes with it for transcriptional activation (150).

At the subcellular level AIRE has been localized to nuclear dots distinct from PML bodies (151, 152) (Figure 3); some of the APS I mutations and deletions of the PHD domains have been shown to disrupt this localization (140).

Figure 3. AIRE nuclear dots visualized with immunofluorescence staining. This picture is reproduced from Ramsey et. al 2002 (140) with the kind permission from the Oxford University Press.

In some studies of AIRE transfected mammalian cells a cytoplasmic fibrillary pattern colocalizing with vimentin and α-tubulin has also been described (143, 151, 152), but since this has never been observed on the “physiologically” AIRE expressing mTECs and monocyte derived cells (153, 154) it is probably an in vitro artefact of AIRE overexpression in cell lines. AIRE contains a monopartite nuclear localization signal (NLS) that interacts with importin-α to induce transport to the nucleus through nuclear pores (105).

Although in vitro reporter assays have shown that AIRE is able to activate transcription when tethered to DNA (103, 142), and in one case also without such tethering (150), no chromatin immunoprecipitation has been performed to demonstrate a physiological binding of the AIRE protein to the sequences to which it has been proposed to bind in vitro (144, 146, 147). It is therefore
not yet clear whether such a transcriptional activation normally happens through direct binding to DNA or through indirect interactions with other elements of the transcriptional complex. Furthermore, it has been shown that AIRE may also have a transrepressing activity by sequestering the coactivator CBP from transcriptional complexes on the NF-kB promoter (155, 156), an effect very similar to that previously described for the transrepressing activity of the glucocorticoid receptor (157). In addition to CBP, AIRE is also able to sequester other unidentified proteins of the transcription complex (156).

In cell fractionation experiments AIRE has been colocalized with the nuclear matrix fraction but not with chromatin and ribonucleoprotein fractions (156, 158), suggesting that the AIRE nuclear bodies are not sites of active transcription but rather constitute a scaffold that organizes the chromatin into clusters of transcriptionally active genes (22, 156, 159). The function of AIRE may therefore be distinct when localized in the nuclear bodies and when free in the nucleoplasm, where a direct role in transcriptional regulation is more likely. The nucleoplasmic fraction is far less abundant than the fraction in the nuclear bodies (158).

The sites of expression of AIRE are still controversial, but it is well established that this gene is expressed in mTECs, monocytes and monocyte derived DCs and macrophages (153, 154, 160, 161). Aire expression is highest in the MHCIIhiCD80hiUEA-1+ subset that is characterized by promiscuous expression of self-antigens (17, 22, 24). Some investigators claim that Aire is expressed in all the target organs of APS I (162, 163). Since one of the methods used in those studies is RT-PCR on whole organ RNA extracts, it is likely that the low level of Aire expression in several organs is due to the presence of rare Aire-expressing tissue macrophages and/or DCs.

Factors that regulate Aire expression

Aire expression in the thymus starts at day 14 of embryonic development in the mouse and is induced by thymic cross-talk with early developmental stages of DN thymocytes (160). In the adult thymus Aire expression in mTECs is induced by negatively selected apoptotic thymocytes (160). Similarities in the phenotype of several different knockout strains with that of Aire deficient mice have led to the hypothesis that the missing genes were somewhere upstream in the signaling pathways that induce Aire expression in mTECs. The missing genes include RelB, LTα, (lymphotoxin) NIK (NF-kB inducing kinase), TRAF6 (tumor necrosis factor receptor-associated factor 6), and NF-kB2 (15, 16, 154, 160, 164, 165); none of these results have yet been confirmed by a thorough study of the signal transduction pathways leading to Aire expression, and furthermore, in many of these knockout mice the lack of Aire expression might be due to a lack of development of Aire-
expressing UEA-1+ mTECs (166), which is not a feature of Aire deficient mice, in which the numbers of these cells are increased (167). Moreover, TRAF6−/− mice and NIK deficient mice display decreased numbers of CD4+CD25+ regulatory T cells in the thymus, whereas T reg development is normal in Aire−/− mice (168-170), suggesting that in addition to the expression of self-antigens in mTECs a certain number of mTECs also is necessary for a proper selection of T regs. For the LTβ receptor an effect on Aire expression has recently been confirmed in murine insulin-expressing thymic medullary epithelial cell lines (25). On the other hand, another group has observed transcriptional effects of LTβ receptor agonistic monoclonal antibody injection in mTEC subsets that do not express Aire, suggesting that LTα may modulate Aire expression levels but is not in itself sufficient to induce Aire expression (171).

In monocytes and in myelomonocytic leukemia cells GM-CSF (granulocyte-monocyte colony stimulating factor) has been shown to induce Aire expression (172, 173) through the MAPK (mitogen-activated protein kinase) signal transduction pathway (173); Aire expression increases during in vitro DC differentiation from monocytes (172). The Aire promoter contains functional binding sites for the transcription factors AP-1, NF-Y and Sp1; both methylation of CpG islands in the Aire promoter and histone acetylation seem to be involved in regulating Aire expression (174). Recently Ets family transcription factors have been shown to bind to the Aire promoter and induce transcription in reporter assays; ets-1 and ESE-1 in particular, which are expressed in the thymic medulla, are good candidates for inducing Aire expression in mTEC and tDCs (175). In human DCs TSLP has been identified as a factor inducing strong upregulation of AIRE (176). TSLP is a cytokine produced by different types of epithelial cells that is able to induce maturation of DCs to produce IL-10 and IL-6 but not IL-12, and thereby enables them to promote Th2 differentiation; this is thought to be particularly important in the gut to avoid a potentially dangerous immune response against the normal bacterial flora (177). The induction of AIRE expression by TSLP might therefore be involved in the development of such an antiinflammatory phenotype in DCs.

**Aire deficient mice**

Three different lines of Aire deficient mice have been generated so far (167, 168, 178). In the first report (178) exon 6 was targeted to mimic the most common Finnish APS I mutation. Surprisingly these mice had a very mild phenotype, with no destruction of any of the APS I target organs. However, they displayed lymphocytic infiltrates and autoantibodies against multiple organs and a very low fertility, which is the only clinical feature in common
with APS I. Apparently Aire deficient mice have only a partial loss of tolerance, and lack either an environmental trigger or other autoimmunity susceptibility genes that interact with Aire to cause disease (168, 179). The target organs appear to be different from those of APS I. The liver is the most common target in Aire deficient mice (50%), but is targeted in only 16% of APS I patients (122, 178). On the other hand the adrenal cortex, which is attacked in 80% of APS I patients (122), has been found to be infiltrated in only 18% of Aire deficient mice (unpublished observation). In an investigation of the immune cells of Aire deficient mice, it was found that the numbers of B cells and of CD4+ and CD8+ T cells, and their basal activation, did not differ from those in Aire+/- mice. In the thymus a normal distribution of DN, DP, CD4 SP and CD8 SP subsets was found. The Vβ repertoire of the T cells was similar to that in Aire+/- mice in the thymus, but it was found to be different and skewed in some Vβ families when T cells from Aire-/- spleens were investigated. The most interesting finding was a hyperproliferation of Aire-/- lymphocytes to hen egg lysozyme (HEL) after immunization and an in vitro recall response (178).

In a second report on Aire deficient mice, attention was focused on the Aire expression in mTECs and its role in central tolerance (167). A phenotype similar to that of the first Aire deficient line was described, with an increasing number of autoimmune targeted organs with aging. Bone marrow chimera experiments suggested a role for Aire-/- irradiation resistant cells in the development of lymphocytic infiltrates in Aire-/- mice. Microarray analysis of differential gene expression on Aire-/- and Aire+/- mTEC showed a decreased expression of several, but not all organ specific antigens in Aire-/- mTECs. It was therefore proposed that Aire regulates promiscuous expression of organ specific antigens in mTECs and that when Aire is lost, promiscuous antigens cannot be expressed at proper levels, whereby autoreactive T cells escape negative selection (Figure 4).
One particularly interesting antigen found to be downregulated in Aire<sup>-/-</sup> mTECs was insulin, which has been reported to be an autoantigen both in the diabetes prone NOD mouse strain and in human type 1 diabetes (26, 27). Diabetes is also present in 13-20% of APS I patients and is often associated with production of anti-insulin antibodies (89, 101). In this study, however, none of the adrenocortical autoantigens were found to be differentially regulated by Aire, although Addison’s disease is one of the most common APS I manifestations. Furthermore, this and other studies showed that several other promiscuously expressed self-antigens were not regulated by Aire in mTECs (22, 159, 167). This raises the question whether the proposed mechanism might be relevant for some of the minor disease manifestations of APS I but not for the diagnostic triad; and in particular it is difficult to couple the mechanism to CMC.

Elegant studies on Aire deficient mice crossed with TCR transgenic mice recognizing a neo-self-antigen under the control of the rat insulin promoter (RIP) or the thyroglobulin promoter have clearly shown a defect in the negative selection of autoreactive T cells in the absence of Aire (169, 180, 181), and that this defect is carried by radioresistant cells (180). Interestingly the neo-self-antigen was found to be differentially regulated in Aire<sup>-/-</sup> mTECs in the same way as insulin in the RIP-HEL transgene (181), but not in the RIP-Ova transgene (169), suggesting that additional mechanisms to promiscuous
self-antigen expression might also be involved; in fact an intermediate level of negative selection was observed in Aire heterozygote mice of the RIP-HEL TCR transgene compared to Aire$^{+/+}$ and Aire$^{-/-}$ mice, but Aire heterozygote mice displayed normal negative selection in the RIP-Ova TCR transgene, which might be related to the different negative selection mechanisms affected in these two models. The fact that the RIP promoter was regulated by Aire in only one of these transgenes indicates that Aire does not regulate insulin expression by direct binding to its promoter and that the chromosomal site of integration of RIP may be relevant for this regulation.

In addition to causing clonal deletion of autoreactive T cells, mTECs and thymic DCs have also been reported to cause positive selection of CD4$^+$CD25$^+$ T regs. In spite of this, Aire deficient mice display normal numbers and a normal function of T regs in the thymus, spleen, and lymph nodes (168-170), suggesting that Aire does not affect thymic selection of T regs.

Recently autoreactivity against two antigens normally expressed in Aire$^{-/-}$ mTECs has been identified, namely against $\alpha$-fodrin in salivary glands (168) and against pancreas-specific protein disulfide isomerase in the exocrine pancreas (170), providing further evidence that promiscuous self-antigen expression is only one of the self-tolerance mechanisms regulated by Aire. When crossed with diabetes prone NOD mice, surprisingly Aire deficient mice do not develop diabetes but develop exocrine pancreas insufficiency (170, 179), in spite of the very low insulin expression in Aire$^{-/-}$ mTECs; furthermore, Aire deficient mice do not develop anti-insulin antibodies nor antibodies against any of the described APS I autoantigens (182, 183). Although a correlation of insulin expression with AIRE expression levels has been confirmed in human thymi (184, 185), AIRE polymorphisms do not modify diabetes susceptibility in Finnish type 1 diabetes patients carrying the VNTR insulin allele that notoriously decreases insulin expression levels in the thymus (186). These data question the relevance of Aire mediated regulation of promiscuous expression in the thymus in the disease phenotype of APS I and of Aire deficient mice. It has been suggested that other relevant genes for negative selection are differentially regulated in Aire deficient mTECs, such as H2-M, H2-O, cathepsins and chemokines (159, 169), and Aire$^{-/-}$ mTECs have also been shown to display a less efficient in vitro antigen presentation to T cells (169). Kuroda et al. transplanted MHC-incompatible thymic stroma from either Aire sufficient or Aire deficient mice into nude mice and observed an autoimmune phenotype after reconstitution only in recipients of Aire deficient thymi. Interestingly, in this model the only effector T cells that are able to respond to the peripheral antigen-presenting cells of the host are those that have been selected by the host’s bone marrow-derived cells in the thymus, and not those selected by the transplanted thymic stroma, which carries a wrong MHC. This finding suggests that Aire expression in mTECs can affect negative selection “in trans”
on bone marrow derived tDCs, perhaps by regulating chemokine expression and therefore the migration of thymocytes in the thymic medulla (7, 187).

The function of Aire in negative selection and central tolerance is now well-established, although its underlying mechanisms are still controversial. In particular it is still an open question through which mechanisms AIRE mutations cause the main disease manifestations of APS I. The expression of Aire in monocytes and in peripheral DCs together with the hyperproliferation of Aire deficient lymphocytes after immunization with an exogenous antigen suggests that Aire might also have an important function in peripheral tolerance, which is the main subject of this thesis (Figure 5).

**Figure 5.** The main target organs for autoimmunity in APS I are the parathyroid and adrenal glands. Previous studies of APS I patients have identified the target autoantigens produced by autoreactive B cells. The role of the deficient gene in APS I has been studied in central tolerance where defective negative selection of thymocytes leads to the escape of autoreactive T cells. Since Aire in addition is expressed in APCs of the periphery the main focus of our studies is on the regulatory mechanisms controlling peripheral B and T cells.
PRESENT STUDY

Aim

The general aim of this research project was to study the self-tolerance mechanisms regulated by Aire, using Aire deficient mice, with particular focus on peripheral tolerance.
Materials and methods

The following is a summary of the materials and methods used in these studies. A more detailed description is given in the individual papers.

Animals (Papers I-IV)

The generation of Aire (B6.129S4-Aire^tm1Pltn) deficient mice has been described previously (178). The mice used in studies I, III, and IV (papers I, III and IV) were back-crossed to C57BL/6 seven times and subsequently subjected to the Speed Congenic procedure for three additional rounds of back-crossing, selecting those males that had the least Sv129 genetic variation. The mice used in study II (paper II) were back-crossed to C57BL/6 for six generations and only wild type littermates from breeding of heterozygote parents were used as controls for Aire deficient mice. In studies I and III congenic C57BL/6 Aire deficient mice were crossed with OT-II TCR transgenic C57BL/6 mice (used with the kind permission of Dr. F. Carbone) and male littermates from heterozygote parents and OT-II fathers were used for the experiments. For some experiments in study IV congenic C57BL/6 Aire deficient mice were crossed with C57BL/KsJ mice and the heterozygote F1 generation was back-crossed to generate F2 littermates for the experiments; for some purposes littermates of the F6 generation of back-crossings to C57BL/KsJ were used.

The animals were 7-29 weeks of age at the time of the experiments, except in study II, where they were 15-24 months old. They were bred either at the Animal Department of the Rudbeck Laboratory, Uppsala (paper I-III) or at the Biomedical Centre, Uppsala (paper IV). They had free access to tap water and pelleted food throughout the experiments. The use of the animals was in accordance with international guidelines (NIH publications 85-23) and was approved by the local animal ethics committee.

Diabetes induction with multiple low dose streptozotocin injections (Paper IV)

Diabetes was induced by intraperitoneal injections of streptozotocin (STZ; Sigma Chemicals, 40 mg/kg body weight; dissolved in NaCl) on five consecutive days. Blood glucose was measured weekly with a glucometer (Abbot Medisence). Mice were considered diabetic when the blood glucose was equal to or higher than 12 mmol/L. The animals were sacrificed either on day 14 or on day 21 of the study.
Proliferation assay (Paper I)

CD11b+, CD11c+ and B220+ APCs were purified from collagenase-treated spleens and lymph nodes by positive selection using anti-CD11b conjugated beads (Miltenyi Biotec), FITC-conjugated anti-CD11c, or anti-B220 antibody (BD Pharmingen), followed by a magnetically labeled anti-FITC antibody (Miltenyi Biotec), and subsequent MACS column separation (Miltenyi Biotec). Responder OT-II transgenic CD4+ cells were purified from lymph nodes and spleens using a negative selection kit (Miltenyi Biotec). CD11c+ or CD11b+ cells were co-cultured with CD4+ OT-II responder cells and increasing amounts of Ova or Ova peptide in proliferation medium containing RPMI 1640 (Invitrogen), 10% fetal calf serum (FCS, Hyclone), 5% NCTC-109 (Invitrogen), 1% penicillin-streptomycin (Sigma), 1% glutamine (Sigma), and 5x10^{-5} M β-mercaptoethanol (Sigma). Cells were pulsed with 1 μCi [3H]-thymidine (Amersham) 16 h prior to harvesting followed by scintillation counting.

Antigen presentation to T cell hybridomas (Paper I)

Spleen cells from Aire^{+/+} or Aire^{-/-} mice were incubated at 37°C for 90 min. Non adherent cells were removed by three washes with RPMI medium. Adherent cells were pulsed with ovalbumin for 1h at 37°C followed by three washes with medium. The cells were incubated for 36 h with T cell hybridomas recognizing different trypsin fragments of Ova (gift from Dr. L. Karlsson). Blocking experiments were performed with the addition of 0.1 μg/well anti-VCAM-1 (vascular cell adhesion molecule-1) antibody or isotype control (BD Pharmingen). Supernatants were collected and analyzed for IL-2 secretion by ELISA (BD Pharmingen).

Microarray analysis (Paper I)

Dendritic cells were purified from Aire^{+/+} or Aire^{-/-} spleens with anti-CD11c microbeads (Miltenyi Biotec) and MACS column separation (Miltenyi Biotec). Total RNA was isolated with TRIzol (Invitrogen) and subsequently repurified using the RNeasy total RNA isolation kit (Qiagen). Double stranded cDNA was produced, using 10 μg RNA and the Superscript Choice System (Invitrogen). cDNA was purified with phenol/chloroform/isoamyl alcohol (Ambion) followed by ethanol precipitation. Ten micrograms cDNA was used to generate cRNA in an in vitro transcription system, using the RNA labeling kit (ENZO) for 6 h at 37°C to incorporate biotinylated ribonucleotides. cRNA samples were purified using the RNeasy kit and then precipitated with ethanol. cRNA samples were fragmented according to Affy-
metrix protocols and 15 µg were mixed with hybridization solution (Affymetrix) and hybridized on the mouse MG_U74Av2 gene chips for 16 h. The chips were then stained with streptavidin-phycoerythrin in the Fluidics Station 400 (Affymetrix) and later the fluorescence was read with the GeneArray Scanner (Affymetrix). Data files were generated using the Affymetrix Microarray Suite 5.0. To compare three CD11c⁺ Aire⁻/⁻ samples as controls and three CD11c⁺ Aire⁺⁺ samples as experimental samples, we set the scaling target value to 2500 in all probe sets for every chip. Nine pairwise comparison files were generated (with comparison of three wild type chips against all three knockout chips), each normalized to all probe sets. Fold change for each probe set was generated using the ratio of Aire⁻/⁻ to Aire⁺⁺ signals from the normalized signal values.

Flow cytometry (Papers I-IV)
Spleens, lymph nodes, thymi and livers (papers I-II) were homogenized to single cell suspensions, red blood cells from spleens were lysed with ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA at pH 7.2) and the cells were resuspended in FACS (fluorescence activated cell sorting) buffer (PBS, 2% FCS, 0.05% sodium azide). Blood samples and peritoneal cells (Papers I and IV) were mixed directly with FACS buffer. Bone marrow cells (Paper II) were flushed out from femurs with PBS containing 50% FCS by using a 19-gauge needle and a 2 ml syringe; they were then washed once and resuspended in FACS buffer. The cells were stained for 30 minutes at 4°C using fluorochrome conjugated antibodies from BD Pharmingen, FITC, PE, PerCP and allophycocyanin for sample collection with a FACScalibur flow cytometer (papers I-III), and in addition Pacific Blue for collection with a FACSARia flow cytometer (paper III); some antibodies were biotin conjugated and detected with fluorochrome conjugated streptavidin, using streptavidin alone as control. The data were analyzed with CellQuest-Pro or FACS Diva software (Becton Dickinson).

Immunofluorescent stainings (Papers II and IV)
Frozen livers and adrenal glands (paper II) or frozen pancreas (paper IV) was cryostat sectioned. The sections were washed in PBS and blocked for 30 minutes with 1% bovine serum albumin, and 2% goat or donkey serum (Sigma) in PBS. The slides were incubated with primary antibody or 1:50 diluted mouse serum overnight at 4°C. Slides were washed in PBS, incubated with FITC-conjugated secondary antibodies for 45 minutes, washed, and mounted in either Vectashield (Vector Laboratories) or Mowiol mounting medium (Calbiochem) and examined with a LEICA DMRB microscope.
For detection of autoantibodies in mouse sera the secondary antibody used was donkey anti-mouse IgG-FITC (Jackson Immunoresearch).

Immunohistochemical stainings (Paper II)
Metallophilic macrophages were detected with FITC-conjugated MOMA-1 antibody (Serotec) and marginal zone B cells using biotinylated anti-CD1d antibody (BD Pharmingen). Secondary antibodies for immunohistochemistry, anti-FITC F(ab’) horseradish peroxidase (HRP) or anti-biotin F(ab’) alkaline phosphatase (AP) were from DAKO. Vector Blue Alkaline Phosphatase Substrate (Vector Laboratories) and DAB peroxidase substrate (DAKO) were used for development of immunohistochemistry stains. Stainings were done on cryosections of spleens which were fixated in acetone for 3.5 minutes and blocked with 2% goat serum in PBS. Thereafter the slides were incubated with primary antibodies for 1 h at room temperature. Slides were washed in PBS and incubated with HRP and AP-conjugated secondary antibodies for 1 h. After washing with PBS, substrates for HRP and then AP were added. Photographs were taken with a LEICA DMRB microscope and LEICA DC 200 camera.

Clinical chemistry and hormone measurements (Papers II and IV)
S-Ca\textsuperscript{2+} (paper II) was measured on 1:10 diluted sera using a colorimetric calcium assay (Sigma). Mouse parathyroid hormone (paper II) was measured with sandwich ELISA on undiluted sera (Immutopics, CA). S-corticosterone (paper II) was analyzed with use of 1:10 diluted serum samples with the competitive OCTEIA corticosterone assay (Immunodiagnostic systems). Alanine aminotransferase (paper II) was determined with a kinetic UV method according to the International Federation of Clinical Chemistry recommendations, using a Konelab 30i analyzer and reagents according to instructions at the clinical chemistry department of the Swedish University of Agricultural Sciences in Uppsala. Insulin (paper IV) was measured in sera collected from STZ treated mice upon killing, using a rat insulin ELISA kit (Mercodia) according to the manufacturer’s instructions. Thyroxine (paper IV) was measured with the enzyme immunoassay kit from MP Biomedicals, following the manufacturer’s instructions.
RNA isolation and reverse transcriptase reaction (Papers I, III, and IV)

Total RNA from tissues or cells was extracted using an RNeasy kit (Qiagen) (papers I, and IV), including an Rnase free Dnase treatment (Qiagen), or with an Aurum™ total RNA Mini Kit (Bio-Rad) (paper III) according to the manufacturer’s instructions. cDNA synthesis was performed either with a Superscript kit (Invitrogen) (paper I) or an iScript cDNA synthesis kit (Bio-Rad) (papers III and IV).

RT-PCR (Papers I and III)

PCR for Aire expression (paper I) on cDNA from Aire^{+/+} mice was performed with use of Taq Polymerase (Invitrogen) with exon-5 and exon-9 specific primers for 35 cycles at 95°C for 1 minute, 57°C for 1 minute, and 72°C for 2 minutes. Mammary tumor virus superantigen (mtv SAG) expression was detected with thymic or splenic cDNA samples from OT-II Aire^{+/+} or OT-II Aire^{-/-} mice, and 1 unit Platinum® Taq Polymerase (Invitrogen). The amplification was performed with a MyCycler (Bio-Rad). After 1 minute and 30 seconds of denaturation at 94°C, 39 rounds of amplification were performed under the following conditions; 30 seconds at 94°C, 30 seconds at 56°C and 1 minute at 72°C, followed by a final polymerization step at 72°C for 7 minutes. GAPDH was amplified as a control housekeeping gene using the same temperature protocol as for mtv SAG.

Quantitative real time PCR (Papers III and IV)

In study III quantitative PCR of the RAG2 gene and of GAPDH as housekeeping gene was performed using 2X IQ™ SYBR® Green Supermix (Bio-Rad) and an iCyclerIQ (Bio-Rad). Cycle thresholds were obtained with iCycler IQ™ Optical Software Version 3.1 from Bio-Rad. In study IV expression of MIF (macrophage migration inhibitory factor), TNFα, IL-10, and β-actin as housekeeping gene was quantified in STZ exposed macrophages using SYBR Green JumpStart Taq ReadyMix (Sigma) and a LightCycler-System (Roche). Expression levels were normalized to that of the housekeeping gene using the $2^{-\Delta\Delta CT}$ method.

In vitro streptozotocin cytotoxicity on macrophages (Paper IV)

Macrophages were collected in 5 ml PBS from Aire^{+/+}, Aire^{+/+} and Aire^{-/-} mice by peritoneal lavage, washed, and cultured in DMEM and 5% FCS
supplemented with either medium, 10 mM STZ, 10 mM STZ + 2 mM nicotinamide, 10 mM STZ + 5 mM nicotinamide, or 10 mM STZ + 10 mM nicotinamide. After 24 h of culture at 37°C macrophages were stained for apoptosis using an AnnexinV staining kit (BD Pharmingen) according to the manufacturer’s protocol, using annexinV-FITC, propidium iodide (PI), and anti-CD11b-APC (BD Pharmingen); the data were collected with a FACScalibur flow cytometer and analyzed with the CellQuest pro software (Becton Dickinson). The percentage of dead CD11blo macrophages was calculated by summing the AnnexinV+PI-, AnnexinV+PI+ and AnnexinV-PI+ gates.

Statistical methods (Paper I-IV)

The Mann Whitney U-test (paper II) and Student’s t-test (paper I-IV) were used to test the hypothesis of no difference between the two groups Aire+/+ and Aire-/- in a set of outcome variables. The Mann-Whitney U-test was applied when the data were not of the interval type, or when the data were not approximately distributed, or when the variances in the two groups were not approximately homogeneous. This test was used here for testing the hypothesis of a general difference in distributions between Aire+/+ and Aire-/-, which can be interpreted as a general difference. When the above assumptions were satisfied, the variables were analyzed with Student’s t-test, which was used to test the hypothesis of a general difference in mean values between the Aire+/+ and Aire-/- individuals.

Splenitic extramedullary hematopoiesis (paper II) was estimated on a three grade scale and the frequency of grades was compared between Aire sufficient and Aire deficient mice with the Kruskal-Wallis test. The frequency of lymphoma (paper II) was compared with the one sided Fisher’s exact test, under the assumption that the frequency of lymphoma is higher in Aire-/- mice, since marginal zone B cell lymphoma has not been observed in aged Sv129 and C57BL/6 strains and it was absent in the Aire sufficient group in this study. Diabetes susceptibility curves, histopathology scores and frequency of autoantibodies (paper IV) were analyzed with two sided Fisher’s exact test.

To test the hypothesis of a general association between development of lymphoma, lymphocytic infiltrates in the liver, and B cell infiltrates in the thymus (paper II), Spearman rank order correlation was used. Linear regression was applied to analyze the CD4/CD8 ratio and %V5hi of CD4+ T cells (paper III). The variables included in the model were age as covariate, genotype as factor and interaction between age and genotype; for these statistical analyses SAS version 9.1 was used. P values ≤0.05 were considered statistically significant.
Results and discussion

Alterations in gene expression of dendritic cells from Aire−/− mice cause increased activation of T cells (Paper I).

The expression of Aire in monocyte derived DCs suggests a role for Aire in peripheral tolerance. In a previous study we found that Aire−/− lymphocytes showed increased proliferation during recall response to HEL in vitro (140). To investigate whether Aire−/− DCs play a role in the hyperproliferation of Aire−/− lymphocytes we studied the in vitro proliferation of Ova peptide specific OT-II TCR transgenic T cells cultured with antigen and either Aire−/− or Aire+/+ APCs. We found higher proliferation in the cultures with Aire−/− CD11c+ cells and particularly with CD11b+ cells (Figure 6), but no difference from Aire+/+ when using B220+ B cells as APCs.

Figure 6. OT-II CD4+ T cell proliferation measured as [3H]-thymidine incorporation on day 3, 4, and 5 of stimuli from Aire+/+ (filled circles) or Aire−/− (open circles) CD11b+ APCs from spleen and lymph nodes in the presence of either OT-II peptide (left panel) or Ova (right panel)

Microarray analysis revealed differential expression of 68 genes in Aire−/− CD11c+ cells, among which VCAM-1 was upregulated twofold. A flow cytometric analysis showed an increased number of CD11c+ and CD11b+ cells in Aire−/− blood, peritoneum, spleen, and lymph nodes. Furthermore, the VCAM-1 upregulation was confirmed at the protein level in a subpopulation of CD11c−CD11b+ DCs (Figure 7), whereas the classical costimulatory molecules B7.1 and B7.2 were normally expressed.
VCAM-1 overexpression was not dependent on stimuli from activated autoreactive T cells, since it was also present in Aire\(^{-/-}\) mice crossed with OT-II TCR transgenic mice in which most of the T cells recognize a foreign Ova peptide and in addition as the serum cytokine levels were normal. We analyzed the number of blood monocytes and VCAM-1 expression on monocytes from APS I patients and found twice as large a number of monocytes with higher VCAM-1 expression compared to age and sex matched healthy controls. Finally we investigated IL-2 production by means of Ova peptide specific T cell hybridomas stimulated by either Aire\(^{-/-}\) or Aire\(^{+/+}\) plate binding APCs and found higher IL-2 production when using Aire\(^{-/-}\) APCs. Since the T hybridomas were independent of CD28 signaling, we addressed the possible involvement of VCAM-1 by using anti-VCAM-1 blocking antibodies and showed that the difference in IL-2 production disappeared.

In conclusion, Aire expression in peripheral DCs is necessary for proper control of T cell activation through a mechanism in partly dependent on the regulation of VCAM-1 costimulation.
Aged Aire deficient mice display altered hematopoiesis of monocytes in the bone marrow and of red blood cells in the spleen, as well as increased activation of marginal zone B cells and development of marginal zone lymphoma (paper II).

The first reports on young Aire deficient mice have described a very mild phenotype with no development of clinical disease. In this study we investigated the question whether aging might contribute to the development of a clinical phenotype similar to APS I.

Compared to Aire\(^{+/+}\) females, Aire\(^{-/-}\) females were found to weigh significantly less and to be megalosplenic, but no such differences were seen between Aire\(^{-/-}\) and Aire\(^{+/+}\) males. None of the APS I autoantigens were recognized by antibodies in sera from Aire\(^{-/-}\) mice. Clinical evaluation showed normally functioning of the adrenal cortex and the parathyroid glands in Aire\(^{-/-}\) mice but leakage of liver enzymes in some of these mice. We found no oral or vaginal growth of \(C.\) \textit{albicans} in Aire deficient females. The lymphocytic infiltrates in the livers of Aire deficient mice were further characterized by immunofluorescent stainings and flow cytometry and were found to be mainly composed of B cells. In addition, abnormal B cell infiltrates were identified in the thymus in 3/8 Aire\(^{-/-}\) mice.

Two interesting features were observed in Aire\(^{-/-}\) spleens, namely very low or absent splenic extramedullary hematopoiesis in 7/10 Aire\(^{-/-}\) mice and hyperplasia or lymphoma of marginal zone B cells in 4/11 such mice (Figure 8, lower right panel).

\textit{Figure 8.} Immunohistochemical staining of Aire\(^{+/+}\) and Aire\(^{-/-}\) spleens for MOMA-1 (metallophilic macrophages, in brown) and CD1d (MZB, in blue).
Immunohistochemical stainings also revealed normal numbers of red pulp and marginal zone macrophages but increased numbers of metallophilic macrophages in Aire$^{-/-}$ mice (Figure 8, lower left panel). A flow cytometric analysis revealed an expansion of marginal zone B cells with an activated phenotype also in Aire$^{-/-}$ mice without lymphoma.

The hematological analysis showed a tendency to a decreased hematocrit and lower red blood cell counts but normal hemoglobin levels in Aire$^{-/-}$ mice. Differential cell counts of bone marrow cells showed a normal bone marrow hematopoiesis.

The hematopoiesis of monocyte precursors in the bone marrow was found to be altered in Aire deficient mice, with an increased proliferation and a more rapid exit into the circulation, giving rise to the increased monocyte counts seen in the blood and probably also to the increased numbers of metallophilic macrophages in the spleen.

In conclusion, aged Aire$^{-/-}$ mice do not develop the clinical features of APS I, but they display increased activity of the marginal zone of the spleen, probably as a result of increased stimulation of marginal zone B cells by metallophilic macrophages through cytokine secretion or antigen uptake. The altered cytokine milieu in the splenic marginal zone causes a depression of the extramedullary hematopoiesis in the spleen that does not lead to anemia by virtue of a normal red blood cell hematopoiesis in the bone marrow. These results point to a function of Aire in the development of monocyte derived cells which in turn affects peripheral tolerance.

Superantigen mediated TCR revision is increased in the spleens of OT-II TCR transgenic Aire deficient mice (Paper III)

In study III we investigated the effects of Aire deficiency on superantigen mediated selection in the thymus and spleen of Aire OT-II TCR transgenic mice. The C57BL/6 mouse strain is known to carry mtv-8 and mtv-9 endogenous superantigens which are able to bind to the V$\beta$5 chain displayed on the transgenic TCR of OT-II mice.

The expression of endogenous mtv superantigen was similar in Aire$^{+/+}$ and Aire$^{-/-}$ OT-II thymus and spleen. In the thymus we did not see any reduction in negative selection, but we observed decreased numbers of transgenic V$\beta$5$^{hi}$V$\alpha$2$^{hi}$ CD4$^{+}$ single positive cells, which also displayed a less mature phenotype in Aire$^{-/-}$ OT-II mice since the Qa2 maturation marker was expressed at lower levels (Figure 9).
Figure 9. Mean fluorescence intensity of Qa2 expression on CD4SP Vβ5.2 hi thymocytes from Aire+/+ (solid dark gray) and Aire−/− (gray line) mice.

Apoptosis of transgenic thymocytes was similar in Aire+/+ and Aire−/− OT-II mice and there was no difference in the intrinsic TCR signaling, suggesting that the decreased numbers of SP cells in the thymi of Aire deficient mice were due to an earlier exit of CD4 SP T cells from the thymus.

In the spleen the number of transgenic Vβ5 hiα2 hi T cells was decreased in Aire−/− OT-II mice, whereas the number of CD4+Vβ5 lo T cells was increased. The latter population has previously been found to derive from superantigen induced RAG re-expression and TCR revision of Vβ5+ T cells both in a transgenic and a non-transgenic setting. RAG expression was increased in Aire−/− OT-II mice (Figure 10), as also was the number of activated CD49d+CD4+Vβ5 lo T cells, confirming that there was an increase in TCR revision.

Figure 10. Quantitative real time PCR of RAG2 expression in thymus and spleen from OT-II Aire+/+ and OT-II Aire−/− mice.
Furthermore, the CD4^+ V\beta^5^- revised T cells displayed a higher frequency of revision into V\beta11 but not into V\beta3 in Aire^-/- OT-II mice, where V\beta11 has been reported to be selected by mtv-8 and mtv-9 whereas V\beta3 was not, suggesting that there was a superantigen mediated selective pressure on the increased TCR revision.

The early egress of single positive transgenic T cells from Aire^-/- OT-II thymi cannot be attributed to a difference in negative selection performed by the endogenous superantigens, since there was no difference in the apoptosis of the single positive cells. This is not surprising, since mtv-8 and mtv-9 superantigens are not so efficient in performing negative selection on H2-IA, and need H2-IE, which unfortunately is mutated in the C57BL/6 strain (188). It is more likely that this phenomenon is caused by faster migration of Aire^-/- OT-II thymocytes through the thymic medulla, perhaps caused by a differential expression of chemokines in Aire^-/- mTECs, as has been proposed by others (159, 179). The mechanisms that induce superantigen mediated TCR revision have not yet been characterized, but it is known that costimulation through CD28 is involved (189, 190). Our earlier finding that Aire^-/- DCs show an upregulated expression of the costimulatory molecule VCAM-1 suggests that Aire^-/- DCs might be responsible for the increased TCR revision seen in Aire^-/- OT-II mice, but this issue needs further investigation.

Aire deficiency causes increased susceptibility to streptozotocin induced diabetes via a macrophage dependent mechanism (Paper IV)

Diabetes induced by multiple low dose streptozotocin (MLDSTZ) injections is an autoimmune disease model in which tolerance is broken by an exaggerated response to a danger signal caused by a toxic substance. In contrast to the spontaneous diabetes developed by the NOD mouse strain, where insulin is an important autoantigen that generates both a T and a B cell response, in MLDSTZ diabetes no anti-insulin antibodies are produced (191). We chose to use this diabetes model in order to study the influence of Aire on autoimmune disease development in a context where disruption of peripheral tolerance mechanisms is more important than central tolerance generated by promiscuous expression of insulin in the thymus.

When we induced diabetes with MLDSTZ injections, Aire^-/- mice were found to be most susceptible both in C57BL/6 and in the F2 generation of C57BL/6 crossed with C57BL/KsJ mice. Among the F2 mice, in addition to the susceptibility, the severity of the diabetes as judged from glucose (Figure 11) and serum insulin levels was also highest in Aire^-/- mice.
Although the susceptibility of Aire<sup>-/-</sup> mice was not significantly different from that in Aire<sup>+/+</sup> mice, only the Aire<sup>-/-</sup> mice developed anti-islet cell antibodies and they displayed a weight gain instead of a weight loss during the diabetes development that was not caused by hypothyroidism, suggesting that their diabetes development had a distinct etiology.

There was no difference in the STZ suppression of insulin release from isolated islets from Aire<sup>+/+</sup>, Aire<sup>+/-</sup> and Aire<sup>-/-</sup> mice; by contrast Aire<sup>-/-</sup> macrophages were more susceptible to STZ cytotoxicity than Aire<sup>+/+</sup> and Aire<sup>+/-</sup> macrophages. When we measured STZ induced activation of macrophages in terms of cytokine production, we found the highest activation in Aire<sup>-/-</sup> macrophages, which produced higher levels of the proinflammatory cytokines MIF and TNFα and lower levels of the anti-inflammatory cytokine IL-10; also, higher MIF levels were produced by Aire<sup>-/-</sup> than by Aire<sup>+/+</sup> mice. We propose a model in which the different susceptibilities to diabetes are caused by differential uptake or metabolism of STZ by macrophages depending on the Aire expression levels (Figure 12).
When Aire expression is absent, the uptake/metabolism of STZ is highest and causes considerable activation but also cell death, which means that the inflammation is only transient and does not lead to an efficient T cell response. When Aire expression is intermediate the activation is increased compared to that in Aire$^{+/+}$ macrophages, but there is no cytotoxicity and therefore a more pronounced T cell response against $\beta$-cells develops.

These results suggest that the function of Aire in the control of peripheral tolerance through myeloid antigen presenting cells is of great importance in the prevention of autoimmune disease.
General discussion

The studies presented in this thesis are the first ones to address the role of Aire in APCs and in peripheral tolerance. That Aire has a function in mTECs and in negative selection in the thymus has previously been established, but the mechanisms are still controversial and the connection between the central tolerance defect and the disease phenotype of Aire deficient mice and of APS I patients is still not clear. The present results open up the possibility that at least some of the disease manifestations of APS I might be caused by a defect in peripheral rather than central tolerance.

We have shown that Aire influences the phenotype of several different monocyte derived APCs, which in turn affect different branches of the immune response. In study I we found that the numbers of myeloid derived antigen presenting cells were increased in the blood, peritoneum, spleen, and lymph nodes of Aire deficient mice, and furthermore that blood monocytes were increased in APS I patients. Aire−/− CD11c−CD11b+ DCs have an increased ability to activate naïve T cells, which is in part mediated by an upregulated expression of the costimulatory molecule VCAM-1. In study II we showed that Aire−/− monocyte precursors had an accelerated development in the bone marrow, which gave rise to increased numbers of blood monocytes and of metallophilic macrophages in the spleen. This might be responsible for the activated phenotype of the marginal zone B cells and the high frequency of marginal zone lymphoma development in aged Aire deficient mice. In study III we observed an increased superantigen mediated TCR revision in OT-II Aire−/− TCR transgenic T cells, perhaps caused by the altered phenotype of Aire−/− DCs. In study IV we demonstrated that Aire−/− macrophages were more sensitive to the activating and cytotoxic effects of STZ as a result of higher uptake and/or metabolism, whereas Aire+/− macrophages are only more sensitive to the activating effects; this, in turn leads to a higher susceptibility in Aire+/− mice to diabetes induction with MLDSTZ. In order to confirm this model we plan to repeat the experiments with lower doses of STZ and we expect to find the highest susceptibility to diabetes in Aire−/− mice if this explanation is correct.

The upregulated expression of VCAM-1 that we have observed in Aire deficient DCs might be targeted with anti-VLA-4 (very late antigen 4) blocking antibody therapy, which has been used successfully to treat multiple sclerosis and Crohn’s disease (3). In APS I it might be effective in treating the life-threatening chronic autoimmune hepatitis that affects 20% of the patients (92).

Although we now have enough evidence that Aire affects peripheral tolerance, we do not know yet whether this is directly involved in the disease manifestations of APS I or in the production of autoantibodies and lymphocytic infiltrates seen in Aire deficient mice. RT-PCR investigations of APS I target organs have been reported by some investigators to detect Aire ex-
pression, suggesting that Aire-expressing cells, perhaps tissue macrophages are present in these organs. Tissue macrophages have been found to have an important function in the testis and ovary, where they take up apoptotic cells, and studies have suggested that macrophages are involved in the regulation of endocrine function by secreting cytokines (192, 193); this is witnessed by the infertility seen in macrophage deficient op/op mice (194). Interestingly, Aire deficient mice also have a very low fertility and become infertile with aging, and hypogonadism is manifested in 60% of APS I female patients but only in 14% of such male patients at 30 years of age (89), probably because of the protection provided by the blood-testis barrier. Aire deficient mice have normally functioning APS I target glands (paper II), but they might have a subclinical phenotype that becomes evident only in response to specific stimuli; in order to study this issue in the adrenal cortex we performed an ACTH (adrenocorticotrophic hormone)-stimulation test and measured the corticosterone levels before and 1 h after the ACTH injection. Unexpectedly, Aire deficient mice produced significantly more corticosterone than Aire+/+ mice after administration of ACTH (Figure 13).

![Figure 13. Serum corticosterone levels in Aire+/+ and Aire-/- mice before and 1h after ACTH injection.](image)

The basal corticosterone levels were normal, suggesting that the exaggerated corticosterone response to ACTH stimulation was not due to increased stress in Aire-/- mice (Figure 11). When we measured the serum thyroxine levels on day 21 after MLDSTZ injections we found a significant increase in Aire+/+ compared to Aire-/- mice, but not in Aire+/+ mice (Figure 14).
These surprising results may suggest that Aire deficient tissue macrophages have an increased ability to stimulate endocrine glands to hormone production in response to endogenous stimuli (ACTH) or danger signals (STZ). In the case of the thyroid gland the effect is evident in Aire\(^{+/−}\) rather than Aire\(^{−/−}\) mice, perhaps for the reason that Aire\(^{−/−}\) macrophages succumb to the cytotoxic effects of STZ, as described in paper IV.

It is tempting to hypothesize that Aire has an important function in the tissue macrophages of the most common target glands in APS I, i.e., the parathyroids and the adrenal cortex, and a minor function in the macrophages of other glands that are less frequently subject to autoimmune destruction. In the absence of Aire, tissue macrophages stimulate or perhaps are unable to inhibit excess hormone production through cytokine secretion, and the endocrine cells therefore become hyperactive, with increased metabolism and hormone secretion. The demand for increased hormone production will increase the expression of the identified APS I autoantigen targets CYP450cSCC, CYP450c21 and CYP450c17 in the adrenal cortex. Hyperfunction of a gland may in itself be a danger signal to the immune system, since it might indicate a tumoral transformation of the endocrine cells, but it is not enough solely to induce an autoimmune response. The increased ability of Aire deficient DCs to activate naïve T cells, together with the increased escape of autoreactive T cells from thymic selection and the hyperfunction of the target glands, may be the explosive combination that causes the autoimmune destruction of the APS I target glands. Interestingly, early hormonal replacement therapy in subclinical phases of endocrine autoimmune disease has been reported to cause amelioration and sometimes disease remission (195-198); a possible explanation for this might be that suppression of endogenous hormonal production by the administered hormone by feedback mechanisms, will decrease the danger signal from the gland and thereby diminish autoimmunity.
APS I patients without CMC, for example Iranian Jewish patients, have a milder disease with fewer manifestations (85). CMC is often but not always one of the first manifestations in APS I patients (89); this might suggest that the *Candida* infection provides a danger signal that initiates the autoimmune response, but it is dispensable since autoimmunity may be achieved even in the absence of CMC. To address this question we plan to investigate whether injection of *Candida* cell-wall components may induce a clinical disease in Aire deficient mice. This may also explain the mild phenotype of Aire deficient mice, since *C. albicans* is not a commensal and it does not cause spontaneous infections in Aire deficient mice (paper II).

It has recently been reported that anti-IFN\(\lambda\) antibodies are very early diagnostic markers in APS I patients (134). Compared to two healthy controls, one APS I patient was found to display an upregulated expression of IFN\(\lambda\) in immature DCs developed *in vitro* from their blood monocytes. We have observed a 20-fold upregulation of an interferon inducible gene in Aire deficient peritoneal macrophages stimulated with lipopolysaccharide (unpublished observation). These results are preliminary and investigations of additional patients and mice are needed, but if the findings are confirmed they might be very relevant to an understanding of the pathogenesis of APS I. Metallophilic macrophages have been reported to be potent IFN\(\lambda\) producers during viral stimulation *in vivo* in C57BL/6 mice (199). We hypothesize that an increased IFN\(\lambda\) production in the splenic marginal zone by metallophilic macrophages might lead to the increased BAFF levels that we have observed in Aire\(^{-}\) sera (unpublished observation), and that this chronic activation of marginal zone B cells will ultimately result in lymphoma development in Aire deficient mice (paper II). IFN\(\lambda\) treatment of patients with chronic hepatitis C infections has been found to cause depression of hematopoiesis, and a similar effect has been observed in mice (200-202); IFN\(\lambda\) production by metallophilic macrophages in Aire deficient mice might therefore also explain the low or absent extramedullary hematopoiesis in the spleen (paper II). Interestingly there are several reports of development of autoimmune hypothyroidism and type 1 diabetes in chronic hepatitis C patients treated with IFN\(\lambda\), and there are also reports of development of CYP450c21 autoantibodies but without clinical symptoms of Addison’s disease (203-205).

IFN\(\alpha\) has been found to be an important factor in the induction of the systemic autoimmune disease SLE (206). In spite of this, APS I patients do not present features of systemic autoimmunity, probably because they develop neutralizing autoantibodies against IFN\(\alpha\) that prevent a systemic effect and only allow a local action of the cytokine. Furthermore, in SLE IFN\(\alpha\) production is induced by an increased load of chromatin and RNA autoantigens from apoptotic and necrotic cells that form immune complexes with autoantibodies and are internalized by pDCs via Fc\(\gamma\)RIIa. In the endosome nucleic acid autoantigens are released from the immune complexes, they bind to TLR9 and TLR7 and stimulate IFN\(\alpha\) production (206). In this case IFN\(\alpha\)
production is more probably due to a direct effect of Aire on other APC subsets than pDCs and less dependent on autoantigen stimuli. Self perpetuation of the production of anti-interferon autoantibodies has been described, where IFNα is expressed in macrophages in the germinal centers as a result of its action on follicular dendritic cells, which present antigens to the hypermutating B cells (134). It will therefore be highly relevant to study IFNα expression in germinal centers from Aire deficient and Aire sufficient mice.

In conclusion, Aire is involved in the regulation of negative selection of thymocytes (Figure 15, left panels). In this thesis we provide evidence that Aire has additional important roles in peripheral tolerance by regulating the function of antigen presenting cells (Figure 15, right panels). Aire deficiency leads to exaggerated activation signals to T and B cells, resulting in increased T cell activation, autoantibody production, and lymphoma. Exploring the mechanisms underlying the development of APS I may provide insights into key pathways for treatment of autoimmunity.
Figure 15. A model describing the hypothesized function of Aire in the thymus and in the periphery. Due to a defect in central tolerance self-reactive T cells escape negative selection. It is likely that pathogens, e.g. Candida infections or periods of stress may initially induce danger signals in endocrine target organs and draining of autoantigens to the regional lymph nodes. When encountering their autoantigen in peripheral lymphoid organs they become hyperactivated due to a defect peripheral tolerance involving increased VCAM-1 expression of Aire⁻ APCs. In addition Aire deficient APCs of endocrine organs seem to have defects in regulatory functions of hormonal responses of endocrine cells resulting in exaggerated stimulated responses. The stimulated increased functionality of endocrine cells will result in increased levels of APS I autoantigens, that is enzymes responsible for hormonal synthesis or release. Thus endocrine organs are now targets for hyperactivated autoreactive T and B cells.
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