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Characterization of Human AIRE Promoter and Analysis of AIRE Expression in Monocyte-Derived Dendritic Cells and Thymomas

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ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original publications, referred to in the text by their roman numerals I-V.

I **Murumägi, A**., Vähämurto, P., and Peterson, P. Characterization of Regulatory Elements and Methylation Pattern of the Autoimmune Regulator (AIRE) Promoter. *Journal of Biological Chemistry*. 2003; 278: 19784 - 19790.

II **Murumägi, A**., Silvennoinen, O., and Peterson, P. Ets transcription factors regulate AIRE gene promoter. *Biochemical and Biophysical Research Communications*. 2006; 348: 768-774.

III Sillanpää, N., Magureanu, CG., **Murumägi, A**., Reinikka, A., Manninen, A., Lahti, M, Ranki, A., Saksela, K., Krohn, K., Lahesmaa, R., and Peterson, P. Autoimmune regulator induced changes in the gene expression profile of human monocyte-dendritic cell-lineage cells. *Molecular Immunology*. 2004; 41:1185-1198.

IV Meager, A., Visvalingam, K., Peterson, P, Möll, K, **Murumägi, A**., Krohn, K, Eskelin, P., Perheentupa, J., Husebye, E., Kadota, Y., and Willcox, N. Anti-Interferon Autoantibodies in Autoimmune Polyendocrinopathy Syndrome Type 1. *PLOS Medicine*. 2006; 3: e289

V Ströbel, P., **Murumägi, A**., Klein, R., Luster, M., Lahti, M., Krohn, K., Schalke, B., Nix, W., Golf, R., Rieckmann, P., Toyka, K., Rosenwald, A., Müller-Hermelink, H.K., Pujol-Borrell, R., Meager, A., Willcox, N., Peterson, P., and Marx, A. Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinopathy syndrome type I (APS-I). *Journal of Pathology*. In press.

ABBREVIATIONS

AIRE/Aire	the human/mouse autoimmune regulator gene
AIRE/Aire	the human/mouse autoimmune regulator protein
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APC	antigen presenting cell
APS	autoimmune polyglandular syndrome
ATP	adenosine triphosphate
5-azaCdr	5-aza-2´-deoxycytidine
bp	base pair
CD	cluster of differentiation
CMC	chronic mucocutaneous candidiasis
CpG	cytosine guanine dinucleotide
DC	dendritic cell
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EMSA	electrophoretic mobility shift assay
EOMG	early-onset MG
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
HMT	histone methyltransferase
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IFN	interferon
IL	interleukin
IPEX	immuno dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
FOXP3	forkhead box P3
kb	kilobase
MBP	methyl-CpG-binding protein
MG	myasthenia gravis
MHC	major histocompatibility complex
mTEC	medullary thymic epithelial cell
MoDC	monocyte-derived dendritic cell
mRNA	messenger RNA
N	amino
NF-κB	nuclear factor κB
P450c17	steroid 17-α-hydroxylase
P450c21	steroid 21-hydroxylase
P450scc	cholesterol side-chain cleavage enzyme
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHD	plant homeodomain
PMA	phorbol myristate acetate
PKC	protein kinase C
OMIM	online mendelian inheritance in man
QPCR	quantitative real-time RT-PCR
RT-PCR	reverse transcriptase polymerase chain reaction
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
TCR	T cell receptor

Th	T helper
TNF	tumor necrosis factor
Treg	regulatory T cell
TSA	trichostatin A
TSLP	thymic stromal lymphopoietin

ABSTRACT

AIRE (autoimmune regulator) is a transcription factor which plays an essential role in central tolerance by directing the expression of peripheral autoantigens in the thymus. Mutations in *AIRE* gene cause a rare organ-specific autoimmune disease APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy), also known as APS-1 (autoimmune polyglandular syndrome type 1). The APECED is characterized by destructive autoimmune diseases of the endocrine organs, chronic mucocutaneous candidiasis and ectodermal disorders. AIRE protein is mainly expressed in thymus and to a lesser degree in lymph nodes, spleen and fetal liver. In the thymus AIRE is found in two populations of antigen presenting cells, in medullary epithelial cells and cells of monocyte-dendritic cell lineage, both cell types being involved in the negative selection of autoreactive T cells.

The aim of this study was to understand the mechanism of *AIRE* gene regulation and the downstream effects of its expression. Characterization of *AIRE* promoter indicated that at the transcriptional level *AIRE* is regulated by ubiquitously expressed transcription factors Sp1, NF-Y and AP-1. In addition, Ets transcription factor family members were identified as positive regulators of *AIRE* transcription. It was also found that epigenetic modifications at the chromatin level, DNA methylation and histone deacetylation, are involved in the regulation of *AIRE* gene expression. Analysis of *AIRE* expression during the differentiation of monocyte-derived dendritic cells demonstrated that *AIRE* mRNA was up-regulated already in the early stages of differentiation. Furthermore, microarray studies of AIRE-induced gene expression in stable AIRE-transfected cell line revealed that the changes in gene expression profile resembled the pattern identified during the maturation of dendritic cells.

Moreover, the occurrence of type I interferon (IFN) autoantibodies was analysed in a large set of APECED patient samples. High titer autoantibodies mainly against IFN-α subtypes and IFN-ω were found in all APECED patients sera. Neutralising autoantibodies against type I IFNs were detected in patients before the other autoantibodies characteristic for APECED and for some cases before the onset of clinical disease components. Analysis of *AIRE* expression in thymomas, which are the epithelial tumors of the thymus, revealed the lack of *AIRE* in 95% of thymoma patients. In addition, thymoma patients also lacked typical autoantibodies and disorders for APECED.

The results from current studies identified the essential promoter elements and transcription factors involved in *AIRE* transcriptional activation. Analysis of AIRE function in the periphery suggested that AIRE might be involved in dendritic cell maturation process. The physiological importance of AIRE in the periphery is not completely understood, and these studies provide new insights into the molecular and immunological mechanisms involved in AIRE function.

INTRODUCTION

The activation of the immune system to defend the body against invaders is based on a very efficient immunological defence, which relies on specific recognition of molecular patterns or antigens by the cells of the innate or adaptive immune systems. The breakdown of tolerance mechanisms can lead to the development of an autoimmune response against self-molecules, cells or tissues. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is an autoimmune disease characterized by destructive autoimmune diseases affecting many organs, mainly endocrine glands. The disease is caused by mutations in a single gene, the autoimmune regulator (*AIRE*).

AIRE gene, identified in 1997 by two independent research teams, plays an important role in the development of central tolerance to several autoantigens and participates in the negative selection of autoreactive T cells by directing the expression of peripheral autoantigens in the thymus. It has been suggested that AIRE directs the expression of hundreds of promiscuously expressed genes in medullary epithelial cells in the thymus.

The purpose of this study was to characterize the *AIRE* promoter and to determine the key transcription factors regulating *AIRE* transcription in order to gain insight into how *AIRE* expression is regulated. To study the role of *AIRE* in the periphery, its expression pattern was investigated in monocyte-derived dendritic cells (MoDCs). Additionally, the possibility of whether *AIRE* is involved in the development of autoimmune diseases observed in thymoma patients was investigated.

REVIEW OF THE LITERATURE

1. IMMUNE SYSTEM AND SELF-TOLERANCE

The primary function of the immune system is to protect the host against invading pathogens and microorganisms. In order to fulfil this task the immune system uses a complex array of protective mechanisms to control and eliminate the invaders. Classically, the immune response is divided into innate and adaptive immunity. The innate immune system provides a first line of defence against microorganisms, although it cannot always eliminate infectious microorganisms (Janeway and Medzhitov 2002). The lymphocytes of the adaptive immune system provide more specific response and, in addition, memorize their defence strategy, thereby ensuring that subsequent infections with the same organism will be handled more efficiently. The two immune responses, often described as separately working units of the immune system, usually act together and are interrelated (Ochsenbein and Zinkernagel 2000, Chaplin 2006).

1.1 Activation of T cell immune response

The initiation and progression of an appropriate immune response is controlled by the tight regulation of lymphocyte activation. The primary step in this process is the recognition of the antigen by T cell receptor (TCR) expressed on T cell. TCRs are composed of two transmembrane subunits α and β or structurally similar γδ polypeptides, which are associated with a membrane-bound complex of proteins collectively known as CD3, together constituting the TCR signaling complex (Davis et al. 2003). The αβ lineage T cells, which represent a major T cell population, can be divided into helper (CD4+) and cytotoxic (CD8+) T cells, according to the expression of CD4 or CD8 coreceptor molecules. CD4+ T helper (Th) cells can be further subdivided into two distinct classes, Th1 and Th2 cells. Th1 cells contribute to cell-mediated immunity by combating intracellular bacterial infections via secretion of interleukin 2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β) which leads to the activation of macrophages (Mosmann and Coffman 1989). Th2 cells, which mainly produce IL-4, IL-5 and IL-10, mediate humoral immune responses by activating B cells to produce antibodies, which eliminate extracellular pathogens (Mosmann and Coffman 1989). It is believed that the balance between these two T cell subtypes determines the outcome of infectious and autoimmune diseases (Crane and Forrester 2005). T cells can recognize antigens only when they are presented as peptide fragments bound to major histocompatibility complex (MHC) molecules (Germain 1994). MHC class I molecules are ubiquitously expressed on the cell surface of nearly all somatic cells whereas MHC class II molecules are exclusively expressed by specialized antigen presenting cells (APCs), including dendritic cells (DCs), B cells and macrophages, nonprofessional epithelial or endothelial APCs such as thymic epithelial cells or IFN-γ exposed keratinocytes (Brodsky and Guagliardi 1991). In order to guarantee the stable interaction between T cell and APC, an antigen-independent co-stimulatory signal is required for the proper T cell activation (Liu and Janeway 1992). This co-stimulatory signal is provided by co-stimulatory molecules expressed on APC and it functions to promote T cell clonal expansion, cytokine secretion and effector function (Salazar-Fontana and Bierer 2001). In the absence of co-stimulatory signal, T cells fail to respond efficiently and are functionally inactivated and resistant to subsequent activation to the antigen. Based on their function, the co-stimulatory molecules are divided into three main families, the B7:CD28 superfamily, a TNF:TNFR superfamily, and CD2 superfamily (Tangye et al. 2000, Sharpe and Freeman 2002, Watts 2005). The most studied T cell co-stimulatory pathway constitutes the B7-1/B7-2:CD28/CTLA-4 cascade (Greenwald et

al. 2005). Signals through the B7:CD28 superfamily are major coordinators of the critical balance between the stimulatory and inhibitory signals required for the proper immune response to pathogens and for maintaining self-tolerance. In order to stabilize the binding of T cell to APCs, T cells also express other membrane receptors known as accessory or adhesion molecules (Crow 2006). Most of the adhesion molecules belong to the integrin and selectin families and CD44. For example the LFA-1 integrin, expressed on the T cell surface, and its ligand, ICAM-1, expressed on the surface of the APC, are key mediators of this interaction, and TCR stimulation promotes stable T cell–APC contact by rapidly enhancing LFA-1 functional activity (Pribila et al. 2004, Lebedeva et al. 2005).

1.2 Basic mechanisms of tolerance

Although the main function of the immune system is to protect the host from pathogens, another important function lies in its mechanism to distinguish self from nonself. Immunological self-tolerance is defined as a stable state in which immune system does not react destructively against self-molecules, cells or tissues (Pugliese 2004). In order to achieve and maintain tolerance the immune system has evolved several mechanisms which operate both centrally and in the periphery. The importance of various tolerance mechanisms is underlined by the fact that the failure of self-tolerance can lead to the autoimmune responses, cellular or tissue destruction, which ultimately can manifest as an autoimmune disease (Pugliese 2004).

1.3 Thymus and its role in the establishment of central tolerance

The thymus is an essential organ for the T cell maturation and establishment of self-tolerance. Histologically, the thymus is composed of many lobules, each of which is divided into outer cortical and inner medullary area (Boyd et al. 1993). The cortex contains mainly immature thymocytes, few epithelial cells and macrophages, with which the immature thymocytes are closely associated. In contrast, the medulla contains mainly epithelial cells and mature thymocytes, in addition to the macrophages and DCs. The characteristic structure in the human thymus medulla is Hassall´s corpuscles, which are formed by concentric layers of epithelial cells (Boyd et al. 1993). The importance of these clumps of epithelial cells was recently demonstrated by Watanabe et al. 2005 (Watanabe et al. 2005). So far it has been thought that Hassall´s corpuscles act in the removal of apoptotic thymocytes and in the maturation of developing thymocytes. Watanabe et al. showed that human Hassall's corpuscles express thymic stromal lymphopoietin (TSLP), a chemokine that activates thymic DCs that in turn lead to the generation of regulatory T cells (Tregs) within the thymus (Watanabe et al. 2005). This finding suggests that Hassall´s corpuscles play an important role in the DC-mediated central tolerance.

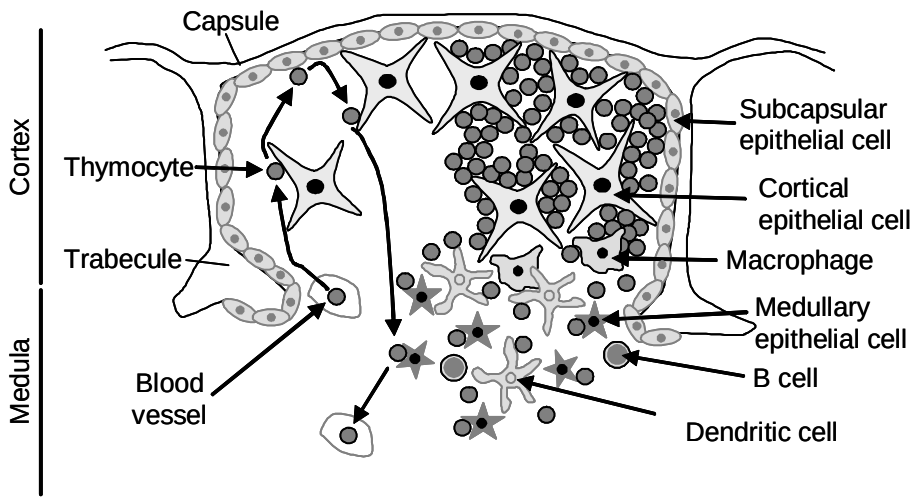

Figure 1. Schematic presentation of thymus architecture. The main cell types and the stepwise cell-to-cell interactions along the migratory route of developing T cells are shown (modified from Kyewski et al. 2002).

The dynamic relocation of developing lymphocytes into, within and out of the multiple environments of the thymus represents the well studied development of T cells, which consists of several processes. During the process of development, the phenotype of thymocytes significantly changes and developing thymocytes can be divided into double negative (DN), double positive (DP) or single positive (SP) cells, depending on their expression of CD4 or CD8 markers. The main phases of thymocyte development are the entry of lymphoid progenitor cells into the thymus and their proliferation as DN cells; the generation and positive selection of CD4+CD8+ DP thymocytes in the thymus cortex; the following differentiation into SP thymocytes and their negative selection in the medulla; and finally, the export of mature T cells from the thymus (**Figure 1**) (Petrie 2003, Gray et al. 2005, Takahama 2006). To ensure the correct order of these processes, the developing thymocytes and thymic stromal cells must communicate with each other both in cell-cell contact and remotely. This lympho-stromal interaction is a bilateral coordination or crosstalk between architectural stromal cells and migrating thymocytes (van Ewijk et al. 1994, Takahama 2006). In this way the developing thymocytes have to create their own path by interacting with stromal cells and by changing the stromal environment for further development. Among such crosstalk signals, chemokines that are produced by thymic stromal cells in their individual microenvironment have the crucial role in guiding the direction of migratory thymocytes. In turn, the developing thymocytes will find their way by sequentially expressing different receptors for these chemokines. Recent studies of mice that are deficient for certain chemokines or chemokine receptor have underlined the important role of chemotactic guidance in controlling T cell development in the thymus. For example in mice deficient for CCR7 or its ligands mature SP thymocytes are arrested in the cortex and do not accumulate in the medulla (Ueno et al. 2004). These mutant mice are defective in forming the medullary region of the thymus.

The role of central tolerance mechanisms is to ensure that the mature T cells that are exported from the thymus are functional and self-tolerant. Deletion of autoreactive thymocytes through the negative selection is considered as a main mechanism for the establishment of central tolerance (Nossal 1994, Anderson et al. 1996, Starr et al. 2003, Pugliese 2004). The negative selection occurs within the thymus medulla, where self-antigen-MHC complexes are

presented to developing lymphocytes by medullary thymic epithelial cells (mTECs) and DCs. Any thymocyte showing strong reactivity will be forced to undergo apoptosis. As a result of this strict selection mechanism only 1-3% of thymocytes survive and are exported from the thymus (Takahama 2006). In past years accumulating evidence has been obtained that a subset of the medium- to high-affinity autoreactive developing T cells are positively selected and differentiated into Tregs (Sakaguchi 2004). This functionally distinct subpopulation of CD4+ T cells constitutively expresses the IL-2 receptor α chain (CD25) and transcription factor *FoxP3*, and has a special function to suppress autoreactive T cells in the periphery (Sakaguchi 2004).

In recent years much attention has been given to thymic medulla, in particular to the mTECs as these cells express a large number of peripheral tissue-restricted self-antigens (Derbinski et al. 2001, Gotter et al. 2004). This unique property of thymic mTECs is termed promiscuous gene expression and it functions to promote self-tolerance to otherwise tissue-specific proteins thus preventing organ-specific autoimmunity (Derbinski et al. 2001). The range of promiscuously expressed molecules covers almost every tissue of the body (Kyewski and Klein 2006). Some of the promiscuously expressed tissue-specific antigens are known as target autoantigens in autoimmune diseases such as insulin, glutamic acid decarboxylases (GAD65 and GAD67), and a protein tyrosine phosphatase-like protein IA-2 in type 1 diabetes (T1D); thyroid peroxidase and thyroglobulin in autoimmune thyroid disease or myelin basic protein in multiple sclerosis (Gotter et al. 2004). The promiscuously expressed genes often co-localize in chromosomal clusters and it has been suggested that they might constitute up to 10% of the whole genome (Gotter and Kyewski 2004). Studies with Aire-deficient mice have demonstrated that the loss of Aire function correlates with the loss in expression of a subset of promiscuously expressed proteins, thus suggesting that Aire is involved in the regulation of expression of specific self-antigens involved in thymic selection (Anderson et al. 2002).

1.4 Mechanisms of peripheral tolerance

Although central tolerance is considered as a main mechanism of tolerance induction, it is not efficient enough to eliminate all potentially autoreactive T cells, which have even been described in healthy individuals (Walker and Abbas 2002). These potential autoreactive cells are kept under control by peripheral tolerance mechanisms operating in lymphoid and nonlymphoid organs, which function as supplementary to the central tolerance and are considered crucial to prevent autoimmunity (Walker and Abbas 2002, Pugliese 2004). In general, the peripheral tolerance mechanisms can be divided based on the mode of action into T cell intrinsic, where autoreactive T cells are directly targeted, and T cell extrinsic, where autoreactive T cells are targeted indirectly through additional cells such as DCs and Tregs (Walker and Abbas 2002).

One of the main mechanisms operating to eliminate autoreactive T cells occurs through activation-induced cell death (AICD) (Walker and Abbas 2002), in which activation through the TCR results in apoptosis. In peripheral T cells AICD is caused by the induction of expression of the Fas (CD95) or TNF receptor (Fas et al. 2006). The physiological importance of Fas/Fas ligand (*FasL*) signaling has been revealed by the finding that in humans, mutations in either the *Fas* or the *FasL* gene impair the elimination of activated peripheral T cells causing an autosomal dominant disorder called autoimmune lymphoproliferative syndrome (ALPS) (Fisher et al. 1995). T cell anergy is another mechanism to neutralise autoreactive T cells in the periphery. By the definition, anergy is referred to as a state of nonresponsiveness to activation in the absence of co-stimulatory signals (Schwartz 1993). Anergy leads to the functional inactivation of T cells, which will be unresponsive to secondary stimulation, even

if this includes both TCR and co-stimulatory signals (Macian et al. 2004). The extrinsic mechanism of peripheral tolerance involves active suppression of T cell responses by Tregs, which are produced in the thymus (see above) and induced in the periphery in an antigen specific fashion (Sakaguchi 2004).

1.5 Dendritic cells and their role in peripheral tolerance

Dendritic cells (DCs) are leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells (Hart 1997). These professional APCs are widely distributed throughout the body. DCs originate from CD34+ progenitor stem cells in the bone marrow which migrate via the blood stream into the peripheral tissues where they encounter essential growth factors such GM-CSF, IL-4, IL-15, TNF-α, TGF-β and IL-3 secreted by various cell types such as endothelial cells, mast cells, keratinocytes and fibroblasts (Mohamadzadeh and Luftig 2004, Reis e Sousa 2006). These growth factors determine the fate of the immature DCs to differentiate into myeloid or lymphoid precursor cells. Myeloid precursors differentiate into immature myeloid DCs whereas lymphoid precursors differentiate into plasmacytoid DCs. Myeloid DCs constitute the dominant subtype in the periphery and are responsible for the efficient antigen capture and presentation to T cells. Plasmacytoid DC subset is a prevalent DC subtype in the thymus but is also found in the periphery.

DCs play an important role in the priming of adaptive immune responses. The capture of antigens and the initiation of immune responses are carried out by DCs at different developmental stages, defined as immature and mature (Steinman 1991, Steinman and Nussenzweig 2002, Reis e Sousa 2006). Immature DCs, found mostly in nonlymphoid tissues, continuously sample the antigenic environment. These cells are specialized in antigen capture and processing, while being unable to stimulate T cells. After the recognition of antigen, DCs migrate out of nonlymphoid tissues into the lymph nodes, where they reside in the T cell areas. During their migration, the DCs mature to become extremely efficient at presenting antigens and stimulating T cells. Only mature DCs have the ability to prime an immune response. DCs display processed antigenic peptides on MHC II molecules to CD4+ T cells (Dubois et al. 1997), which then become activated along with the co-stimulatory signals (e.g., CD80, CD86), which are delivered by DC in lymphoid organs. In addition, the interaction between T cells and DCs is mediated by several accessory receptors and their ligands for example CD40 and CD40L and adhesion molecules (Cella et al. 1996). Activated myeloid DCs release IL-12, which modulate and stimulate the production of IFN-γ from T cells whereas plasmacytoid DCs produce high levels of type I IFNs (Mackey et al. 1998).

In addition to their role in initiating immune response against pathogens, DCs play an important role in the induction tolerance. DCs present self-antigen via MHC class molecules in the thymic medulla. Studies have shown that if MHC II molecules are only expressed by the thymic cortical epithelium and not by DCs residing in the medulla, there is a high probability for an autoimmune disease (Reis e Sousa 2006). Therefore, DCs have a critical role in the educative process of thymic T cells to self-antigens. DCs capture and present self-antigens that are tissue-specific, for example peptides derived from insulin producing β-cells of the pancreas (Kurts et al. 1996). In addition, DCs can tolerize autoreactive T cells in the periphery by inducing deletion, anergy or by expanding Tregs (Steinman and Nussenzweig 2002).

1.6 Autoimmune diseases

Autoimmune diseases arise as a result of failure in tolerance mechanisms, which leads to the activation of autoreactive T cells, B cells, or both. Abnormal thymic selection or regulation of autoreactive lymphocytes and abnormalities in the way the self-antigens are presented to the immune system are considered as most likely triggers for a rise in autoimmune response. The development of autoimmunity is complex including both genetic and environmental factors. Both these factors influence the overall reactivity and quality of the immune system cells (Marrack et al. 2001, Rioux and Abbas 2005). In addition, both factors might be involved in determining which antigens, and thus which organs, are targets in autoimmune response. Most autoimmune diseases are polygenic in which several susceptibility genes contribute to disease development. However, mutations in a single gene can also cause autoimmunity. For example, mutations in *FoxP3* gene, which encodes a transcription factor necessary for Treg development cause a rare syndrome named immune dysregulation, polyendocrinopathy X-linked syndrome (IPEX; (Wildin et al. 2001). As another example, defects in *AIRE* gene result in autoimmune disease named APECED (see below). Other monogenic autoimmune diseases include autoimmune lymphoproliferative syndromes (ALPS, OMIM 601859) type I and II (Fisher et al. 1995).

Overall, autoimmune diseases affect up to 5% of the population (Marrack et al. 2001). Often autoimmune diseases are classified as organ-specific and systemic diseases (Davidson and Diamond 2001). Organ-specific autoimmune diseases are characterized by an immune response targeting a specific organ and in some diseases even specific region within that organ as is the case for β-cells of the pancreatic islets in T1D (Marrack et al. 2001). Other examples include Hashimoto´s thyroiditis and Grave´s disease, both mainly affecting the thyroid gland. Although nearly every organ in the body can be a target in autoimmune response, endocrine glands including the thyroid, the pancreatic islets, and the adrenal cortex, are more susceptible to autoimmunity, and when viewed as a group, are responsible for the 50% of organ-specific autoimmune disease cases in Europe and the USA (Anderson 2002). Systemic autoimmune diseases are characterized by an immune response directed against ubiquitous self-antigens resulting in destruction of many tissues. Examples include systemic lupus erythematosus (SLE) and primary Sjögren´s syndrome, in which tissues such as skin, kidneys and brain are affected.

2. APECED

2.1 Clinical features of APECED

APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; OMIM 240300), also known as autoimmune polyglandular syndrome type 1 (APS-1), is a rare monogenic autoimmune disorder caused by mutations in the *AIRE* gene (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997). APECED is organ-specific autoimmune disease where many tissues, mainly endocrine glands are affected (Perheentupa 2002). The disease is defined by the presence of at least two of the three major clinical manifestations: adrenocortical failure (Addison´s disease), hypoparathyroidism and chronic mucocutaneous candidiasis (CMC) (Perheentupa 2002). In addition, APECED patients develop variable combinations of other autoimmune conditions such as T1D, autoimmune thyroid disease, autoimmune hepatitis, alopecia, teeth and nail anomalies, vitiligo or keratopathy (**Table 1**) (Ahonen et al. 1990). Most APECED patients have up to seven different disease manifestations, and additional disease components may appear throughout the lifetime (Ahonen et al. 1990, Perheentupa 2002). The disease usually starts in childhood with the manifestation of CMC, followed by the development of autoimmune

hypoparathyroidism and more than half of the patients also develop Addison´s disease (Ahonen et al. 1990).

Table 1. The occurrence of APECED disease components among 68 Finnish patients. Adapted from Ahonen et al. (1990).

Disease	Prevalence %
Mucocutaneous candidiasis	100
Hypoparathyroidism	79
Addison´s disease	72
Gonadal failure*	60
Hypothyroidism	4
Type 1 diabetes	12
Enamel hypoplasia	77
Alopecia	72
Vitiligo	13
Nail dystrophy	52
Malabsorption	18
Autoimmune hepatitis	12
Autoimmune gastritis	13

* Calculated for postpubertal individuals

APECED patients display a variety of autoantibodies against intracellular key enzymes present in the affected organs such as adrenal gland, gonads and placenta (Peterson et al. 1998). The major autoantigens in APECED are steroidogenic enzymes 17-α-hydroxylase (P450c17), 21-hydroxylase (P450c21) and cholesterol side-chain cleavage enzyme (P450scc), which all belong to the steroidogenic P450 superfamily (Krohn et al. 1992, Winqvist et al. 1992, Uibo et al. 1994). The expression of P450c21 is restricted to adrenal cortex, while P450c17 and P450scc are also expressed in gonads (Peterson et al. 1997). The occurrence of these antibodies predicts the appearance of adrenocortical and ovarian failure and therefore they serve as predictive markers for APECED (Ahonen et al. 1987). Additionally, various other tissue-specific proteins have been identified as targets for autoimmune response in APECED patients, including GAD65 and GAD67, insulin, thyroid peroxidase and thyroglobulin (**Table 2**). Interestingly, APECED patients may have organ-specific autoantibodies even when there are no signs of the disease (Söderbergh et al. 1996).

Table 2. Common autoantigens and targeted organs in APECED patients. Adapted from Meriluoto et al. (2001), Heino et al. (2001) and Peterson et al. (2004).

Autoantigen	Targeted organ	Disease
P450c21, P450c17α, P450scc	Adrenal cortex	Addison´s disease
P450c17α, P450scc	Gonads	Gonadal failure
GAD65/GAD67, ICA, IA-2 tyrosine phosphatase- like protein, insulin	Pancreas	Type 1 diabetes
Thyroid peroxidase, thyroglobulin	Thyroid gland	Hypothyroidism
P450 CYP1A2, P450 CYP 2A6, P450 CYP1A1, P450 CYP2B6 AADC	Liver	Autoimmune hepatitis
SOX9, SOX10	Skin	Vitiligo
Tyrosine hydroxylase	Scalp	Alpecia
Tryptophan hydroxylase	Gastrointestinal tract	Malabsorption

APECED belongs to the group of endocrine autoimmune diseases collectively termed autoimmune polyglandular syndromes (APS) (Eisenbarth and Gottlieb 2004). APECED (as APS-1) and APS-2 differ in their prevalence, time of onset and inheritance but share several features in their clinical picture (Eisenbarth and Gottlieb 2004). APS-2, also called Schmidt´s syndrome is defined as a combination of Addison´s disease with thyroid autoimmune disease and/or T1D and is more common than APECED (Guisan et al. 1969).

2.2 Genetics of APECED

APECED is inherited in an autosomal recessive manner. However, in one Italian family with APECED, mutation in *AIRE* has been described which acts in a dominant negative fashion (Cetani et al. 2001). The disease has been reported worldwide, although it is more prevalent among genetically isolated populations such as Finns (1: 25000), Sardinians (1: 14500) and Iranian Jews (1: 9000) (Ahonen 1985, Zlotogora and Shapiro 1992, Rosatelli et al. 1998). Among Finns and Iranian Jews, the disease is presumably inherited from one or two founder individuals. Moreover, the phenotype of the disease in both populations is slightly different which has been proposed to be the result of different mutations in the *AIRE* gene (Björses et al. 1996). In contrast to complex autoimmune diseases, APECED is not associated with any particular human leukocyte antigen (HLA) haplotype and both sexes are equally affected, in accordance with the autosomal recessive pattern of inheritance (Ahonen et al. 1988). Opposite to APECED, APS-2 as well as isolated Addison´s disease, show strong association with the MHC locus and are more common in females (Partanen et al. 1994).

To date more than 50 different mutations located over the coding region of *AIRE* gene have been identified. Most of the mutations are either nonsense or frameshift mutations resulting in a faulty protein or single amino acid-changing missense mutations (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997, Heino et al. 2001, Halonen et al. 2002, Meloni et al. 2002, Vogel et al. 2003, Meloni et al. 2005). Often the mutations are found in the functional protein domains of AIRE. Two mutations are more common, the R257X, a C-to-T substitution that changes arginine to a premature stop codon in exon 6 and a 13-bp deletion (967-979del13bp) in exon 8 (Nagamine et al. 1997). The R257X mutation is the predominant mutation among Finnish patients, found in 83% of APECED cases in Finland (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997, Bjorses et al. 2000), whereas the 967-979del13bp is the most prevalent mutation in North-American, British and Norwegian APECED patients (Wang et al. 1998, Heino et al. 1999a). APECED belongs to the Finnish disease heritage, which consists of a group of monogenic diseases that are enriched in the Finnish population due to founder effect and genetic isolation (Peltonen et al. 1999).

2.3 AIRE gene and protein

AIRE gene was identified by positional cloning in 1997 by two independent groups (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997). The *AIRE* gene consists of 14 exons spanning approximately 13 kb of genomic DNA. At the same time with the characterization of *AIRE* gene, a putative promoter containing a TATA box and a GC box was identified immediately upstream of the first exon (The Finnish-German APECED Consortium 1997). In addition, a putative CpG island associated with the 5´-end of the gene was observed (The Finnish-German APECED Consortium 1997). Two years after the identification of human *AIRE* gene several research groups cloned and characterized its mouse homologue (Blechschmidt et al. 1999, Mittaz et al. 1999, Wang et al. 1999). The murine *Aire* gene is located on chromosome 10 and shares highly conserved structural organization and sequence homology with its human counterpart. At the protein level, human

and murine *AIRE* share 71% similarity (Blechschmidt et al. 1999, Mittaz et al. 1999, Wang et al. 1999).

Along with the characterization of the mouse *Aire* gene, Mittaz et al. (1999) carried out a sequence analysis of the human and mouse *AIRE* promoter. They identified four homologous regions between *AIRE/Aire* promoters in a 600 bp region upstream 5´ of the translation start site. Based on computational analysis, conserved binding sites for several transcription factors were identified. These included binding sites for basal transcriptional complex such as TATA box, an inverted CCAAT box and AP-4 site. In addition, they noticed that both *AIRE/Aire* promoter regions contained conserved binding sites for thymus specific transcription factors such as Ets-1 and GFI1 and also binding sites for transcription factors that are essential in hematopoesis. They also reported five GC boxes in human *AIRE* promoter whereas mouse promoter, contains none of them.

The *AIRE* gene encodes a 545 amino acid proline-rich protein with a molecular weight of approximately 58 kDa (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997). The AIRE protein contains several motifs indicative of a transcriptional regulator. In the N-terminus AIRE harbours a conserved nuclear localization signal (NLS) and a homogenously staining region (HSR) domain; the SAND domain is located in the middle, and two plant homeodomain or PHD-type zinc fingers are located in the C-terminus of the protein (**Figure 2**). In addition, AIRE contains also four LXXLL motifs (Nagamine et al. 1997, The Finnish-German APECED Consortium 1997).

Figure 2. Schematic representation of the AIRE protein. HSR, homogenously staining region; L, LXXLL motif; SAND, Sp100, AIRE, NucP41/75 and DEAF-1/supressin; PHD zinc finger motif, plant homeodomain type motif; PRR, proline-rich region.

The PHD- type zinc fingers are mainly found in proteins involved in transcriptional control at the chromatin level (Aasland et al. 1995) These include proteins involved in chromatin-mediated regulation of transcription such as Mi-2 autoantigen and transcription intermediary factor 1 (TIF1) (Ge et al. 1995, Le Douarin et al. 1995). More than 400 proteins harbouring this protein motif have been identified (Capili et al. 2001). The importance of PHD finger domains for the normal functioning of the AIRE protein is underlined by the finding that the major mutations in AIRE reside in both PHD fingers (Heino et al. 2001). The LXXLL motif (where L is leucine and X any amino acid) was originally identified in coactivators of nuclear receptors (Heery et al. 1997) but it is present also in several other proteins that do not directly interact with nuclear receptors including transcription factors and coactivators such as CBP and p300 (Plevin et al. 2005). It has been demonstrated that the LXXLL motifs mediate interactions that can activate or repress transcription (Plevin et al. 2005). AIRE protein contains also a DNA binding domain called SAND (Sp100, AIRE, NucP41/75 and DEAF-1/supressin) (Gibson et al. 1998). SAND domain is found in several nuclear proteins which function in chromatin-dependent transcriptional control (Gibson et al. 1998). This domain coexists almost always with other functional protein domains, such as chromatin-associated and protein interaction-mediating motifs (Bottomley et al. 2001). Amino acids 1-207 in the N-terminal part of AIRE mediate its homodimerization (Pitkänen et al. 2000). In the N-terminus, AIRE harbours a highly conserved 100-amino acid HSR domain, which has been also found

in Sp100 and Sp140 proteins (Gibson et al. 1998). HSR domain has been shown to function as a dimerization domain in Sp100 and related proteins (Seeler et al. 1998).

The presence of domains in AIRE protein, which in other proteins are known to be involved in mediating their function in transcription, has suggested a similar role for AIRE. Several studies have demonstrated that AIRE has transcriptional transactivation properties (Björses et al. 2000, Pitkänen et al. 2000). AIRE can activate transcription from a reporter gene when fused to a heterologous DNA binding domain in the GAL4 system (Björses et al. 2000, Pitkänen et al. 2000) and it can also activate the IFN-β minimal promoter (Pitkänen et al. 2001, Pitkänen et al. 2005). The importance of this functional feature of AIRE physiology was underlined by the result that APECED-causing mutations inhibited AIRE-mediated transactivation in both GAL4 and IFN-β systems (Pitkänen et al. 2005). Attempts to identify AIRE interacting protein partners have successfully demonstrated AIRE interaction with the common coactivator CREB-binding protein (CBP) (Pitkänen et al. 2000). Further studies of the functional relevance of this interaction showed that the CBP protein acts as a coactivator for AIRE in the GAL4 and IFN-β systems (Pitkänen et al. 2005). Again, in case of APECED-causing mutations this coactivation was inhibited, demonstrating the importance of this interaction for AIRE function (Pitkänen et al. 2005).

At the subcellular level, AIRE has been found in the cell nucleus as a speckled pattern in domains resembling promyelocytic leukemia (PML) nuclear bodies (Björses et al. 1999, Heino et al. 1999b, Rinderle et al. 1999). These nuclear bodies are known to be associated with several transcriptionally active proteins. When transfected into the tissue culture cells, the protein forms a pattern of colocalisation with intermediate filaments or microtubules in the cytoplasm (Björses et al. 1999, Heino et al. 1999b, Rinderle et al. 1999). Also, in the cytoplasm the colocalization with the vimentin has been reported (Björses et al. 1999).

2.4 *AIRE* expression pattern

AIRE is mainly expressed in the thymus. A lower level of expression has been found in other immunological tissues including the spleen, lymph nodes and fetal liver (Björses et al. 1999, Nagamine et al. 1997, The Finnish-German APECED Consortium 1997). *AIRE* gene expression has also been detected in human differentiated DCs and peripheral blood monocytes (Kogawa et al. 2002). In the thymus, *AIRE* is found in two types of APCs, in mTECs and in cells of monocyte-dendritic cell lineage (Heino et al. 1999b). Interestingly, *AIRE* is not expressed in target organs of autoimmune destruction including adrenal cortex, adult liver and pancreas (Björses et al. 1999, Heino et al. 1999b). The mouse *Aire* has a similar expression pattern as its human homologue, although it has been detected also outside the immune system in a variety of organs including the brain, liver, kidney, pancreas, intestine, gonads, thyroid and adrenal glands (Blechschmidt et al. 1999, Heino et al. 2000, Halonen et al. 2001).

2.5 Function of AIRE in the establishment of self-tolerance

Aire-deficient mouse models have been a valuable tool in understanding AIRE function and the pathogenesis of APECED (Anderson 2002, Ramsey et al. 2002, Kuroda et al. 2005). Aire-deficient mice develop multiple organ-specific autoantibodies, multiorgan lymphocytic infiltrates, and infertility, thus mimicking the human APECED disease.

AIRE plays an essential role in the development of central tolerance to several autoantigens and participates in the negative selection of autoreactive T cells by directing the expression of

peripheral autoantigens in the thymus (Peterson et al. 2004). The expression of peripheral autoantigens in the thymus, also known as a promiscuous gene expression, is a unique property of thymic epithelial cells, especially mTECs (Klein and Kyewski 2000, Derbinski et al. 2001). AIRE supposedly directs the expression of hundreds of promiscuously expressed genes in mTECs. Anderson et al. demonstrated that thymic epithelial cells from Aire-deficient mice failed to promiscuously express many organ-specific autoantigens otherwise found in the thymus, therefore autoreactive T cells were able to escape negative selection and enter the peripheral immune system, eventually causing the development of autoimmunity (Anderson et al. 2002) It is currently not known how AIRE controls the expression of all these genes, although it has been hypothesized that AIRE might participate in higher-order complexes and affect transcription without directly contacting the DNA (Derbinski et al. 2005). In this way it can modulate the transcription of a particular promoter according to the composition of the particular transcriptional complex (Derbinski et al. 2005).

3. REGULATION OF GENE EXPRESSION

3.1 Levels of regulation

Our body consists of hundreds of different cell types, each having a specific role that contributes to the overall functioning of the organism. Every single cell contains the same genes, but only particular genes are expressed. The purpose of the regulation of gene expression is to achieve proper spatial and temporal expression of functional proteins. Deficient control of gene expression can often lead to disease such as cancer. The expression of eukaryotic genes is regulated at multiple levels, including transcription initiation and elongation, mRNA processing, transport, translation and stability. However, most of the regulation is believed to occur at the level of transcription initiation, where it is decided whether a particular gene will be expressed or not (Orphanides and Reinberg 2002, Maston et al. 2006).

DNA is packed into a highly organized nucleoprotein structure called chromatin. In dividing cells, the chromatin can be seen as individual chromosomes. In non-dividing cells, chromatin is distributed diffusely throughout the nucleus and is organized into condensed heterochromatin or more open (decondensed) euchromatin. Heterochromatin refers to the transcriptionally inactive genome and euchromatin corresponds to genome regions that contain actively transcribed genes. Chromatin structure, which is modified by histones, plays important role in the regulation of gene expression by allowing transcription factors to access genes. Modification of histones and thus nucleosome by chromatin-modifying and remodeling complexes determines whether a particular chromatin area will be active or inactive. Transcriptional regulation of gene expression depends on *cis*-acting regulatory sequences interacting with *trans*-acting DNA-binding transcription factors, which function either to enhance or repress transcription. The *cis*-acting regulatory sequences include the promoter, enhancers, silencers, insulators, or locus control regions. Transcription and chromatin modifications are closely interlinked, as the changes in local chromatin structure are required for the transcriptional activation (Orphanides and Reinberg 2002).

For the production of functional mRNA several RNA-processing steps have evolved such as capping, splicing, polyadenylation and RNA editing of the initial RNA transcript. Capping, the addition of a cap structure at the 5´- end of the transcript protects it from degradation and also serves as a binding site for proteins involved in export of the mature RNA into cytoplasm. In addition, splicing out of introns and generation of a 3´- end by the addition of a poly(A) tail to the pre-mRNA are essential steps in the production of the translatable mRNA

(Proudfoot et al. 2002). The stability of a particular mRNA is determined by specific interactions between its structural elements and RNA-binding proteins that can be general or mRNA-specific (Guhaniyogi and Brewer 2001). The mRNA turnover can contribute to rapid changes in gene expression in response to changing environmental or developmental conditions, but can also enable the cell to maintain the levels of a translatable transcript (Guhaniyogi and Brewer 2001).

Another important step in the regulation of expression level is post-translational modification of a protein. These include acetylation, hydroxylation, carboxylation, glycosylation, methylation, phosphorylation and cleavage, which can modulate the activity and spatial-temporal distribution of proteins in cells and tissues, generating huge diversity and complexity of gene products (Yang 2005). Furthermore, proteins are finally degraded by the ubiquitin-proteasome pathway, a process involving specific marking of a protein by ubiquitin, which is the signal for the proteasome mediated degradation (Glickman and Ciechanover 2002).

3.2 Transcriptional regulatory elements

The transcription of eukaryotic protein-coding genes is mediated by a complex network of factors that include sequence-specific DNA-binding proteins, transcriptional coregulators, chromatin-remodeling factors, enzymes that covalently modify histones and other proteins, and the basal transcriptional machinery (Smale and Kadonaga 2003, Maston et al. 2006).

A significant amount of the regulatory information that specifies the transcriptional program of each gene is encoded in the DNA sequence, such as in promoters and enhancers. However, the main target sequence for the factors that control the initiation of transcription is the core promoter. The core promoter elements are typically located in the near vicinity of the transcription start site, ~ -45 to +40 bp from the transcription start site (Smale 2001, Smale and Kadonaga 2003). The core promoter serves as a platform for the assembly of basic transcription machinery and polymerase II initiation complex, which together specify the start site of transcription and ultimately initiate the transcription of the gene (Thomas and Chiang 2006). The first identified core promoter element was the TATA box (consensus sequence TATA(A/T)A(A/T) (Yean and Gralla 1997). The TATA box is surrounded by GC-rich sequences and is recognized by the TATA-box-binding protein (TBP) subunit of the transcription factor IID (TFIID) complex. It is located about 30 bp upstream from the transcription start site. Other well known core promoter elements include Initiator element (Inr), downstream promoter element (DPE), transcription factor IIB (TFIIB) recognition element (BRE), and motif ten element (MTE), which all, except for BRE, are the binding sites for the TFIID complex (Lim et al. 2004). A recent statistical analysis of ~ 10 000 predicted human promoter sequences revealed that the core promoter sequence motifs might not be as common as generally believed (Gershenzon and Ioshikhes 2005). The analysis was based on four core promoter elements (TATA, Inr, DPE, and BRE) and the results indicated that the Inr was the most prevalent element, which was present in almost half of all analyzed promoters. DPE and BRE elements were each found in about one fourth of promoters, whereas TATA boxes were present in only one eighth of promoters. The most intriguing was the finding that approximately a quarter of all promoters analyzed had none of these four elements, suggesting that other additional yet undiscovered core promoter elements might exist.

The proximal promoter is a region immediately upstream from the core promoter, usually from -50 to -200 bp relative to the transcription start site. The proximal promoter region contains multiple binding sites for the sequence specific transcription factors, such as GC-

boxes (consensus sequence GGGCGG), which are recognized by the ubiquitous transcription factors Sp1 and Sp3, and CCAAT boxes (consensus sequence GGCCAATCT), which are recognized by the NF-Y transcription factor family proteins.

3.2.1 CpG islands

CpG islands are stretches of unmethylated DNA with a higher frequency of CpG dinucleotides (in which cytosine occurs immediately 5´ to guanine) compared to the entire sequence of human genome (Gardiner-Garden and Frommer 1987). CpG islands have been identified in organisms with large genomes, such as vertebrates and some higher plants. Data from computational analysis of the human genome sequence has revealed the presence of approximately 29000 CpG islands (Lander et al. 2001). In general, CpG islands are short GC-rich regions with an average GC content of 60 - 70%. More than 95% of CpG islands are smaller than 1800 bp, and more than 75% are smaller than 850 bp (Lander et al. 2001). Approximately half of all human and mouse genes contain a CpG island (Antequera and Bird 1993). These include housekeeping genes, which show wider expression pattern, and 40% of the genes with a tissue- or cell-type-specific expression pattern. The majority of the CpG islands associated with genes are located within the 5´-end regions of the genes or within the first coding exon sequences (Costello and Plass 2001, Yamashita et al. 2005). However, CpG islands do not occur exclusively within the promoter region of the gene, but have also been found in the coding regions and their position relative to transcriptional start sites varies (Jones 1999). For example, the pro-opiomelanocortin (*POMC*) gene has two separate CpG islands (Gardiner-Garden and Frommer 1994). A 5´ CpG island surrounding the *POMC* transcription start site and a 3´ CpG island which lies approximately 5 kb downstream covering the third exon of the gene (Gardiner-Garden and Frommer 1994). Other similar examples include the human gene *APOE*, *p16*, *PAX6* and *MYOD1* (Jones 1999).

3.2.2 Enhancers and silencers

Enhancers are positive *cis*-acting regulatory elements that increase the basal level of transcription and are typically located at long distances from the core promoter. They function independently of their distance and orientation relative to the promoter (Maston et al. 2006). Enhancers are often composed of a cluster of transcription factor binding sites (within 200-300 bp region), which work cooperatively to enhance transcription. In contrast to the core promoter elements, which are bound by ubiquitous transcription factors, the enhancer elements can be recognized also by tissue-specific transcription factors (Maston et al. 2006). Silencers are sequence-specific negative regulatory elements that function to silence or reduce the transcription of a gene. Typically they function as enhancers independently of their orientation and distance from the promoter (reviewed in Ogbourne and Antalis 1998). They can be located as part of a proximal promoter, as part of an enhancer, or as an independent regulatory element far from their target gene, in its intron or in its 3´-untranslated region (Maston et al. 2006). Silencers are recognized and bound by negative transcription factors called repressors. In some cases the proper functioning of the repressor protein requires the recruitment of negative cofactors also called as corepressors (Maston et al. 2006).

3.3 Epigenetic control of gene expression

Epigenetic mechanisms provide an “extra” layer of transcriptional control that regulates how genes are expressed, however, without a change in DNA sequence (Wolffe and Matzke 1999). Well characterized epigenetic mechanisms that have significant effect on gene expression are DNA methylation and various changes in chromatin configuration, i.e. histone acetylation and

methylation. These processes are crucial for the normal development and growth of cells, as the disruption of the balance of epigenetic network can cause several diseases, including cancer and genetic disorders. Recently, combining genomics and epigenetics, a new discipline called epigenomics has evolved (Callinan and Feinberg 2006). Epigenomics aims to study epigenetical modifications at the whole genome level, creating DNA methylation maps for all human genes in all major tissues.

3.3.1 Chromatin

DNA is packaged into a highly organized and dynamic protein-DNA complex called chromatin. The fundamental repeating unit of chromatin is the nucleosome, which is composed of an octamer of the four core histones (two copies each of histones H2A, H2B, H3, and H4) and 147 base pairs of DNA wrapped twice around the histone octamer (Luger et al. 1997, Strahl and Allis 2000). Nucleosomes are connected by small segments of linker DNA and further assembled into higher-order structures, which are stabilized by the histone H1. Chromatin serves as a template for various essential biological processes including transcription, replication, recombination and cell cycle progression. Nucleosomes are organized, mobilized and remodeled by chromatin modifying enzymes and chromatin remodeling complexes which as a result will determine the accessibility of the underlying DNA to the regulatory factors (Felsenfeld and Groudine 2003). In general, it is believed that the chromatin remodeling and modifying complexes function in concert and in a temporal order to regulate gene expression.

Chromatin remodeling enzymes exist as multi-subunit complexes that use the energy of ATP hydrolysis to disrupt or alter the association of histones with DNA, thus making DNA more accessible for various regulatory proteins (Vignali et al. 2000, Kadam and Emerson 2002). Based on their protein compositions and functions, these ATP-dependent remodeling complexes can be divided into several classes. These include the SWI/SNF (BAF), imitation-switch (ISWI), INO80, sick with rsc/rat (SWR1) and Mi-2/CHD groups (Saha et al. 2006). These classes of remodeling complexes are highly conserved throughout eukaryotes, each one being specialized on a particular chromatin task.

3.3.2 Histone modifications

Histones, the building blocks of a nucleosome, are small positively charged proteins. Each core histone is composed of a conserved structured domain and an unstructured N-terminal tail domain, which extends out from the nucleosome core. Histone tails are essential for the higher-order folding of the chromatin and provide binding sites for the non-histone regulatory proteins. They are subject to various covalent post-translational modifications that include acetylation, methylation, phosphorylation, ubiquitination, sumoylation and poly-ADP-ribosylation (Strahl and Allis 2000). Modifications of histone tails has a profound effect on chromatin structure by affecting the local environment (by changing the charge of histones, which allows decondensation); facilitating the binding of non-histone proteins, such as transcription factors, to access DNA and regulate gene expression; or allowing the interaction of various chromatin-remodeling complexes (Jenuwein and Allis 2001, Grewal and Moazed 2003, Iizuka and Smith 2003, Martin and Zhang 2005). The addition or removal of covalent modifications on histone proteins is performed by a group of proteins collectively termed as chromatin modifying complexes. Most studied chromatin modifying complexes are histone acetyltransferases (HATs) and histone deacetylases (HDACs).

The diversity of histone tail modifications and the combinatorial potential allow storing the epigenetic information within the histone tails. It has been postulated that these modifications create a "histone code", which determines whether a particular gene will be active or inactive (Jenuwein and Allis 2001). According to this hypothesis the modification marks on histone tails are read by proteins, which specifically recognise distinct modifications and accordingly regulate downstream functions such as transcription.

3.3.3 Histone acetylation and deacetylation

Histone acetylation and deacetylation generally correlate with transcriptional activation and repression, respectively (Eberharter and Becker 2002). Acetylation of the lysine residues at the N-terminal histone tails results in the neutralisation of their charge and this leads to the weakening of histone-DNA contacts making DNA more accessible to transcription factors. The main acetylation sites include five highly conserved lysines (K) in histone H3 (K9, K14, K18, K23 and K27), four in H4 (K5, K8, K12 and K16), plus less conserved sites in histones H2A (K5, K9) and H2B (K5, K12, K15, K20) (Carrozza et al. 2003, Sims et al. 2003). Histone acetylation, a transfer of acetyl groups from acetyl coenzyme A (acetyl-CoA) onto histone acceptors (i.e. the ∋-amino groups of conserved lysine residues within the core histones), is carried out by a class of enzymes termed as histone acetyltransferases (HATs). Several HAT families are identified, including TFIID subunit TAF250, p300/CBP and GNAT superfamily (GCN5-related *N*-acetyltransferases) (reviewed in Marmorstein and Roth 2001, Carrozza et al. 2003). GNAT includes proteins involved transcriptional initiation such as Gcn5 and PCAF (reviewed in Vetting et al. 2005). The p300/CBP family HATs function as coactivators for multiple transcription factors (Ogryzko et al. 1996). GCN5 family HATs are known to activate a distinct subset of genes, whereas for example the p300/CBP family HATs act as global transcriptional activators. Several HATs like PCAF, GCN5 and TAFII250 contain a bromodomain, which is also present in some chromatin-associated proteins (Horn and Peterson 2001). The bromodomain, also known as acetyl-lysine binding domain, specifically recognizes and binds to the acetylated lysines (Zeng and Zhou 2002). Usually HATs form complexes with many cofactors that allow either gene-specific or genome-wide activity. Histone acetylation plays a crucial role also in other relevant biological processes such as DNA replication, DNA repair and cell-cycle progression (Carrozza et al. 2003, Kurdistani et al. 2004).

The opposite process to histone acetylation is histone deacetylation, the removal of acetyl groups from histone tails. This is catalyzed by histone deacetylases (HDACs) (reviewed in de Ruijter et al. 2003). In cells, HDACs are often part of large multiprotein complexes that are recruited to promoter sequences through their interaction with specific DNA-binding transcription factors. Based on sequence similarity to the yeast founding members mammalian HDACs are divided into three classes. Class I HDACs have homology to the yeast global transcriptional regulator Rpd3 and include HDAC1, HDAC2, HDAC3, and HDAC8 (Khochbin et al. 2001, Gregoretti et al. 2004). Class II contains yeast deacetylase Hda1 homologous histone deacetylases HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10. Seven members are known from class III HDACs and their catalytic domains are similar to yeast NAD^+-dependent deacetylase Sir2 (Gray and Ekström 2001, de Ruijter et al. 2003). Different HDACs have different functions, which is implicated also by the fact that for example class I HDACs are ubiquitously expressed and found in the nucleus, while class II HDACs have tissue-specific expression patterns and shuttle between the nucleus and cytoplasm on certain cellular signals (Khochbin et al. 2001, Marks et al. 2003).

3.3.4 Histone methylation

Histone methylation is involved in various biological processes, including transcriptional regulation, heterochromatin formation and X-chromosome inactivation (reviewed in Kouzarides 2002). Histones are methylated either on their arginine (R) or lysine (K) residues. Arginines can be mono- or dimethylated within the tails of histone H3 (R2, R17, R26) and H4 (R3). Histone lysine methylation takes place on histones H3 (K4, K9, K27, K36 and K79) and H4 (K20). In addition to mono- and dimethylation, histone lysines can also be trimethylated (Santos-Rosa et al. 2002). Recently was discovered that the PHD-type zinc finger domain, found in a variety of proteins including AIRE, functions as a highly specialized methyl-lysine binding domain (Li et al. 2006, Peña et al. 2006, Shi et al. 2006, Wysocka et al. 2006). PHD finger domain was found to bind specifically to the trimethylated K4 residue on histone H3 (H3K4) and link it to the activation of gene expression or repression. Histone methylation is carried out by histone methyltransferases (HMTs), which use S-adenosyl-methionine (SAM) as the methyl group donor (Zhang and Reinberg 2001, Kouzarides 2002). HMTs are grouped into two families: histone lysine methyltransferases (HKMTs), which catalyze the methylation of lysine residues; and protein arginine methyltransferases (PRMTs), which catalyze the methylation of arginine residues (Margueron et al. 2005). The lysine methyltransferases are divided into two main classes, including SET domain-containing (named after proteins in which they were found - Drosophila Su(var)3-9, Ez and Trithorax) and non-SET-domain containing lysine methyltransferases (Kouzarides 2002). In humans, the SET domain containing HMTs include SUV39H1 and SUV39H2 (Firestein et al. 2000). This class of enzymes is required for the proper formation of heterochromatin and they are responsible for the catalysis of histone H3K9 methylation. The methylation of H3K9 is associated with the assembly of heterochromatin and to the stable silencing of genes (Freitag and Selker 2005). However, recent findings also indicate that H3K9 trimethylation is also associated with actively transcribed genes (Vakoc et al. 2005).

Until recently, the histone methylation has been suggested to be a stable and irreversible process. With the identification of the enzyme that can reverse the histone methylation, this view has changed. Shi et al. have identified and characterized the first histone lysine demethylase (Shi et al. 2004). This enzyme called lysine-specific demethylase (LSD1), a member of the family of polyamine oxidases, can demethylate a specific lysine (K4) at histone H3 by using an amine oxidase reaction (Shi et al. 2004). However, LSD1 is able to demethylate mono- and dimethylated H3K4, but not tri-methylated H3K4 and thus it is hypothesized that another yet to be discovered enzyme can remove tri-methyl groups. Another family of proteins with the histone demethylase activity was identified this year (Tsukada et al. 2006). Tsukada et al. have demonstrated that a novel JmjC domain-containing protein JHDM1 (JmjC domain-containing histone demethylase 1) specifically demethylates histone H3 at lysine 36 (H3K36) (Tsukada et al. 2006).

3.3.5 DNA methylation

DNA methylation is the most common eukaryotic DNA modification and constitutes one of the best-understood epigenetic phenomena. In eukaryotes, DNA methylation involves the addition of a methyl group to the carbon 5 position of the cytosine ring resulting in 5-methylcytosine (**Figure 3**) (Singal and Ginder 1999). This reaction is catalyzed by DNA methyltransferases (DNMTs), mostly in the context of the sequence 5´-CG-3´, which is also referred to as a CpG dinucleotide (Singal and Ginder 1999). The 5-methylcytosines are chemically unstable and can spontaneously be deaminated to thymines. As a result of this conversion, the number of CpG dinucleotides in the genome has during evolution gradually

decreased. Most of them are located in CpG islands. Methylation of promoter CpG islands plays a crucial role in gene silencing, genome imprinting, X-chromosome inactivation and carcinogenesis (Jaenisch et al. 1998, Feil and Khosla 1999, Jones and Laird 1999, Bird 2002).

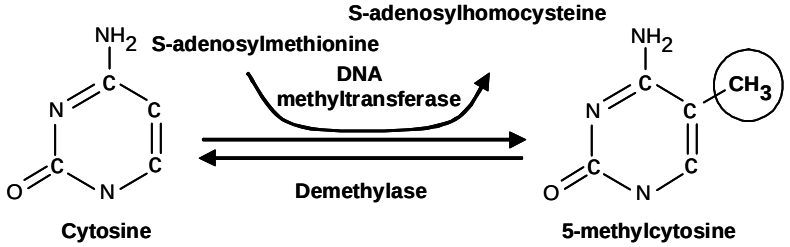

Figure 3. Schematic representation of the cytosine methylation.

Properly established and maintained DNA methylation patterns are absolutely essential for mammalian development and normal functioning of the organism. The mammalian DNA methylation machinery has two main components, the DNA methyltransferases (DNMTs) that establish and maintain DNA methylation patterns, and the methyl-CpG-binding proteins (MBDs), which are involved in 'reading' the methylation mark (Bestor 2000). Mammalian cytosine methyltransferases can be divided based on their preferred DNA substrate into *de novo* and maintenance methyltransferases (Bestor 2000). The *de novo* methyltransferases are DNMT3A and DNMT3B, which function to establish new methylation patterns of the genome during embryonic development. The maintenance methyltransferase DNMT1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication, in other words, it functions to maintain the methylation pattern in somatic cells. In opposite to DNMT3A, DNMT3B and DNMT1, a fourth DNA methyltransferase, DNMT2 is not catalytically active and therefore not involved in maintaining methylation patterns. Another family member, DNMT3L is a DNMT-related protein, which does not have methyltransferase activity, but it has been demonstrated that DNMT3L can modulate the catalytic activity of DNMT3a and DNMT3b by physically associating with them (Aapola et al. 2002, Suetake et al. 2004).

3.3.5.1 Role of DNA methylation in the control of gene expression

A strong correlation between DNA methylation and the silencing of the gene expression has been known already for more than two decades (reviewed in Santos et al. 2005). This epigenetic phenomenon can regulate gene activity either at the local level, through effects in a single promoter or enhancer, or through global mechanisms that influence many genes within an entire chromosome or genome. In **Figure 4** a schematic presentation of the main methylation mechanisms is shown.

Two general mechanisms are known how DNA methylation inhibits gene expression, both of them are considered to be biologically relevant. First, DNA methylation can down-regulate gene expression by preventing the binding of the basal transcription machinery and of ubiquitous transcription factors to their recognition sequences (Tate and Bird 1993, Attwood et al. 2002) Several transcription factors such as AP-2, NF-κB, c-Myc/Myn, and the cyclic AMP-dependent activator CREB, contain CpG residues in their consensus sites and bind weakly to methylated recognition elements (Kass et al. 1997). In contrast, the activities of

other factors such as Sp1 and CTF, which bind to CpG containing response elements, are independent of CpG methylation (Hapgood et al. 2001). Many transcription factors however, have no CpG dinucleotide residues in their binding sites. The second mechanism is more complex and opposite to the first one, as it involves proteins that are attracted to, rather than repelled by, methylated CpG sites (Kass et al. 1997, Newell-Price et al. 2000). According to this mechanism, DNA methylation is followed by recruitment of MBPs that preferentially recognize methylated DNA. In turn, MBPs associate with HDAC and chromatin remodelling complexes causing stabilization of condensed chromatin. Six mammalian MBPs have been characterized so far, named MBD1-MBD4, MeCP2 and KAISO (Klose and Bird 2006). All of them, except KAISO, contain a specific binding domain, the so-called methyl-CpG-binding domain (Newell-Price et al. 2000, Klose and Bird 2006). KAISO recognizes methylated DNA through its zinc-finger domains (Prokhortchouk et al. 2001). All MBPs are involved in methylation-dependent silencing of gene expression. MBPs preferentially bind to methylated CpG islands and recruit chromatin remodeling co-repressor complexes, thus blocking the access needed by transcription factors for the initiation of transcription (Newell-Price et al. 2000, Attwood et al. 2002). In addition, it has been shown that MeCP2 can associate with the histone methyltransferase activity by specifically methylating K9 of histone H3 (Fuks 2005).

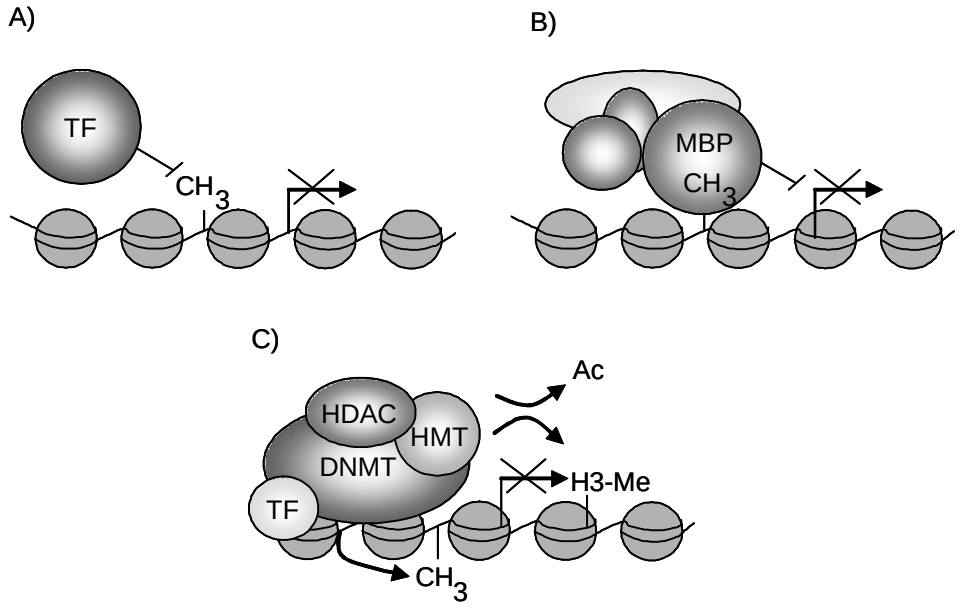

Figure 4. Mechanisms how DNA methylation can repress gene expression. A) DNA methylation of CpG sites in the cognate DNA-binding sequences of some transcription factors (TF) inhibits DNA binding by blocking activators from binding targets sites. B) Methyl-CpG-binding proteins (MBPs) directly recognize methylated DNA and recruit co-repressor molecules to silence transcription and to modify surrounding chromatin. C) In addition to their DNA methyltransferase activities, DNMT enzymes are also physically linked to histone deacetylase (HDAC) and histone methyltransferase (HMT) activities. In this case, the addition of methyl groups to DNA is coupled to transcriptional repression and chromatin modification. Modified from (Klose and Bird 2006).

In recent years it has become evident that DNA methylation and chromatin modifications are interlinked in the control of gene expression. New findings suggest that in addition to their catalytic role, the DNMTs have a non-enzymatic role in transcriptional silencing (Fuks 2005, Klose and Bird 2006). DNMTs can interact with HMTs and HDACs, thereby indicating that the DNMT-mediated gene silencing is associated with the chromatin modification (Fuks

2005). It has been proposed that the DNMTs have a dual function in silencing gene expression, directly through transcriptional repression and indirectly through epigenetic modification of cytosine (Klose and Bird 2006).

Interestingly, the density of methylation may correlate with the expression efficiency of various promoters, as a weak promoter can be silenced by only a few methylated CpG sites, whereas a higher density of methylation is required to repress a strong promoter (Boyes and Bird 1992). Even though distantly methylated sequences can contribute to repression, the level of repression is considered to be enhanced if the promoter itself is methylated (Nan et al. 1997). The existence of multiple MBPs with repressive properties supports the argument that these may be important mediators of the methylation signal, but their involvement in specific processes that require transduction of the DNA methylation signal is still unknown.

CpG methylation has been suggested to be involved in the control of tissue-specific gene expression and has been shown to suppress transcription of several tissue-specific genes, such as galectin and tissue transglutaminase gene (Newell-Price et al. 2000). For example, methylation of the human β-*globin* locus has been shown to occur in a tissue-specific manner (van der Ploeg and Flavell 1980), demonstrating a positive correlation between hypomethylation and gene expression in different tissues. With few exceptions, CpG islands on autosomal gene promoters do not become methylated in differentiated cells. For example, the CpG island in the muscle determination gene *MyoD1* is not methylated in nonexpressing tissues such as the brain (Jones 1999). Although specialized genes are variably methylated in differentiated cells, the exact correlation with expression has remained open.

AIMS OF THE STUDY

1) To isolate and characterize the function of the proximal human *AIRE* promoter region and identify key transcription factors regulating *AIRE* gene expression.

2) To examine the expression and function of *AIRE* in the MoDCs.

3) To determine the occurrence of anti-type I IFN autoantibodies in APECED patients' sera.

4) To examine the expression of *AIRE* in different thymoma subtypes.

PATIENTS, MATERIALS AND METHODS

1. Patients and control samples (IV and V)

For study IV, serum samples were derived from 77 Finnish, 16 Norwegian and one Pakistan patients with APECED. Clinical diagnosis of APECED was based on the presence of at least two of the most common disease components (Addison´s disease, hypoparathyroidism and CMC). As healthy and disease controls, sera from 10 *AIRE*-heterozygous unaffected first-degree relatives was analyzed. All samples were taken with informed consent and local ethical committee approval.

For study V, altogether 215 thymoma patients were included. Thymomas were subtyped according to the World Health Organization classification into type A (formerly termed "medullary"), type AB (formerly called "mixed") and type B (formerly called "cortical") (Rosai and Sobin 1999). In addition, B type thymomas were further subdivided into B1, B2 and B3 subtypes (Rosai and Sobin 1999). The diagnosis of autoimmune diseases was based on common clinical findings and the detection of respective serum autoantibodies. Normal thymic tissue removed during cardiac or thyroid surgery from immunologically healthy persons and non-neoplastic thymectomy specimens from patients with EOMG was used as controls.

2. *In silico* analysis (I and II)

Computer programs Match (http://www.gene-regulation.com) and MatInspector (http://www.genomatix.de) were used to identify putative transcription factor binding sites in the *AIRE* promoter sequence. The EMBOSS program (http://www.ebi.ac.uk/emboss/cpgplot) was used for the CpG island prediction within *AIRE* promoter sequence.

3. Cloning of human *AIRE* promoter fragments (I and II)

A 1235 bp human *AIRE* gene promoter fragment was isolated by PCR amplification from human genomic DNA and subcloned into the pHIV-LTR-luc plasmid (provided by Prof. Kalle Saksela, University of Helsinki, Finland) encoding firefly luciferase reporter gene and sequenced. The -1235 bp *AIRE* promoter construct was used as a template to synthesize a series of 5`-deletion reporter gene constructs. The primers were designed on the basis of the sequence of the human *AIRE* gene promoter (Nagamine et al. 1997) and included restriction enzyme sites for *Not*I in their 5´- end and for *Bam*HI in 3´-ends. Mutations were introduced into predicted promoter elements on *AIRE* promoter -1235 bp and -450 bp constructs using the GeneEditor *in vitro* site-directed mutagenesis system (Promega) and QuickChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing.

4. Cell culture (I-III)

HeLa (human cervical carcinoma cell line), COS-7 (SV40-transformed green monkey kidney cells) and TEC1A3 (epithelial cell line) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and a 100 units/ml penicillin-streptomycin mixture (BioWhittaker). THP-1 (human acute monocytic leukemia cell line) and U937 (human leukemic monocyte lymphoma cell line) cells were maintained in RPMI medium supplemented with 10% FCS and antibiotics.

5. Transfections and reporter gene assay (I and II)
COS-7 and TEC1A3 cells were transfected using ExGen500 transfection reagent (Fermentas) and HeLa cells were transfected using the calcium phosphate co-precipitation method. The U937 cells were transfected by electroporation with BioRad Gene Pulser apparatus at 250 V, 960 μF, with 0.4 cm cuvettes. Transfected cells were lysed after 24 h. Luciferase activity was measured using a luminometer with the Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. Results were normalized based on β-galactosidase activity of the lysates, determined by ONPG (O-nitrophenyl-β-D-galactopyranoside) as a substrate. To study the response of the -1235 bp *AIRE* promoter construct to phorbol myristate acetate (PMA), HeLa cells were stimulated with PMA (20 ng/ml) 24 h after transfection in 1 % serum starvation media for 6 h.

6. Electrophoretic mobility shift assay (I, II and IV)
For EMSAs nuclear extracts from HeLa and TEC1A3 were used. Oligonucleotides were annealed in equimolar amounts and end-labeled by T4 polynucleotide kinase with [γ-^{32}P] ATP. The binding reactions were performed by incubating nuclear extracts with poly(dI-dC) on ice for 15 min followed by additional 15 minutes incubation with appropriate ^{32}P-labelled oligonucleotide. Reactions were resolved in a 4.5% nondenaturing polyacrylamide gel and visualized by autoradiography. For competition experiments, a 100-fold excess of the corresponding unlabeled oligonucleotide was added, and for supershift experiments corresponding antibody was added to the preincubation mixture.

7. *In vitro* DNA methylation (I)
In vitro methylation of the -1235 bp reporter construct was performed with *Sss*I methylase (New England BioLabs) according to the manufacturer's instructions. After the purification through a Wizard DNA Clean-Up system (Promega) the methylated plasmid DNA was used for transfection.

8. Bisulfite genomic DNA sequencing (I)
For the bisulfite treatment experiments, genomic DNA was extracted from TEC1A3, HeLa, THP-1, and U937 cells following the standard protocols. Genomic DNA was denatured in 0.3 M NaOH at 37°C for 15 min. After the addition of 3 M sodium bisulfite and 10 mM hydroquinone, samples were mixed, overlaid with mineral oil, and incubated at 50 °C for 16 h. The modified DNA was purified through the Wizard DNA Clean-Up system (Promega) and denatured. The DNA was precipitated and resuspended in TE. The bisulfite-modified DNA was amplified by PCR using *AIRE* promoter specific primers and subsequently, the PCR products were cloned into pCRII-TOPO vector (Invitrogen) and analyzed by sequencing.

9. 5-aza-2´-deoxycytidine and trichostatin A treatments (I)
DNA methyltransferase inhibitor, 5-aza-2´-deoxycytidine (5-azaCdr) (Sigma), was added to TEC1A3, HeLa and U937 cells at final concentrations from 0.2 to 15 μM for 72 h. Histone deacetylase inhibitor, trichostatin A (TSA, 100 ng/ml, Sigma) was added for 24 h alone or at the end of the 5-azaCdr treatment. Subsequently, the RNA was extracted from the cells using the Total RNA Isolation system (Promega) according to the manufacturer's instructions. RNA was used for first-strand cDNA synthesis with the First-Strand cDNA Synthesis kit (Fermentas) and used for quantitative real-time RT-PCR.

10. Generation of MoDCs (III and IV)
Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of normal unrelated healthy donors using Ficoll-Paque Plus (Amersham Biosciences) gradient

centrifugation. The PBMCs were washed twice in PBS supplemented with 0.5% FCS and 2 mM EDTA and resuspended in this buffer. Monocytes were enriched from the PBMCs with monoclonal anti-CD14 antibody-coated microbeads (Miltenyi Biotec) and the MACS column system (Miltenyi Biotec). Immature DCs were obtained by supplementing the culture media with 1000 U/ml IL-4 and 800 U/ml GM-CSF (R&D Systems) for 7 days. On day 7, monocyte-conditioned medium was added and the cells were further cultured for 3 days to obtain mature DCs.

11. DNA microarray (III)

11.1 Filter array

Radiolabelled cDNA was generated from mRNA and hybridised to double-spotted Atlas Array™ Human Haematology/Immunology nylon membranes according to manufacturer's instructions (AtlasPure, Clontech Laboratories). Briefly, the membranes were pre-hybridised in Express-Hyb and preheated sheared salmon testes DNA for 30 min at 68 °C. The pool of labelled probes was denatured together with C_0t-1 DNA by boiling for 2 min in a water bath followed by incubation on ice for 2 min. The hybridization was performed for 16 h at 68 °C. The membranes were then washed three times in 2× SSC, 1% SDS and once in 0.1× SSC, 0.5% SDS for 30 min at 68 °C. Phosphor screens were exposed to the membranes at room temperature for 72 h. The screens were scanned with Phosphorimager (Molecular Dynamics) and the hybridization pattern was analysed using ImageQuant (Molecular Dynamics) and Microsoft Excel software (Microsoft).

11.2 Custom-made cDNA microarray

A custom cDNA chip having 1972 separate transcripts spotted in at least triplicates was obtained from the University of Turku, Finland. Cy3 and Cy5 labeled probes were synthesized using 15 μg of total RNA. The hybridization was performed at 68°C for 18 h. After washes, fluorescent intensities of Cy3 and Cy5 were measured using ScanArray Express HT laser scanner (Packard BioScience) and the expression data was quantitated with the GeneSpring software program (Silicon Genetics). The default background correction and normalization methods were used as proposed by the software for cDNA chips (LOWESS).

12. Quantitative real-time RT-PCR (I, III-V)

Quantitative real-time PCR was performed with the LightCycler instrument (Roche Applied Science) using a ready-to-use one-step QuantiTect SYBR Green RT-PCR kit (Qiagen). Reactions were set up in 15 μl of final volume containing 2 μl of sample cDNA or standards, 0.5 μM primers, and 7.5 μl of 2x RT-PCR master mix. The specificity of amplification was subjected to melting curve analysis. The relative amount of the AIRE transcript was normalized to the amount of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript in each cDNA. Primer sequences to amplify the AIRE or GAPDH were as follows: AIRE-F, CCCTACTGTGTGTGGGTCCT; AIRE-R, ACGTCTCCTGAGCAGGATCT; GAPDH-F, CTGAGCTAGACGGGAAGCTC; and GAPDH-R, TCTGAGTGTGGCAG GGACT.

13. Flow cytometry (III)

The analysis of cell surface antigens was performed using flow cytometry (FACScan, Becton Dickinson). After washing cells were resuspended in staining buffer (1×PBS, 3% inactivated FCS, 0.09% NaCl) and fluorescent-labelled antibody was then added. After incubation at 4 °C in the dark for 30 min, the cells were washed twice, fixed in 1% formaldehyde and analysed for fluorescence. The cells were stained with fluorescein isothiocyanate (FITC) labelled monoclonal antibodies: anti-HLA-DR, anti-CD25, anti-CD30L and anti-CD40 (PharMingen).

14. Western blotting (III)

Western blotting for ERK, p38 and JNK was done according to the manufacturer´s instructions (Cell Signalling Technology). The proteins were separated on SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane and blocked with 5% non-fat dried milk in TBS 0.1% Tween-20. The membranes were incubated with primary antibody diluted in TBS 0.05% Tween-20 followed by secondary biotinylated antibody and streptavidin-biotin horseradish peroxidase conjugate. Immunodetection was done using enhanced chemilumiscence method (Amersham Pharmacia Biotech).

RESULTS

1. CHARACTERIZATION OF AIRE PROMOTER (I and II)

1.1 Isolation of the 5´-flanking region of *AIRE* gene and identification of *AIRE* minimal promoter (I)

In order to gain insight into how the transcription of *AIRE* gene is regulated, the 5´-region of *AIRE* promoter was cloned and its key regulatory elements were subsequently identified. The human *AIRE* promoter region, containing 1235 bp upstream from the translation start site, was PCR amplified from human genomic DNA. The sequence corresponded to the genomic sequence in GenBank (accession number AB006684). This -1235 bp promoter fragment was subcloned in front of the luciferase reporter gene and used in subsequent reporter gene assays performed in COS-7 (I) and epithelial cell line TEC1A2 (I) and HeLa (II) cells. The -1235 bp reporter construct showed strong promoter activation in transiently transfected COS-7 and TEC1A3 cells, which indicated the presence of *cis*-acting elements in *AIRE* promoter region. In order to map the elements involved in *AIRE* transcriptional control, a series of deletions were introduced into 5´-end of the -1235 bp reporter construct. The luciferase assay with five different deletion constructs demonstrated that the elements required for the minimal *AIRE* promoter activity reside within the 350 bp upstream of the translation start site.

1.2 Characterization of transcription factors binding to *AIRE* promoter (I and II)

In order to identify and localize various regulatory elements within the *AIRE* proximal promoter we performed *in silico* analysis using the Match and MatInspector programs. The analysis led to the prediction of several *cis*-elements, including a TATA-box, a GC-box, an inverted CCAAT box, one binding site for transcription factor AP-1 and three binding sites for the Ets family transcription factors. In **Figure 5** all identified *AIRE* promoter elements are marked by their position. The putative Ets binding sites were named according to their relative distance from the translational start site as Ets-A, Ets-B and Ets-C.

Electrophoretic mobility shift assay (EMSA) combined with supershift assays were used to identify the binding of transcription factors to the predicted response elements on *AIRE* promoter. As a source of protein, HeLa and TEC1A3 nuclear extracts were used. The binding of AP-1 transcription factor complex was identified using the oligonucleotide corresponding to the putative AP-1 response element on *AIRE* promoter. EMSA results also revealed the specific binding of the transcription factor complex NF-Y to the CCAAT box and the binding of transcription factor Sp1 to the GC box corresponding oligonucleotides, respectively. The specificity was confirmed by performing competing experiments using unlabelled or mutated oligonucleotides, and antibodies against each investigated transcription factor. To determine the functional role of the TATA box and other putative transcription factor binding sites in *AIRE* transcriptional activation, site-directed mutations were introduced into each promoter element in the context of the -1235 bp luciferase reporter construct. Luciferase assay results showed that the mutation of the TATA box decreased *AIRE* promoter activity to 48% in COS-7 and to 69% in TEC1A3 cells, respectively. The disruption of the GC box, the CCAAT box, and AP-1 binding sites resulted in marked decrease of *AIRE* promoter activity in both COS-7 and TEC1A3 cells, demonstrating that these promoter elements are essential for *AIRE* gene regulation.

EMSA results also showed specific DNA-protein complexes using the putative Ets-site specific oligonucleotides. Furthermore, the effect of Ets factors on *AIRE* transcriptional

activation was studied using the -1235 bp and -450 bp *AIRE* promoter luciferase constructs. Transient cotransfection of Ets-1, Ets-2 and ESE-1 expression vectors with *AIRE* reporter constructs in HeLa cells resulted in *AIRE* transcription activation, with the highest *AIRE* promoter activation obtained by Ets-1 cotransfection. These results indicated that Ets factors are potential transcriptional regulators for *AIRE* promoter. In addition, the Ets-mediated activation of *AIRE* promoter was confirmed by luciferase experiments using Ets-site mutated reporter constructs (single or combined mutations). Based on the mutagenesis analysis it was found that only Ets-A and Ets-B sites are functional and able to specifically bind endogenous Ets transcription factors present in the HeLa cells. Taken together, the promoter studies demonstrated that *AIRE* transcription is regulated by ubiquitous transcription factors such as Sp1, NF-Y, AP-1 and Ets-family members.

```
-450 GAGGGCACCTGTCTCGGCTTTGCCCCATTCGAGCAGGGCCCTCGCCGAGGCAGGACAGGG
           Ets-C
-390 CCACATTCGGAAGTGAGAGTTCTCTGAGTCCCGCACAGAGCGAGTCTCTGTCCCCAGCCC
                          AP-1                Ets-B
-330 CCAAGGCAGCTGCCCTGGTGGGTGAGTCAGGCCAGGCCCGGAGACTTCCCGAGAGCGAGG
                                 Ets-A                      CCAAT box
-270 GAGGGACAGCAGCGCCTCCATCACAGGGAAGTGTCCCCGCGGGAGGCCCTGGCCCTGATT
           GC-box                                   TATA box
-210 GGGCGCCGGGGCGGAGCGGCCTTTGCTCTTTGCGTGGTCGCGGGGGTATAACAGCGGCGC
-150 GCGTGGCTCGCAGACCGGGGAGACGGGCGGGCGCACAGCCGGCGCGGAGGCCCCACAGCC
 -90 CCGCCGGGACCCGAGGCCAAGCGAGGGGCTGCCAGTGTCCCGGGACCCACCGCGTCCGCC
 -30 CCAGCCCCGGGTCCCCGCGCCCACCCCATG
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Figure 5. The human *AIRE* proximal promoter sequence. The TATA box, GC box, an inverted CCAAT box and binding sites for the AP-1 and Ets transcription factors are indicated and marked in bold. Translation start site is also shown in bold.

1.3 PMA-mediated *AIRE* promoter activation is regulated through Ets binding sites (II)

Phorbol myristate acetate (PMA) is a known activator of protein kinase C (PKC) and it has been shown to activate several transcription factor complexes, including Ets family members (Foletta et al. 1998). Therefore it was of interest to test whether PMA has any effect on *AIRE* promoter activation and whether Ets factors could be involved in this regulation. Reporter gene assay performed in HeLa cells resulted in 8-fold activation of the -1235 bp *AIRE* promoter construct after 6 hours of PMA treatment. On the other hand, a mutant reporter, where all three Ets sites were disrupted, showed markedly lower basal activity and reduced response to PMA stimulation. In a different experiment where the -1235 bp *AIRE* reporter construct was cotransfected with Ets-1 and Ets-2 and subsequently treated with PMA, a significantly higher level of *AIRE* promoter activity was seen (13-fold and 18-fold activation, respectively). Taken together, these results indicated the involvement of Ets factors in PMA-mediated *AIRE* promoter activation.

1.4 Epigenetic mechanisms are involved in the control of *AIRE* gene regulation (I)

The presence of a CpG island around the start site of *AIRE* promoter was suggested already in 1997 by the Finnish-German APECED Consortium, at the same time with the identification of *AIRE* gene (The Finnish-German APECED Consortium 1997). In accordance with this hypothesis, the analysis of the *AIRE* proximal promoter region indicated the presence of a 390

bp CpG island, which starts from approximately 300 bp upstream of the translational start site and encompasses the first exon. *In vitro* methylation of the -1235 bp promoter construct completely inhibited *AIRE* promoter activity in TEC1A3 and COS-7 cells, suggesting the involvement of promoter methylation in *AIRE* gene regulation. This possibility was studied by the bisulfite genomic DNA sequencing experiment, where the methylation pattern of individual CpG sites within the 390-bp fragment of the *AIRE* promoter was analysed in four *AIRE* non-expressing cell lines including HeLa, TEC1A3 and two monocytoid cell lines, THP-1 and U937. All studied cancer cell lines exhibited methylated CpG pattern, which was particularly obvious in monocytic cell line DNAs. In order to test the potential role of epigenetic modifications such as DNA methylation and chromatin modification in the control of *AIRE* expression, TEC1A3, U937, and HeLa cells were cultured in the presence of demethylating agent 5-azaCdR and HDAC inhibitor TSA. The relative *AIRE* mRNA levels were analysed using the quantitative real-time PCR. Treatment with 5-azaCdR alone resulted in up-regulation of *AIRE* expression in TEC1A3 and U937 cells and was further enhanced by the combined treatment with TSA. The 5-azaCdR treatment up-regulated *AIRE* expression also in HeLa cells, whereas TSA had no effect, suggesting that the level of chromatin modifications in *AIRE* promoter region varies between cell types. In conclusion, these experiments demonstrated that both methylation and histone deacetylation are involved in *AIRE* promoter regulation suggesting that these epigenetic mechanisms play a role in the control of *AIRE* expression.

2. AIRE HAS A ROLE IN MONOCYTE-DENDRITIC CELL MATURATION (III)

Results from Aire-deficient mice revealed the important role of Aire in directing the expression of peripheral autoantigens in mTECs, suggesting self-tolerance mechanism for these autoantigens in the thymus (Anderson et al. 2002, Ramsey et al. 2002). In addition to the thymus, *AIRE* expression has also been reported in peripheral lymphoid organs and in peripheral monocyte-derived DCs (MoDC) (Heino et al. 1999b, Kogawa et al. 2002). However, it remains unclear whether AIRE is involved in the peripheral tolerance mechanisms and what role it plays in the periphery. Therefore the expression of *AIRE* in the MoDCs was studied in more detail.

2.1 *AIRE* expression and AIRE-induced changes in MoDC lineage

AIRE expression was studied in the MoDC lineage using quantitative real-time PCR. During the DC differentiation process, a significant increase in *AIRE* mRNA level was already detectable in immature DCs and remained at the steady-state level during the maturation of DCs.

In order to study the function of AIRE during the DC maturation process, a human monocyte cell culture model was created by stably overexpressing AIRE in monocytoid U937 cells. The relative mRNA levels of 406 genes (~25% detected) in cDNA filter microarrays and 1972 genes (~60% detected) in cDNA chips were analysed for changes in the gene-expression profile related to AIRE overexpression. Altogether, 48 genes (3.7% of detected genes) were up-regulated and 24 (1.8% of detected genes) were down-regulated, by ectopic AIRE expression. The pattern of gene expression observed in AIRE-expressing U937 cells resembled those identified during the maturation process of monocyte- and bone marrow-derived DCs (Granucci et al. 2001, Le Naour et al. 2001, Tureci et al. 2003). AIRE up-regulated a broad range of genes, including genes involved in cellular adhesion (incl. *ICAM-1*), in chemokine and cytokine signaling (incl. *CCL22, IL-6*), genes involved in metabolism and oncogenesis (incl. *transferrin*), genes involved in signal transduction (incl. upstream

activators of ERK1/2- and p38-signaling pathways *MAPKK1*, *MAP2K2* and *MAP2K6*) and several surface antigens (incl. *CD37*) and transcription factors (incl. *RelA*, *RelB*, *JunB*). Transcripts suppressed by AIRE-expression included genes involved in signal transduction (incl. *MAPK4*), genes involved in protein turnover (incl. ubiquitin protein ligase *E3A*). The microarray results for certain genes were confirmed by quantitative PCR. Interestingly, the expression levels of common autoantigens for APECED such as *CYP17*, *CYP21* and *CYP11A1* remained unaffected in the cell culture model and during DC differentiation process.

2.2 The effect of AIRE expression on MAPK pathway

These results suggested that AIRE acts as an activator for the ERK1/2 and the p38 pathways, and as an inhibitor for the JNK pathway. In order to verify this hypothesis, AIRE-positive cells (stably transfected U937 cells) and AIRE-negative cells (control U937 cells) were analysed for the presence of phosporylated and unphosporylated forms of ERK1/2 using Western blotting. Indeed, AIRE expression could induce the ERK1/2 cascade, as indicated by an increase in the amount of the phosphorylated forms of ERK1/2, whereas the phosphorylation status of p38 and JNK remained unchanged (unpublished observation). In addition, reporter gene and EMSA assays demonstrated that the transcription factor AP-1, a MAP kinase cascade downstream target, exhibited stronger DNA-binding activity in AIRE-positive cells, thus supporting the original finding.

3. IDENTIFICATION OF ANTI-IFN AUTOANTIBODIES IN APECED PATIENTS (IV)

The occurrence of chronic mucocutaneous candidiasis (CMC), which is one of the first clinical manifestations of APECED, has also been reported for thymoma and myasthenia gravis (MG) patients (Meager et al. 1997). High titers of autoantibodies against IFN-α subtypes, IFN-ω and IL-12 have been found both in thymoma and MG patients' sera (Meager et al. 1997, Meager et al. 2003, Zhang et al. 2003). To determine whether these autoantibodies occur also in APECED patients' sera, a large set of Nordic APECED patient samples were analysed.

3.1 Autoantibodies against IFN-α subtypes and IFN-ω in APECED patients

Two cohorts of Nordic APECED patients (60 typical Finnish and 16 Norway APECED patients with both *AIRE* alleles mutated) were screened for the presence of anti-IFN autoantibodies in their sera. High titre IgG autoantibodies to most IFN-α subtypes and IFN-ω were found in all tested APECED patients using ELISA-based and functional IFN neutralising assays. Negative results were obtained from the control group, which consisted of ten unaffected heterozygous first-degree relatives of APECED patients. The analysis of kinetics of anti-IFN autoantibodies in serial samples from 51 Finnish patients demonstrated persistently high levels of neutralising antibody titres against IFN-α and IFN-ω in all patients, with some variations over the age points. The anti-IFN antibodies were found to strongly neutralise the biological activity of the IFN-α as well as IFN-ω. The analysis of autoantibodies against other cytokines demonstrated lower titer autoantibodies against IFN-β and IFN-λs in APECED patients' sera. However, autoantibodies to IFN-γ and other immunoregulatory cytokines, such as IL-10 and IL-12, were less detected and did not neutralise.

3.2 The anti-IFN-α and anti-INF-ω autoantibodies were found in APECED patients before the development of clinical disease

Anti-IFN-α and anti-INF-ω antibodies were present in APECED patients' sera already before the development of characteristic APECED autoantibodies. In some patients the anti-IFN autoantibodies were detectable even before the development of the three main clinical APECED disease components. Anti-IFN antibodies were mostly undetectable in unaffected heterozygous relatives of APECED patients, as well as in APS-2 patients and in patients with endocrine diseases most typical of APECED, suggesting that the presence of these antibodies is related to development of APECED.

3.3 Potentially autoimmunising IFN-α secreting cell types were localized in thymus medulla

In order to find out the potential autoimmunising cell type, the expression and secretion of IFN-α was studied in sections of normal thymus. Numerous IFN-α positive APCs were found in normal thymic medulla. In addition, strong evidence of local IFN secretion in thymic medulla was detected. Since no APECED thymic cells were available, the IFN-α production was studied using quantitative RT-PCR in monocytes and immature and mature DCs cultured from blood originating from a compound heterozygous APECED patient. It was found that IFN-α2 and IFN-α8 exhibited higher expression levels in immature DCs from APECED patient compared to healthy control samples.

4. LACK OF AIRE EXPRESSION IN THYMOMAS DOES NOT LEAD TO THE DEVELOPMENT OF APECED (V)

Thymomas are epithelial tumors of the thymus, which are often associated with autoimmune diseases (Eisenbarth and Gottlieb 2004). The most common autoimmune manifestation in thymoma patients is MG, found in 40% of thymoma cases (Evoli et al. 2002). Other occasionally occurring thymoma-associated autoimmune disorders include bone marrow aplasia and immunodeficiency (Hoffacker et al. 2000). In order to find out whether the autoimmunity seen in thymoma patients involves *AIRE*, its expression and also the expression of common autoantigens for APECED was analyzed in different thymoma samples.

4.1 Expression of *AIRE* in thymoma samples

The expression of *AIRE* and its splice variant *AIRE-2* was studied in a large set of clinical samples, originating from different thymoma subtypes (type A, AB, B1, B2/B3) using quantitative RT-PCR and immunohistochemistry. Both *AIRE* and *AIRE-2* were found to be absent in type A, AB and B2/B3 thymoma samples, whereas *AIRE* expression was detectable in few type B1 thymoma samples. Interestingly, the majority of thymoma samples were *AIRE* negative, but *AIRE* expression was observed in the adjacent residual thymus and in lymph nodes of the same patients.

4.2 The analysis of the common APECED autoantigens in thymomas

The analysis of the common APECED autoantigens by quantitative and semi-quantitative RT-PCR revealed lower expression levels of insulin and CYP1A2 in AIRE negative thymoma samples compared to the control thymus samples, demonstrating that the expression of insulin

and CYP1A2 depends on presence of AIRE. However, only slight reduction was noted for other APECED autoantigens such as CYP17, CYP21, GAD65 and thyroglobulin.

4.3 Autoimmune manifestations and autoantibodies in patients with thymoma or thymic follicular hyperplasia (TFH)

Analysis of serum autoantibodies and occurrence of autoimmune disorders in thymoma patients indicated that 67% of thymoma patients had manifested different autoimmune diseases, from which MG was the most prevalent, accounting for 83% of cases. Also, a correlation was found between the presence of autoantibodies and the observed autoimmune disease in thymoma patients. Interestingly, though most of thymomas lacked *AIRE* expression, none of the three main APECED disease components (adrenocortical failure, hypoparathyroidism and CMC) could be observed in thymoma patients, with few exceptions. Thymoma patients also mostly lacked the characteristic autoantibodies for APECED. The occurrence of serum autoantibodies and autoimmune disorders was also investigated in the early-onset MG (EOMG) patients. The EOMG patients usually develop thymic follicular hyperplasia (TFH). The presence of other autoimmune disorders in EOMG patients was found to be similar to that observed in thymoma patients. Also, EOMG patients were found to have similar autoantibodies like in thymoma patients. Analysis of anti-cytokine autoantibodies in thymoma patient sera demonstrated the presence of neutralising anti-IFN-α and IFN-ω autoantibodies, which were not found in EOMG patients' sera. In addition, anti-IL-12 autoantibodies were detected in thymoma patients' sera.

DISCUSSION

1. Regulation of the human *AIRE* promoter

Study I and II demonstrated that human *AIRE* promoter contains a classical TATA box assigning the promoter to the TATA+ promoter family. According to the recent analysis of promoter databanks, TATA+ promoters form only 10-22% of human promoter sequences (depending of promoter bank analyzed), which is significantly less than previously estimated (34%) (Gershenzon and Ioshikhes 2005). More importantly, functional binding analysis with transcription factors Sp1, NF-Y and AP-1 confirmed the role of these ubiquitously expressed transcription factors in *AIRE* regulation.

Study II demonstrated that Ets transcription factor family members Ets-1, Ets-2 and ESE-1 are positive regulators of *AIRE* transcription. Mutation of the indicated transcription factor binding sites decreased the *AIRE* promoter activity in reporter gene assay compared with the wildtype promoter construct, thereby demonstrating the requirement of these transcription factors for *AIRE* basal promoter activity. The Ets family proteins bind to DNA through a Ets-domain - a sequence that is highly conserved within different Ets proteins, implying that various Ets factors can recognize the same consensus sequence harboring the 5'-GGAA/T-3' core motif (Sharrocks 2001, Oikawa and Yamada 2003). Therefore it is likely that other Ets factors would participate in the regulation of *AIRE* gene transcription. For example, Ets family members such as PU.1 and Spi-B, which are expressed also in the thymus, might be involved in *AIRE* transcriptional control. Combinatorial control of their downstream genes is a characteristic feature of Ets family proteins, involving interactions between Ets proteins and other key transcription factors such as AP-1 (Verger and Duterque-Coquillaud 2002, Oikawa and Yamada 2003). In line with this, the highest *AIRE* promoter activity was seen when Ets-1, Ets-2 and ESE-1 were expressed in combination with AP-1, which indicated the cooperation between these transcription factors on *AIRE* promoter activation (unpublished observation). In addition, promoter studies revealed that PMA could up-regulate *AIRE* promoter in HeLa cells. PMA can activate several transcription factors, including NF-κB family members, AP-1 and Ets proteins, through the PKC pathway (Adunyah et al. 1991, Foletta et al. 1998). In accordance with this, it was shown that PMA induced *AIRE* transcription was dependent on Ets binding sites.

The restricted expression of *AIRE* in mTECs and to a lesser degree in other immune tissues suggests that its expression is tightly controlled. However, the analysis of *AIRE* promoter sequence did not reveal obvious binding sites for the thymus- or hematopoiesis-specific transcription factors. The epigenetic modifications have been previously demonstrated to coordinate cell type-specific expression. In particular, the importance of promoter methylation in the control of tissue-specific expression has been reported. For example, the expression of *SERPINB5*, a serine protease also functioning as a tumor suppressor, is regulated through the CpG island methylation (Futscher et al. 2002). Other examples include human oxytocin receptor (*OTR*) (Kusui et al. 2001) and endothelial nitric-oxide synthase (e*NOS*) genes (Chan et al. 2004). In this light, the CpG island methylation and histone deacetylation within the *AIRE* promoter could be implicated in the regulation of expression in restricted cell types. Indeed, the *AIRE* promoter experiments indicated an important role of genomic methylation in the control of *AIRE* expression. The human *AIRE* promoter contained 47 CpG motifs covering 390-bp region. All CpG positions showed a heavy methylation pattern in tissue-culture cell lines that normally do not express endogenous *AIRE*. As no thymic epithelial cell line expressing endogenous *AIRE* is available, the Study I was limited to

cancer cell lines not expressing endogenous *AIRE*. Therefore, in the future it would be very informative to perform this type of analysis for the samples obtained from various human tissues, including the thymus. Another evidence suggesting that the epigenetic modifications act in *AIRE* cell-specific expression comes from the finding that *AIRE* expression was up-regulated when the cells were treated with the DNMT inhibitor 5-azaCdR and HDAC inhibitor TSA. Although these experiments did not define the exact regions of histone acetylation, they support the view that chromatin methylation modifications, combined with histone acetylation state at the promoter, have a role in the regulation of *AIRE* expression.

Recently, Klamp et al. analyzed the *AIRE* mRNA expression in a large set of normal human tissues and cells, cancer specimen and methylation deficient human cancer cell lines by RT-PCR and quantitative real-time RT-PCR (Klamp et al. 2006). Using such a broad data basis, which included 36 different normal tissue and cell types, their results confirmed the previous findings that *AIRE* is predominantly expressed in the thymus and to a lesser degree in other immunological tissues. The majority of analyzed samples from tissues of nonlymphoid origin, such as breast, uterus, brain, muscle etc., revealed the lack of *AIRE* mRNA expression. More importantly, their data support the original finding of Study I confirming that the promoter CpG methylation contributes to transcriptional control of *AIRE* gene. Using human colon cancer HCT116 cell line which lacks natural DNA methylation activity, it was demonstrated that *AIRE* was expressed at the mRNA level, whereas its expression was absent in the parental cell line (Klamp et al. 2006).

To date the majority of information concerning signaling pathways involved in the regulation of *AIRE* expression originates from mouse studies. Several proteins have been identified as upstream regulators of *Aire* expression. Two studies have shown the involvement of the lymphotoxin pathway in the regulation of *Aire* expression in the mTECs (Boehm et al. 2003, Chin et al. 2003). Chin et al. have demonstrated that *Aire* expression is reduced in lymphotoxin-α (LTα) and lymphotoxin-β receptor (LTβR) -deficient mice, but it was reconstituted when the LTα-deficient mice were treated with an agonist monoclonal antibody directed against LTβR, suggesting that lymphotoxin pathway directs *Aire* thymic expression (Chin et al. 2003). However, in the second study led by Boehm et al., LTβR-deficient mice did not have any detectable change in *Aire* expression level in mTECs, although these mice also developed autoimmunity, a consequence attributed to a defective thymic development (Boehm et al. 2003). Decreased *Aire* expression levels have also been reported in the NF-κB-inducing kinase (NIK) and tumor-necrosis-factor receptor-associated factor (TRAF)6-deficient thymi (Akiyama et al. 2005, Kajiura et al. 2004). All these proteins have been shown to mediate NF-κB signaling through the alternative NF-κB pathway, which leads to the activation of p52-RelB (Mordmüller et al. 2003, Müller and Siebenlist 2003). The potential importance of the NF-κB pathway in *AIRE* expression is further supported by the finding that RelB-deficient thymus lacks *Aire* expression (Heino et al. 2000). However, as the block in RelB signaling pathway in thymus alters the differentiation of the medullary epithelial cells, then the absence of the *Aire* can be due to the abnormal differentiation process and not necessarily the result of direct regulation of *AIRE* gene. Therefore future studies are needed to clarify whether *AIRE* is directly regulated by the NF-κB pathway. Furthermore, in vitro studies have shown that *AIRE* expression in DCs can be induced by the human thymic stromal lymphopoietin (TSLP) (Watanabe et al. 2004), although the relevance of this finding in the context of *AIRE* expression awaits further experiments.

2. Function of AIRE in the periphery

AIRE has an established function in the thymus, but its role in periphery has remained elusive. In order to gain more knowledge on the function of *AIRE* in the periphery, Study III investigated the expression of *AIRE* and AIRE-induced changes in the gene expression profile in a human monocyte cell culture model and during the maturation of MoDCs. The expression of *AIRE* at the mRNA level was found to be increased during the differentiation of MoDCs and stayed at the similar expression level throughout the differentiation process. Expression array analyses of U937 cells stably overexpressing AIRE, and control cells, demonstrated that AIRE-induced changes in gene expression profile resembled phenotypic changes occurring during the maturation of DCs, including the up-regulation of *CCL22*, *CD25*, *ICAM-1* and *RelB* genes (Granucci et al. 2001, Le Naour et al. 2001, Tureci et al. 2003). In contrast, expression levels of typical steroidogenic autoantigens for APECED were not affected by the overexpression of AIRE in cell culture model, nor in *AIRE*-expressing DCs, suggesting that the role played by AIRE in the periphery is different from its established role in the thymus. It was proposed that in the periphery AIRE might regulate the expression of genes known to be up-regulated during the maturation process of MoDCs.

Based on the observation that AIRE is also expressed in peripheral APCs, it has been earlier suggested that AIRE might play a role in peripheral tolerance. Recently, Ramsey et al. demonstrated that indeed AIRE has a role in peripheral tolerance by maintaining self-tolerance through modulating the antigenic response of T cells (Ramsey et al. 2006). Using Aire-deficient mouse model, they showed that Aire-negative DCs activated naïve T cells more efficiently than the wild-type AIRE-positive DCs. As a consequence the Aire-deficient mice were found to have elevated number of DCs in spleen and lymph nodes, thus indicating that Aire is required for proper T cell activation by DCs in the periphery. More recently, the same group published results about the analyzis of 15-24 months-old Aire-deficient mice (Hässler et al. 2006) and their findings imply that Aire controls the development of APCs and marginal zone B cell activation.

3. Anti-IFN autoantibodies are present in APECED patients

Study IV demonstrated that APECED patients had increased levels of autoantibodies against type I IFNs, in particular to the IFN-α subtypes and IFN-ω. Interferons are named for their ability to interfere with viral replication and infection, and play an essential role in both innate and adaptive immunity (Pestka et al. 2004). IFNs are divided based on their sequence, receptor specificity and structure into two main classes: Type I IFNs (IFN-αs, IFN-β, IFN-ε, IFN-κ, IFN-ω, INF-δ and IFN-τ) and Type II IFNs (IFN-γ) (Pestka et al. 2004). Type I IFNs provide a defence against viral infections and their expression is induced at very high levels in plasmacytoid DCs and monocytes following the viral infections or stimulation through Toll-like receptors (Ozato et al. 2002).

Anti-type I IFN autoantibodies were already detected in APECED patients' sera before the development of other typical autoantibodies for APECED, and in some cases occurred even prior to the development of clinical features of APECED, such as CMC. Since these autoantibodies were not detected in unaffected *AIRE* heterozygous relatives of APECED patients, in APS-2 patients and in isolated cases of the endocrine diseases most typical to APECED, it was concluded that they are APECED-specific. Previously it was shown that anti-IFN-α autoantibodies in thymoma-associated MG patients originated from the thymus. Likewise, in APECED patients the specific type of APCs, responsible for the secretion of

IFN-α, was located to the thymic medullary epithelial cells. At present, the reason and the exact mechanism of how APECED patients develop anti-type I IFN autoantibodies, is not known. So far it can be hypothesized that these autoantibodies could be induced by changes in cells producing IFNs, which include potent APCs. For example, DCs that produce IFN-α and IFN-β are strongly implicated in autoimmunisation in psoriasis and T1D and SLE (Banchereau et al. 2004, Nestle et al. 2005). This possibility was supported by the finding that increased expression of type I IFN autoantibodies was found in blood-derived immature DCs in one APECED patient. Taken together, the results of this study indicate that the production of anti-type I IFN autoantibodies is not a consequence of any APECED-related disorder, but most likely type I IFNs are involved in autoimmunisation and/or these antibodies make APECED patients more susceptible to other infections (e.g. *Candida* infection). The findings presented in Study IV suggest that anti-IFN autoantibodies might be a valuable tool in the diagnosis of APECED patients with homozygous mutation in *AIRE*.

4. Thymomas lack *AIRE* expression

Thymomas are often associated with autoimmunity. It was found that ~95% of the studied thymoma subtypes lacked AIRE expression both at the mRNA and protein level, whereas in some cases its expression was detectable in the adjacent residual thymus and in lymph nodes of the same patients. Interestingly, the assumption that lack of *AIRE* expression would lead to the development of autoantibodies and disorders typical for APECED proved to be wrong. In addition, the prototypic AIRE-dependent autoantigens such as insulin and CYP1A2 were also absent in AIRE-negative thymomas. Moreover, the disorders and the organ-specific autoantibodies which occur with the development of APECED were rarely detectable in thymoma patients. With an exception for insulin and CYP1A2, the expression of several other common APECED autoantigens such as GAD65, GAD67 and thyroglobulin was only moderately altered, implying that the lack of AIRE in thymomas might lead to the incomplete self-tolerance induction during thymopoiesis.

In addition, a similar spectrum of autoantibodies and clinical autoimmune disorders occurring in MG was also detected in patients with neoplasia or hyperplasia of the thymus, occurring despite the presence or absence of AIRE. The results also suggested that the comparison of AIRE-positive and AIRE-negative human thymomas might be useful in testing the AIRE-dependence of specific autoantigens. This study demonstrated for the first time that the thymic expression of two APECED autoantigens CYP17 and CYP21 was not dependent on AIRE expression, since they were detected both in AIRE-positive and -negative thymoma samples. In conclusion, the data presented in this study imply that the absence of AIRE in thymoma patients is not the reason for autoimmune manifestations seen in these patients and thymomas can be considered as a model of acquired AIRE deficiency, which provides a platform to study AIRE-dependence of autoantigen expression in human thymic epithelial cells.

SUMMARY AND CONCLUSIONS

The *AIRE* gene is essential for the establishment of central tolerance in the thymus, as patients with loss-of-function mutations in *AIRE* develop a rare organ-specific autoimmune disease called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also known as autoimmune polyglandular syndrome type 1 (APS-1).

In order to study the transcriptional regulation of *AIRE* gene, its promoter fragment was isolated (I). It was found that common transcription factors such as Sp1, NF-Y, AP-1 and Ets-transcription family members Ets-1, Ets-2 and ESE-1 are involved in the regulation of *AIRE* transcription (I and II). *AIRE* promoter which contains a CpG island was shown to be subject to chromatin modifications at the level of methylation and deacetylation, suggesting that these epigenetic control mechanisms participate in the control of *AIRE* gene expression (I).

Analysis of *AIRE* expression in MoDCs revealed that *AIRE* mRNA was up-regulated during the differentiation process and also remained at a similar level in mature DCs (III). To determine the role of AIRE during the DC maturation process a monocyte cell model was created and used in expression array. AIRE-induced changes in gene expression profile were similar to those previously identified during the maturation process of monocyte- and bone marrow-derived DCs, suggesting that in the periphery AIRE might be involved in the maturation of DCs.

Analysis of the presence of autoantibodies to type I IFNs in APECED patients' sera (with both *AIRE* alleles mutated) revealed high levels of neutralising autoantibodies against most of the type I IFN-α subtypes and against IFN-ω in all APECED patients (IV). These autoantibodies were detected before the development of common autoantibodies to APECED, and in some cases before the onset of clinical disease components. Since the observed anti-type I IFN autoantibodies were not found in healthy controls, in heterozygous carriers of *AIRE* mutation or in patients suffering from other endocrine disorders, it was concluded that they were APECED-specific and thus were proposed to be used as valuable diagnostic marker of patients with homozygous mutation in *AIRE*.

Investigation of the expression of *AIRE* and common APECED autoantigens in a large set of thymoma samples indicated the lack of *AIRE* and insulin expression in ~95% of studied samples, while *AIRE* and insulin expression were found to be present in remnant thymic tissue and lymph nodes (V). Despite the absence of *AIRE*, the autoantibodies and clinical disease components common for APECED were not observed in the majority of thymoma patients. Further studies are needed in order to understand these findings.

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ORIGINAL COMMUNICATIONS

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Characterization of Regulatory Elements and Methylation Pattern of the Autoimmune Regulator (AIRE) Promoter*

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Defects in the AIRE gene cause a monogenic autoimmune syndrome APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy), which is characterized by loss of self-tolerance to multiple organs. In concordance with its role in immune tolerance, AIRE is most strongly expressed in thymic epithelial cells and in cells of monocytic-dendritic lineage. The AIRE protein has been shown to function as a transcriptional regulator, however, the mechanisms regulating AIRE gene expression are not known. Here we have characterized the AIRE promoter region by identifying a minimal promoter region within 350 bp of the translation initiation codon. Electrophoretic mobility shift assays and transient transfections with mutated promoter constructs revealed a functional TATA box (−163 to −153) and binding sites for transcription complexes AP-1 (−307 to −296), NF-Y (−213 to −202), and Sp1 (−202 to −189). The presence of a 390-bp CpG island within the proximal promoter suggested that cytosine methylation has a role in transcriptional regulation of AIRE, which was supported by *in vitro* methylation experiments of promoter constructs. Sodium bisulfite sequencing showed a less methylated status of AIRE promoter in the thymic epithelial cell line TEC1A3 compared with HeLa and monocytic cells U937 and THP-1. Real-time PCR analysis showed that treatment with 5-aza-2′-deoxycytidine (5-azaCdR), a DNA methyltransferase inhibitor, up-regulated AIRE transcript levels in TEC1A3, U937, and HeLa cells and that even greater activations in TEC1A3 and U937 cells were observed using combined treatments with deacetylase inhibitor trichostatin A. These results suggest that AIRE gene expression is modulated through modifications in chromatin methylation and acetylation.

Mutations in the autoimmune regulator (AIRE)[1] gene cause an autosomal recessive disease called APECED (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) (1, 2). The syndrome, occurring as a result of defective tolerance to self-antigens, is characterized by endocrine organ-specific autoimmunity and chronic mucocutaneous candidiasis and ectodermal disorders (3).

AIRE encodes a 58-kDa protein with structural motifs indicative of a transcriptional regulator having a conserved nuclear localization signal, two plant homeodomain zinc-finger motifs, four L*XX*LL or nuclear receptor box motifs, a proline-rich region, a SAND domain, and an HSR domain (1, 2, 4). The protein has a tissue-specific expression pattern, being mainly expressed in the thymus, lymph nodes, the spleen, and fetal liver (1, 2, 5, 6). In the thymus, AIRE is expressed in medullary epithelial cells and cells of monocyte-dendritic lineage (5, 7). Subcellularly, AIRE is located in the cell nucleus and in nuclear dots resembling promyelocytic leukemia bodies (7, 8). Supporting AIRE subcellular location and its structural features, AIRE has been shown to act as a transcriptional activator, the process that is mediated through the plant homeodomain zinc fingers (8). AIRE also interacts with the common transcriptional coactivator CREB-binding protein (CBP) through the CH1 and CH3 conserved domains (9). Recent findings in AIRE-deficient mice suggest that the protein influences expression of peripheral self-antigens in the thymus, explaining the mechanism through which immune tolerance can be broken in multiorgan endocrine autoimmune diseases (10, 11).

The role of AIRE in tolerance mechanisms led us to investigate the transcriptional regulation of the AIRE gene. Here we have characterized the human AIRE gene promoter structure identifying a minimal promoter and its transcriptional control elements. We describe the methylation status of the AIRE promoter in several cell lines and demonstrate the up-regulation of AIRE mRNA as a response to 5-aza-2′-deoxycytidine (5-azaCdR) and trichostatin A (TSA) treatments.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells were maintained in Dulbecco's modified Eagle's medium. HeLa and TEC 1A3 (human thymic epithelial cell line) cells were maintained in Eagle's minimum essential medium. Human monocytoid cell lines, THP-1 and U937, were cultured in RPMI 1640. Media was supplemented with 10% fetal calf serum and a 100 units/ml penicillin-streptomycin mixture (BioWhittaker, Europe).

Isolation of AIRE Promoter—A 1.2-kb genomic fragment containing the 5′-end region of the AIRE gene was isolated by PCR amplification from human genomic DNA. Nucleotide sequences for primers are listed in Table I. The 5′-flanking region of AIRE promoter was subcloned into the pHIV-LTR-luc plasmid (Prof. Kalle Saksela, University of Tampere, Finland) encoding firefly luciferase reporter gene and named as pAP1235. The promoter region in the pAP1235 construct was verified by sequencing.

Computer Analysis—Transcription factor binding sites were predicted by using the Match™ program, which uses TRANSFAC 5.0 matrices (core similarity 1.0 and matrix similarity 0.9). The presence of CpG islands was analyzed with the EMBOSS program CpGplot using the algorithms of Gardiner-Garden and Frommer (12). According to this analysis, a CpG-rich region is defined as stretches of DNA in which both

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[1] The abbreviations used are: AIRE, autoimmune regulator promoter; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; 5-azaCdR, 5-aza-2′-deoxycytidine; TSA, trichostatin A; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; RT, real-time; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE I
Primer sequences used in this study

The mutated nucleotides are underlined.

Name	Sequence (5′ → 3′)	Purpose[a]
GR1/1F	CGAGGCCAAGCGAGGGGCTGCCAG	Cloning
GR1/1R	GGACTATCCCTGGCTCACAGGGCC	Cloning
PA1F	TTTGCGGCCGCGAACACTCGTGCCCAGAGACC	Cloning
PA2F	TTTGCGGCCGCCCTGTCCAGGCCACAGCATCC	Cloning
PA3F	TTTGCGGCCGCTAGGGGCTCTCAGCTTGTGTG	Cloning
PA4F	TTTGCGGCCGCCACAGAGCGAGTCTCTGTCCC	Cloning
PA5F	TTTGCGGCCGCCCTCCATCACAGGGAAGTGTC	Cloning
PA6F	TTTGCGGCCGCGGAGCGGCCTTTGCTCTTTGC	Cloning
PA1R	TTTGGATCCGGGCGCGGGGACCCGGGGCTG	Cloning
AIRE TATA box	GGTCGCGGGGGTCGAACAGCGGCGCG	mutagenesis
AIRE AP-1	CTGGTGGGTGAGCAAGGCCAGGCCCG	mutagenesis
AIRE NF-Y	CTGGCCCTGATTAAGCGCCGGGGCGGAG	mutagenesis
AIRE Sp1	GATTGGGCGCCGAAGCGAAGCGGCCTTTGC	mutagenesis
AIRE AP-1	TGGTGGGTGAGTCAGGCCAGGC	EMSA
AIRE AP-1 mut.	TGGTGGGAAAGTCAGGCCAGGC	EMSA
AP-1 consensus	CGCTTGATGAGTCAGCCGGAA	EMSA
AIRE NF-Y	GAGGCCCTGGCCCTGATTGGGCGCCG	EMSA
AIRE NF-Y mut.	GGCCCTGATTAAGCGCCGGGGC	EMSA
NF-Y consensus	GAGATTAACCAATCACGTACGGT	EMSA
AIRE Sp1	GGCGCCGGGGCGGAGCGGCCTT	EMSA
AIRE Sp1 mut.	GGCGCCGAAGCGAAGCGGCCTT	EMSA
Sp1 consensus	ATTCGATCGGGGCGGGGCGAGC	EMSA
BITSA 1 fwd	GGATAGGGTTATATTTGGAAGTGA	BS
BITSA 1 rev	CTAACTCACAAAACCTAAAAACAA	BS
BITSA 2 fwd	TGTTTTTAGTTTTTAAGGTAGTTG	BS
BITSA 2 rev	TACAACAATAAAAAAACACTATCC	BS

[a] EMSA, electrophoretic mobility shift assay; BS, bisulfite sequencing

the moving average of percentage of G plus C nucleotides is greater than 50 and the moving average of observed/expected CpG is greater than 0.6.

Construction of Deletion and Mutant Reporter Plasmids—The pAP1235 plasmid construct containing the 5′-flanking region was used as a template to synthesize a series of deletion reporter gene constructs. The forward and reverse primers containing *Not*I and *Bam*HI restriction sites, respectively, were used in cloning of deletion constructs (Table I and Fig. 1). Site-directed mutagenesis of the pAP1235 construct was performed using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). Mutations were introduced into putative DNA binding sites for the AP-1, NF-Y, and Sp1 transcription factors, as well as into the putative TATA box. Primer sequences used in site-directed mutagenesis are given below in Table I. All mutations were confirmed by DNA sequencing.

Transient Transfections and Luciferase Reporter Assays—Cells (1.5×10^5) were transiently transfected with 2.5 μg of DNA (2 μg of luciferase reporter plasmid, 0.5 μg of β-galactosidase expression vector pEF-BOS (Prof. Kalle Saksela, University of Tampere, Finland)) using ExGen 500 transfection reagent (Fermentas) following the manufacturer's instructions. After 24 h, the cells were lysed to measure the luciferase activity using the Luciferase Assay System (Promega). The luciferase activities were normalized for transfection efficiency according to β-galactosidase activities. The transfections were performed in triplicate.

Electrophoretic Mobility Gel Shift Assay—Nuclear extracts were made from HeLa and TEC1A3 cells with the variation of the method of Dignam *et al.* (13). Briefly, 3×10^7 cells were washed twice with ice-cold phosphate-buffered saline and resuspended in buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 2 μg/ml aprotinin) and left on ice for 15 min. The lysates were passed several times through a 25-gauge needle. The nuclei were recovered by brief centrifugation at full speed and resuspended in buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 1 μg/ml leupeptin, and 2 μg/ml aprotinin), followed by sonication and incubation on ice for 30 min. The reaction mix was centrifuged for 15 min at full speed, and the supernatant was collected and stored at −70 °C until use. Protein concentrations were determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA). The oligonucleotides used in EMSA are listed in Table I. Pre-binding of nuclear extract to poly(dI-dC) (Roche Applied Science) was carried out in a 10-μl reaction volume at 30 °C for 30 min in a buffer containing 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, and 0.05 mg/ml poly(dI-dC)·poly(dI-dC). For competition experiments, unlabeled competing oligonucleotides at 100-fold molecular excess were included in preincubation mixture. For antibody supershift experiments, corresponding antibody (2 μg) was included in the preincubation mixture. Purified ^{32}P-end-labeled, double-stranded oligonucleotide was then added to the reaction mix and incubated at 30 °C for 30 min. The reaction mixtures were subjected to native 4.5% polyacrylamide gel electrophoresis. Following the electrophoresis, the gel was dried and exposed for autoradiography (Kodak Biomax MS-1, Sigma). The Sp1 and AP-1 consensus oligonucleotides were purchased from Promega. The Sp1, pan-Jun, and pan-Fos antibodies were purchased from Santa Cruz Biotechnology. Anti-NF-YA and anti-NF-YB antibodies were a gift from Dr. Roberto Mantovani (University of Milano, Italy).

In Vitro DNA Methylation—*Sss*I methylase (New England BioLabs) was used to methylate AIRE promoter luciferase reporter construct pAP1235. Briefly, plasmid DNA was incubated with 1 unit of methylase per 1 μg of DNA in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9, supplemented with 160 μM *S*-adenosylmethionine. Reactions were carried out at 37 °C overnight. The methylated plasmid DNA was purified through a Wizard DNA Clean-Up system (Promega) and transfected into TEC1A3 and COS-7 cells in parallel with the unmethylated pAP1235 construct.

Genomic DNA Isolation—Genomic DNA was extracted from TEC1A3, HeLa, THP-1, and U937 cells according to Sambrook *et al.* (14). Briefly, the genomic DNA was isolated by cell lysis with proteinase K (Promega) digestion and extraction with phenol/chloroform. After the precipitation, the genomic DNA was digested with restriction enzymes *Sma*I and *Sac*I, separated on a 1.5% agarose gel with 1× TAE buffer (40 mM Tris-HCl, 1 mM EDTA, pH 8), blotted on nylon membrane, and hybridized with the radioactive probe corresponding to the genomic sequence (−568 to −195 bp). After hybridization and subsequent washing, the filters were exposed to autoradiography film.

Sodium Bisulfite Genomic DNA Sequencing—Bisulfite genomic sequencing was performed as described previously (15, 16). The genomic DNA (2 μg) was denatured in 0.3 M NaOH at 37 °C for 15 min. After the addition of 3 M sodium bisulfite (Sigma) and 10 mM hydroquinone (Sigma), samples were mixed, overlaid with mineral oil, and incubated at 50 °C for 16 h. The modified DNA was purified through the Wizard DNA Clean-Up system (Promega) and denatured by addition of 0.3 M NaOH at 37 °C for 15 min. The bisulfite-reacted DNA was precipitated and resuspended in 1 mM Tris-HCl, pH 8, and used immediately or stored at −20 °C. The sequence of interest in the bisulfite-reacted DNA was amplified by PCR in a reaction mixture containing 200 ng of DNA, 0.5 μM primers, 200 μM dNTPs, 1× buffer, 5% Me_2SO, and 1 unit of Hercules polymerase (Stratagene). Each amplification reaction con-

sisted of a 5-min incubation at 95 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 56 °C, 1.5 min at 72 °C, and a final elongation step for 7 min at 72 °C. Primer sequences are listed in Table I. The modified DNA was further amplified by nested amplification, DNA fragments were gel-purified with a Sephaglas™ Bandprep kit (Amersham Biosciences) and cloned into pCRII-TOPO vector (Invitrogen). Approximately 10 clones were sequenced per cell line.

5-AzaCdr and TSA Treatment—DNA methyltransferase inhibitor, 5-azaCdr, was added to cells at final concentrations from 0.2 to 15 μM for 72 h. Deacetylase inhibitor, TSA (100 ng/ml), was added for 24 h alone or at the end of the 5-azaCdr treatment, where indicated. Subsequently, cells were used for RNA isolation using the Total RNA Isolation system (Promega), and 3 μg of total RNA was converted to cDNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences).

Quantitative Real-time PCR—Real-time PCR was performed with the LightCycler instrument (Roche Applied Science) using a ready-to-use one-step QuantiTect™ SYBR® Green RT-PCR kit (Qiagen). Reactions were set up in 15 μl of final volume containing 2 μl of sample cDNA or standards, 0.5 μM primers, and 7.5 μl of 2× RT-PCR master mix. The amplification program for AIRE included an initial denaturation step at 95 °C for 15 min, followed by 55 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 25 s, and extension at 72 °C for 20 s. SYBR® Green fluorescence was measured after each extension step. The specificity of amplification was subjected to melting curve analysis. The relative amount of the AIRE transcript was normalized to the amount of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript in each cDNA. The PCR conditions for GAPDH differed in that the annealing temperature was 58 °C and amplification consisted of 45 cycles. Cloned AIRE and GAPDH cDNAs were used as standards for quantification. Primer sequences to amplify the AIRE or GAPDH were as follows: AIRE-F, CCCTACTGTGTGTGGGTCCT; AIRE-R, ACGTCTCCTGAGCAGGATCT; GAPDH-F, CTGAGCTAGACGGGAAGCTC; and GAPDH-R, TCTGAGTGTGGCAGGGACT. Each of the PCR assays was run in triplicate, and the AIRE and GAPDH copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the LightCycler thermal cycler.

RESULTS

AIRE Proximal Promoter Contains Several Positive Regulatory Elements—We first isolated the human AIRE gene 5′-flanking region using PCR amplification of genomic DNA. The nucleotide sequence of the clone was found to be 100% identical with the genomic sequence in GenBank™ (accession number AB006684). The transcription initiation site, 128 bp upstream of the ATG codon, determined by rapid amplification of cDNA ends method, has been described earlier (1).

To demonstrate the activity of the AIRE promoter, the fragment was subsequently cloned in front of a luciferase reporter gene and transfected transiently into COS-7 and TEC1A3 cell lines. The luciferase activity with the pAP1235 reporter construct was 146- and 44-fold in COS-7 and TEC1A3 cells, respectively, compared with a promoterless control plasmid pBL-KS (Fig. 1), indicating cis-acting elements in the AIRE promoter region. To further map the regions responsible for transcriptional control we made deletion constructs of the pAP1235 plasmid from the 5′-end of the promoter fragment. Luciferase assays with the deletion constructs pAP940, pAP583, and pAP350 gave comparably similar activities in COS-7 cells, whereas the pAP248 construct resulted in significant decrease of the activity to 19% in COS-7 cells when compared with the pAP1235 construct. An even further decrease of the luciferase activity (to ~3%) was seen with the pAP190 deletion construct. Essentially similar results were obtained using the TEC1A3 cell line. As a conclusion of deletion construct analysis, the results showed that the sequence elements required for the minimal AIRE promoter activity reside within the first 350 bp upstream of the translation start site.

Characterization of Factors Binding to the Promoter—Analysis of the 350-bp promoter region with the Match™ program indicated the presence of high score potential binding sites for transcription factor complexes such as a GC box (at position −202 to −188), an inverted CCAAT box (position −212 to −200), and a binding site for transcription factor AP-1 (position −307 to −296).

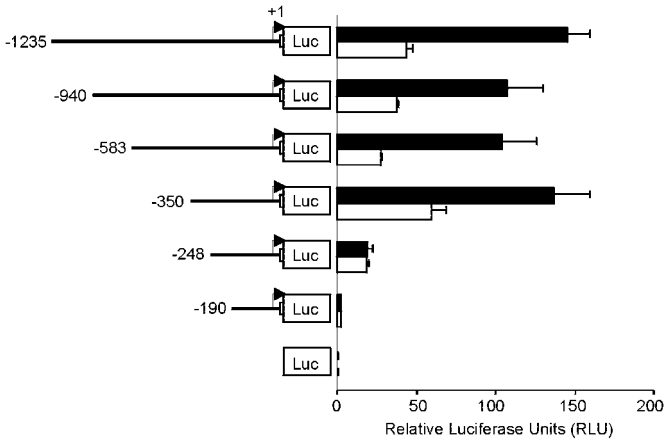

FIG. 1. **Deletion analysis of the human AIRE promoter.** A series of AIRE promoter 5′-deletion constructs were transiently transfected into COS-7 (*black bars*) and TEC1A3 (*light bars*) to identify the minimal promoter region. The *left side* of the figure shows the plasmid constructs with sizes indicated in base pairs; the *right side* shows promoter activity measured in relative luciferase units obtained from each transfection. Luciferase activity is relative to a pBL-KS promoterless control. Results shown are a mean ± S.D. of three experiments performed in triplicate.

To characterize the transcription factors that interact with putative binding sites in the AIRE promoter region, EMSA, coupled with supershift assays, was performed. A shifted band appeared with the AP-1 site double-stranded oligonucleotide (−313 to −291) as a probe (Fig. 2*A*). The complex disappeared by the addition of a 100-molar excess amount of the unlabeled probe but was not affected by addition of the same amount of unrelated oligonucleotide to the reaction mix as a competitor. The mutated oligonucleotide probe from the AP-1 site was unable to form the complex, further underlining the specificity of the AP-1 site. To further characterize the complex, we performed a supershift assay by using antibodies raised against pan-Jun and pan-Fos proteins. The shifted band that appeared with the AIRE AP-1 site was supershifted by the addition of pan-Jun antibody, however, not by pan-Fos antibody (Fig. 2*A*). Similar DNA-protein complexes and supershift results were observed in both cell lines. As a control probe, we used a previously described AP-1 consensus site in complex formation and supershift analysis (Fig. 2*A*).

The relevance of the CCAAT box in AIRE gene regulation was determined with probes coding for the CCAAT box sequence. DNA-binding reactions showed a shifted protein-DNA complex, which was fully abolished by addition of a 100-fold molar excess of cold oligonucleotide (Fig. 2*B*). Only a CCAAT box-specific oligonucleotide inhibited the formation of complex, because the protein-DNA complex was not competed away by a nonspecific competitor as a probe. In contrast, as an indication of protein binding specificity, mutations introduced into the AIRE CCAAT box consensus sequence abolished protein-DNA complex formation. To identify whether the protein within the specific protein-DNA complex was in fact NF-Y, supershift assays were performed with antibodies against A and B subunits of the NF-Y transcription complex. Both anti-NF-YA and anti-NF-YB antibodies were able to supershift the protein-DNA complexes, resembling the supershifted complex obtained with the CCAAT consensus sequence (Fig. 2*B*).

Furthermore, the importance of the GC box in the AIRE promoter was tested. The shifted complex obtained with the AIRE Sp1 probe was similar to the complex obtained with a control consensus Sp1 element (Fig. 2*C*). The complex disap-

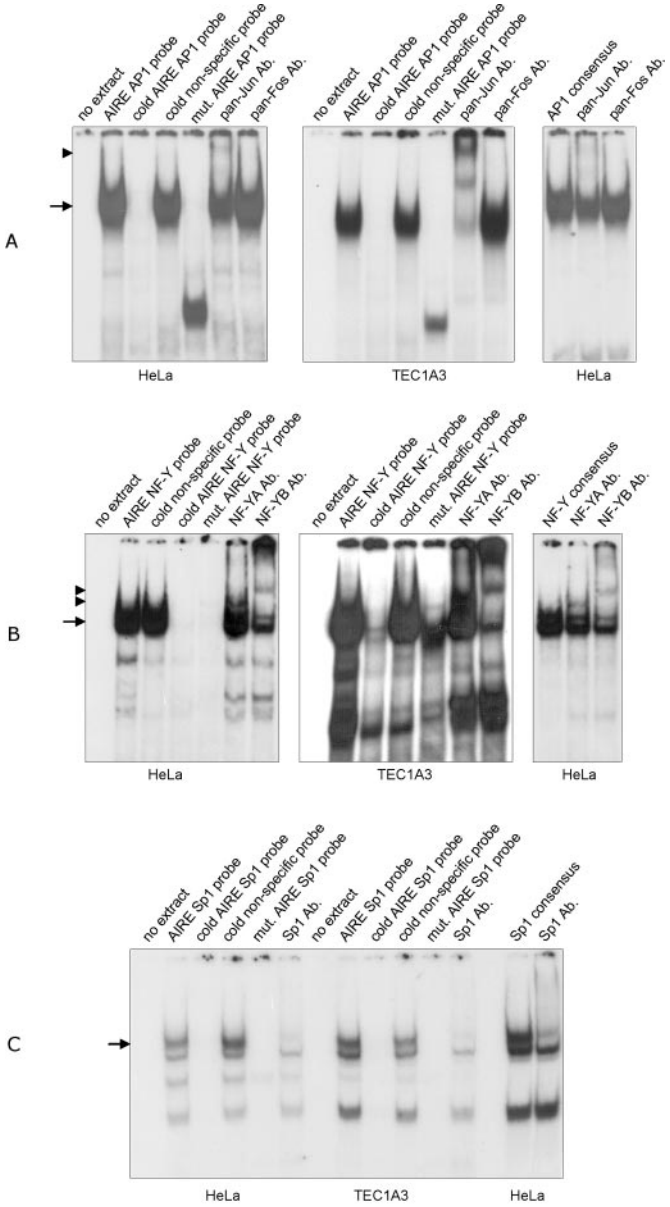

FIG. 2. **Electrophoretic mobility gel shift assay with AIRE promoter binding sites.** *A*, AIRE promoter AP-1 binding site. Competition and supershift assay demonstrated AP-1 binding to its consensus site at the AIRE promoter. Incubation of nuclear extracts from HeLa or TEC1A3 cells with pan-Jun antibody resulted in a supershifted protein-DNA complex, whereas the pan-Fos antibody did not supershift the complex. As a control, the AP-1 consensus probe was used. *B*, AIRE promoter CCAAT box. Competition and supershift assay showed NF-Y-specific binding to the inverted CCAAT box in AIRE promoter in HeLa and TEC1A3 cells. As a control, the NF-Y consensus probe was used. *C*, AIRE promoter GC box. Competition and supershift assay demonstrated the Sp1 binding to GC box in AIRE promoter. Addition of antibody specific for the Sp1 antagonized the protein-DNA complex. The *arrows* indicate specific complexes, and *arrowheads* mark antibody supershifts.

peared with the addition of the unlabeled competitor but was not influenced by excess amount of unrelated oligonucleotide. The mutated AIRE Sp1 oligonucleotide used as probe resulted in no complex formation. The identity of the complex was further confirmed using an antibody specific for the Sp1 transcription factor. The Sp1 antibody was able to specifically interfere with the formation of the protein complex with the AIRE promoter element as well as with the consensus Sp1 element in supershift assays. Taken together, these results indicated that the AIRE promoter contains at least three specific regulatory elements, which form protein-DNA complexes with AP-1, NF-Y, and Sp1 transcription family members.

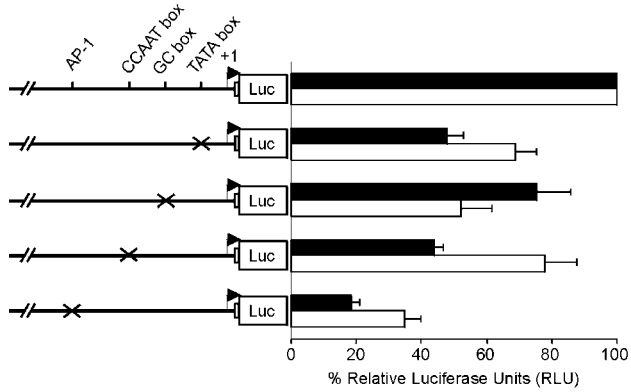

FIG. 3. **Functional analysis of the AIRE-mutated regulatory sequences.** Site-directed mutations were introduced into the binding sites of AP-1, NF-Y, and Sp1 transcription factors and to TATA box (*black bars*, COS-7; *light bars*, EC1A3). *Crosses* indicate the mutation sites. Results shown are mean ± S.D. of three experiments performed in triplicate.

Functional Analysis of TATA Box, AP-1, NF-Y, and Sp1 Regulatory Elements—In addition to the AP-1, NF-Y, and Sp1 regulatory elements, the AIRE promoter contains a TATA box located at the position 33 bp upstream of the transcriptional start site. To study the functional significance of the TATA box and transcriptional complexes in the AIRE promoter, mutations were introduced into the pAP1235 luciferase reporter construct using site-directed oligonucleotide mutagenesis and transfected into the COS-7 and TEC1A3 cell lines (Fig. 3). The mutations introduced into the TATA box decreased AIRE promoter activity to 48% in COS-7 and to 69% in TEC1A3 cells, respectively. Mutations in the CCAAT box reduced the promoter activity to 44% in COS-7 cells, compared with 78% in TEC1A3 cells. Nucleotide substitutions within the GC box resulted in 75% activity in COS-7 and 52% in TEC1A3 cells compared with the full-length pAP1235 construct. Similarly, mutations in the AP-1 binding site decreased the promoter activity to 18% in COS-7 and to 35% in TEC1A3 cells. Thus, the results of these experiments showed that the TATA box, the AP-1 binding site, the inverted CCAAT box, and the GC box are functional. Moreover, the factors binding to these sites are essential for the AIRE gene regulation.

AIRE Promoter Contains CpG Island and the Activity Is Blocked by in Vitro Methylation—We noted that the region surrounding the AIRE proximal promoter is GC-rich. The CpG dinucleotide distribution within the 1.2-kb genomic fragment was statistically analyzed as described by the method of Gardiner-Garden and Frommer (12). Correspondingly, a 390-bp CpG island was located at the AIRE promoter region, starting from about 300 bp upstream of the translational start site and encompassing the first exon (Fig. 4*A*).

The effect of *in vitro* methylation on the promoter of the AIRE gene was tested in a transient expression assay using the pAP1235 promoter construct. After methylation with *Sss*I methylase, which methylates cytosines residues within CpG dinucleotide, the methylated and unmethylated reporter constructs were transfected into TEC1A3 cells and assayed for expression. As a result *in vitro* methylation completely suppressed AIRE promoter activity compared with the unmethylated promoter construct (Fig. 4*B*). Similar results were obtained with experiments using the COS-7 cell line (data not shown).

AIRE Promoter Is Less Methylated in the Thymic Epithelial Cell Line—The methylation status of CpG sites in the AIRE

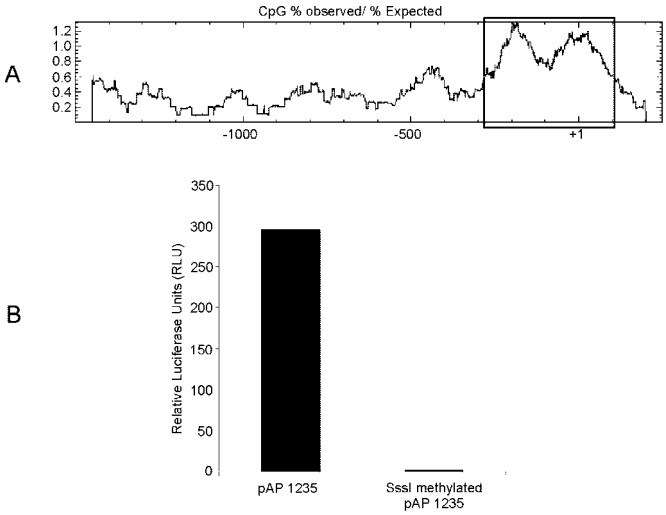

FIG. 4. ***In vitro* methylation inhibits AIRE promoter activity.** *A*, location of the CpG island at the AIRE promoter. The CpG island is marked by a *box*, and *numbers* on the *x*-axis indicate the nucleotide positions relative to the translational start site. *B*, the pAP1235 AIRE promoter construct, either unmethylated, or methylated with *Sss*I methylase, was transiently transfected into TEC1A3 cells. The transcription activity is shown in relative units of luciferase activity.

FIG. 5. **CpG methylation in the AIRE promoter region (−315 to +65) after bisulfite treatment of genomic DNA.** The methylation status of 47 CpG sites in TEC1A3, HeLa, THP-1, and U937 cell lines is shown. Binding sites for AP-1 (−307 to −296), NF-Y (−213 to −202), Sp1 (−202 to −189), and TATA box (−163 to −153) are *underlined*, and the translation start site (*atg*) is in *boldface*.

promoter region was first studied in TEC1A3 and THP-1 cell line genomic DNA using the methyl-sensitive restriction enzyme *Sma*I inside of a methylation-insensitive *Sac*I fragment spanning the AIRE promoter. Subsequent hybridization with the AIRE promoter-specific probe revealed uncleaved *Sac*I fragments, suggesting methylated status of the *Sma*I sites (data not shown).

The DNA from HeLa, TEC1A3, and from two monocyte cell lines, THP-1 and U937, was further analyzed by bisulfite genomic sequencing. Genomic DNA was treated with sodium bisulfite under conditions where cytosines are converted to uracils, while methylated cytosines remain unmodified. The promoter region contained 47 CpG sites, covering a 390-bp fragment of the AIRE promoter. All tested CpG sites showed a heavy methylation pattern in monocytic cell lines (THP-1 and U937 cells, Fig. 5). Results obtained from HeLa cells were consistent with the results from THP-1 and U937 cells, although few CpG sites had a partial methylation pattern. In contrast, in thymus epithelial TEC1A3 cells, 9 CpG sites out of 47 were completely unmethylated and 11 CpG sites possessed a partial methylation pattern. The results indicated a different methylation pattern of AIRE promoter in TEC1A3 cells compared with the other three cell lines.

5-AzaCdR and TSA Treatments Up-regulate AIRE Expression—To test the potential role of DNA methylation and chromatin modification in the control of AIRE expression, a demethylating agent 5-azaCdR and a histone deacetylase specific inhibitor, TSA, were used. TEC1A3 cells were grown in the presence of various 5-azaCdR concentrations, and purified RNA was analyzed by quantitative real-time PCR for AIRE expression in three cell lines (TEC1A3, U937, and HeLa). The 5-azaCdR treatment alone with the highest concentration (15 μM) was able to up-regulate AIRE expression levels 4.5 (TEC1A3)-, 26 (U937)-, and 16.5 (HeLa)-fold when compared with the untreated cells (Fig. 6). Lower 5-azaCdR concentrations also yielded lower activation of the AIRE expression. However, the combination of 0.5 μM 5-azaCdR and TSA was even able to further increase the expression of AIRE in TEC1A3 (6.5-fold) and U937 (49.5-fold) cells, although this was not observed in HeLa cells. The activation of AIRE mRNA, although by a lesser extent, was also seen by TSA treatment

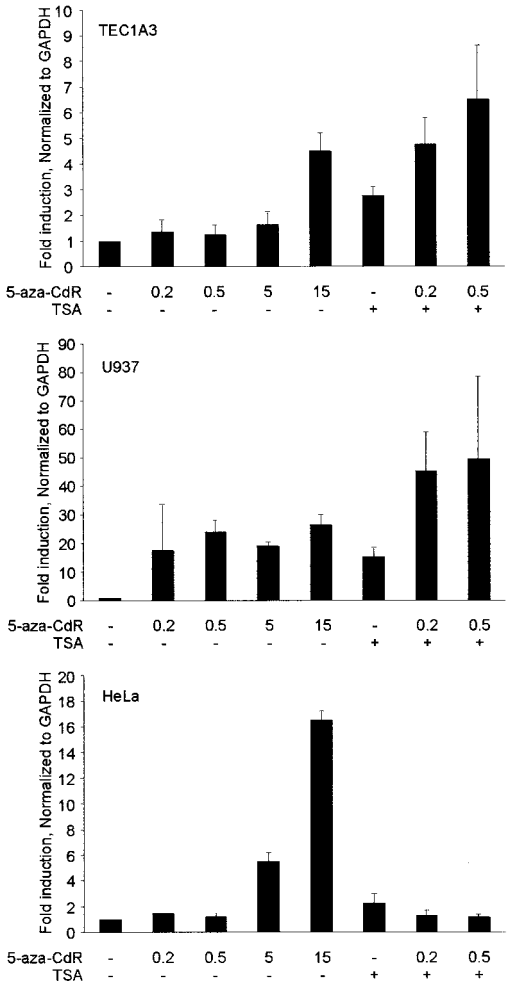

FIG. 6. **Quantitative analysis of AIRE mRNA levels following treatment with 5-azaCdR and TSA.** Real-time quantitative PCR analyses of total RNA isolated from TEC1A3, U937, and HeLa cells treated with various concentrations of 5-azaCdR (0.2, 0.5, 5, and 5 μM) and TSA (100 ng/ml) or in combination. The expression levels of the AIRE transcript were normalized for the expression levels of the GAPDH transcripts in the same cDNA samples.

alone. The results showed that methylation and deacetylase activity inhibition treatments are able to activate the AIRE promoter and that, in TEC1A3 and U937 cells, the combination

of the two treatments resulted in higher AIRE transcript expression levels suggesting that both methylation and histone deacetylation are responsible for the silencing of the AIRE gene.

DISCUSSION

The highest AIRE expression is seen in thymic epithelial cells (5, 6). Mittaz *et al.* (17) concluded from human and mouse AIRE promoter sequence analysis that both human and mouse genes contain conserved binding sites for thymus- and hematopoiesis-specific transcription factors. Analysis of the human AIRE promoter sequence undertaken here did not detect high score binding sites for tissue-specific transcription factors. However, the possibility that tissue-specific transcription factors participate in AIRE transcriptional regulation cannot be excluded, because these factors may interact with other protein complexes binding to AIRE promoter or some transcription factors may bind to the upstream regulatory region. Considering that AIRE controls the expression of other genes, we wanted to elucidate the mechanisms behind the AIRE gene transcriptional control.

The 1235-bp fragment upstream of the initiation codon showed strong promoter activity in COS-7 and in the thymic epithelial cell line TEC1A3 for the expression of reporter gene. The deletional analysis of the 5′-flanking AIRE promoter region demonstrated that the proximal 350-bp region contained all necessary elements for minimal promoter activity, because the pAP350 construct gave comparably similar luciferase results with pAP583, pAP940, and pAP1235 deletion constructs. Strong promoter activity within a relatively short 350-bp upstream region suggested the presence of specific transcription factor binding sites, which was also supported by computer analysis giving high score potential binding sites for transcription factors such as AP-1, NF-Y, and Sp1. We also found that the luciferase expression level in TEC1A3 was consistently lower, being one-third of the expression seen in COS-7 cells. As one explanation, this could be due to the higher metabolic activity of COS-7 cells. Interestingly, however, the luciferase expression of the 248-bp proximal fragment was almost similar in two cell lines suggesting one critical DNA binding element within the −248 to −350 region. The highest scoring element in this region was the AP-1 site suggesting that higher expression of this transcription complex in COS-7 cells might be the reason for the luciferase expression discrepancy in the two cell lines. The further deletion to −190, excluding the CCAAT box, almost abolished promoter activity indicating the role of this site in AIRE promoter regulation.

The EMSA experiments and mutated construct analysis confirmed four important regions for AIRE activity. From these promoter elements, the mutation inside of the AP-1 element had the most dramatic reduction of the promoter activity by decreasing it to 18% (COS-7) and 35% (TEC1A3), clearly demonstrating the responsiveness of AIRE expression from AP-1 complex. The AP-1 complex consists of distinct homodimers or heterodimers composed of various members of the Fos, Jun, and ATF subfamilies (18). Using pan-Jun and pan-Fos antibodies in EMSA, we could show that proteins within the AP-1 complex, binding to the AIRE promoter, belong to the Jun subfamily but not to the Fos transcription factor family. Second, we showed that the CCAAT and GC boxes, located at −212 to −200 bp and −202 to −188 bp, respectively, are important in AIRE activation. The NF-Y transcription factor complex binding to the CCAAT box consists of three subunits, NF-YA, NF-YB, and NF-YC, and all of them are required for efficient DNA binding (19). The interaction between NF-YB and NF-YC allows association of this complex further with NF-YA and subsequent binding to their consensus sequence in DNA (20). Supershift assays with two members of this complex, NF-YA and NF-YB, pointed to the formation of a complete NF-Y complex on the AIRE promoter. Third, AIRE promoter activity is also mediated by the Sp1 transcription complex, because the Sp1-specific antibody interfered with the GC box-shifted complex and because the mutated Sp1 binding site construct had strongly decreased transcriptional activity in the COS-7 and TEC1A3 cell lines. It should be mentioned that two more GC boxes were predicted by computer analysis, albeit with a lower score, at nucleotides −37 to −24 and −131 to −109. Finally, the AIRE promoter contains a canonical vertebrate TATA box allowing for recruitment of a complex containing the TATA-binding protein and RNA polymerase II holoenzyme (21, 22). As expected, the mutations created within the TATA site had significant effect on AIRE promoter activity consistent with its predicted functional role.

The presence of a CpG island suggested the possibility that the AIRE gene might be regulated through changes in methylation status, which was first tested by *in vitro* methylation of the pAP1235 promoter construct. The four cell lines studied exhibited a methylated CpG pattern by bisulfite sequencing, and this was particularly obvious in DNA from HeLa and monocytic cell lines THP-1 and U937. In TEC1A3 cell DNA, several CpG sites appeared unmethylated or partially methylated. Methylation of the Sp1 site has been shown to cause gene silencing (23, 24), and three CpG sites within the Sp1 binding GC box appeared methylated except in TEC1A3 cells where one of the sites was only partially methylated (Fig. 5). AIRE expression level is highest in thymus epithelial cells (5); therefore, the thymic epithelial cell line represents the closest cell culture model for studying AIRE expression. Treatment with 5-azaCdR alone was able to induce higher AIRE mRNA expression levels in TEC1A3 and U937 cells, and the activation was further enhanced when the cells were treated in combination with deacetylase inhibitor TSA. A similar increase in mRNA level after 5-aza-CdR treatment was also observed in the HeLa cell line; however, the TSA treatment did not influence the expression level, suggesting that chromatin modification in the AIRE promoter region may vary between cell types. Synergistic effect of 5-azaCdR and TSA in enhanced re-expression of several hypermethylated genes was first reported by Cameron *et al.* (25). The association of CpG methylation and chromatin modifications has been established in experiments showing that the Sin3-histone deacetylase complex is recruited to chromatin by methylated CpG-binding protein, MeCP2 (26, 27).

This is the first study to reveal the functional control of the AIRE promoter. In conclusion, our results demonstrate that the AIRE promoter contains binding sites for three transcription factor complexes, AP-1, NF-Y, and Sp1, and a functional TATA box. In addition, results of methylation analyses show a highly methylated CpG island inside the AIRE promoter in several cell lines, albeit at lower level of methylation in thymic epithelial cell line DNA. Furthermore, an increase of AIRE mRNA was seen when the cells were treated with 5-azaCdR and TSA, indicating that chromatin methylation modifications, most likely combined with histone acetylation state, may have an important role in AIRE transcriptional regulation.

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Anti-Interferon Autoantibodies in Autoimmune Polyendocrinopathy Syndrome Type 1

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Abbreviations: AD, Addison disease; *AIRE*, *autoimmune regulator*; APC, antigen-presenting cell; APS[number], autoimmune polyendocrinopathy syndrome type [number]; AVINA, antiviral interferon neutralisation assay; CMC, chronic muco-cutaneous candidiasis; DC, dendritic cell; ELISA, enzyme-linked immunoabsorbent assay; HP, hypoparathyroidism; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LU, laboratory units; MEC, medullary epithelial cell; MG, myasthenia gravis; RT-PCR, real-time PCR; SLE, systemic lupus erythematosus

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ABSTRACT

Background

The *autoimmune regulator (AIRE)* gene influences thymic self-tolerance induction. In autoimmune polyendocrinopathy syndrome type 1 (APS1; OMIM 240300), recessive *AIRE* mutations lead to autoimmunity targetting endocrine and other epithelial tissues, although chronic candidiasis usually appears first. Autoimmunity and chronic candidiasis can associate with thymomas as well. Patients with these tumours frequently also have high titre immunoglobulin G autoantibodies neutralising type I interferon (IFN)–α and IFN-ω, which are secreted signalling proteins of the cytokine superfamily involved in both innate and adaptive immunity.

Methods and Findings

We tested for serum autoantibodies to type I IFNs and other immunoregulatory cytokines using specific binding and neutralisation assays. Unexpectedly, in 60/60 Finnish and 16/16 Norwegian APS1 patients with both *AIRE* alleles mutated, we found high titre neutralising immunoglobulin G autoantibodies to most IFN-α subtypes and especially IFN-ω (60% homologous to IFN-α)—mostly in the earliest samples. We found lower titres against IFN-β (30% homologous to IFN-α) in 23% of patients; two-thirds of these (from Finland only) also had low titres against the distantly related "type III IFN" (IFN-λ1; alias interleukin-29). However, autoantibodies to the unrelated type II IFN, IFN-γ, and other immunoregulatory cytokines, such as interleukin-10 and interleukin-12, were much rarer and did not neutralise.

Neutralising titres against type I IFNs averaged even higher in patients with APS1 than in patients with thymomas. Anti–type I IFN autoantibodies preceded overt candidiasis (and several of the autoimmune disorders) in the informative patients, and persisted for decades thereafter. They were undetectable in unaffected heterozygous relatives of APS1 probands (except for low titres against IFN-λ1), in APS2 patients, and in isolated cases of the endocrine diseases most typical of APS1, so they appear to be APS1-specific.

Looking for potentially autoimmunising cell types, we found numerous IFN-α^+ antigen-presenting cells—plus strong evidence of local IFN secretion—in the normal thymic medulla (where *AIRE* expression is strongest), and also in normal germinal centres, where it could perpetuate these autoantibody responses once initiated. IFN-α2 and IFN-α8 transcripts were also more abundant in antigen-presenting cells cultured from an APS1 patient's blood than from age-matched healthy controls.

Conclusions

These apparently spontaneous autoantibody responses to IFNs, particularly IFN-α and IFN-ω, segregate like a recessive trait; their high "penetrance" is especially remarkable for such a variable condition. Their apparent restriction to APS1 patients implies practical value in the clinic, e.g., in diagnosing unusual or prodromal *AIRE*-mutant patients with only single components of APS1, and possibly in prognosis if they prove to predict its onset. These autoantibody responses also raise numerous questions, e.g., about the rarity of other infections in APS1. Moreover, there must also be clues to autoimmunising mechanisms/cell types in the hierarchy of preferences for IFN-ω, IFN-α8, IFN-α2, and IFN-β and IFN-λ1.

The Editors' Summary of this article follows the references.

Introduction

The *autoimmune regulator (AIRE)* gene encodes a two-plant-homeodomain zinc-finger protein that acts as a transcriptional regulator [1–4]. *AIRE* is highly expressed in thymic medullary epithelial cells (MECs), where it is strongly implicated in the expression of some peripheral self-antigens and the induction of tolerance thereto [1–6]; it is also expressed in dendritic cells (DCs) [5], which are very potent antigen-presenting cells (APCs). Over 50 recessive mutations have been identified in the coding region of *AIRE*. In homozygotes or compound heterozygotes, they cause autoimmune polyendocrinopathy syndrome type 1 (APS1; OMIM 240300), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy [2–4,6–8]. However, in at least 2% of clinically typical patients, no mutations are found [8].

Probably through defects in AIRE protein function, in self-antigen expression by MECs, and in self-tolerance induction in autoreactive T cells as they are generated in the thymus, the ensuing autoimmune attack focusses particularly on epidermal and endocrine tissues, and features both T cells and autoantibodies [1–4,7,8]. By definition, the patients have at least two of the "APS1 triad"—hypoparathyroidism (HP), Addison disease (AD), and chronic muco-cutaneous candidiasis (CMC). However, the clinical phenotype is highly variable. Characteristically, patients present with CMC and/or skin disorders, usually early in childhood; these symptoms are followed (sometimes 1–3 decades later) by autoimmune endocrine disorders, which may also target the gonads and/or endocrine cells in the gut, pancreatic islets, and thyroid gland [3,4,7,8]. The candidiasis, which is evident early in nearly all APS1 patients, may be due to defects in *AIRE* signalling pathways involved in the handling of *Candida* sp. infection.

Interestingly, CMC also co-occurs with autoimmunity (albeit against different targets) in occasional patients with thymomas [9]. We have recently found—at diagnosis—high titre immunoglobulin G (IgG) neutralising autoantibodies to interferon (IFN)–α (all 12 subtypes) and IFN-ω, and to interleukin (IL)–12, in many patients with thymoma, with late-onset myasthenia gravis (MG), and especially with thymoma and MG together [10,11]. The human type I IFNs all use the same receptor, and fall into five classes: IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω [12]. Only the IFN-α group contains multiple closely related "subtypes", to which IFN-ω and IFN-β are about 60% and 30% related, respectively [12]. IFN-α, IFN-β, and IFN-ω are inducibly expressed at very high levels in plasmacytoid DCs and monocytes following viral infection or stimulation through Toll-like receptors [12,13], and are key early players in innate immune responses. In addition, both type I IFNs and IL-12 polarise towards pro-inflammatory T helper 1 responses in humans [14,15], and possibly within MG thymomas [16].

The recently identified human "type III IFNs", IFN-λ1, IFN-λ2, and IFN-λ3 (alias IL-29, IL-28A, and IL-28B, respectively), are related structurally to, and share ~15% homology with, type I IFNs [17,18]. They act through a distinct receptor comprising a unique IFNLR subunit and an IL-10Rβ subunit (also part of the IL-10, IL-22, and IL-26 receptors) [17–19]. IL-10 itself is 11%–13% homologous to the type III IFNs [17]. Like the type I IFNs, the IFN-λs are expressed by human peripheral blood mononuclear cells and plasmacytoid DCs upon infection with viruses or stimulation with double-stranded RNA [17,18]. The single type II IFN, IFN-γ, shows essentially no homology to type I or III IFNs [12], and is produced by T cells and natural killer cells.

Autoimmunising processes are very hard to study in humans: there must be valuable clues in the thymomas, in which we find evidence for immunisation against IFN-α and IL-12 [20,21]. This autoimmunisation must be highly selective, since we have rarely found autoantibodies against many other cytokines—even including IFN-β [10]—and never against IFN-γ [11]. Indeed, all of these autoantibodies are also rare in many other infectious, neoplastic, or autoimmune diseases, including sporadic multiple sclerosis and, importantly for the present study, thyroid disease and type 1 diabetes mellitus [11].

In view of these parallels with thymoma patients, we tested for similar anti-cytokine autoantibodies in APS1 patients (and controls); though generally rare, the disease is less uncommon in Finnish (1:25,000), Sardinian (1:14,400), Iranian Jewish (1:9,000), and Norwegian (1:80,000) populations [4,8]. We therefore screened coded serum samples from a well-studied Finnish cohort [8] and from a further smaller group of Norwegian APS1 patients in validated binding enzyme-linked immunoabsorbent assays (ELISAs) and neutralising bioassays against type I, II, and III IFNs, IL-10, and IL-12 [10,11]. We focussed on the genetics, specificity, kinetics, and clinical correlates of the anti-IFN autoantibodies we found, and also tried to locate potentially autoimmunising cell types in normal thymus and reactive tonsils.

Methods

Patients

We studied the 77 Finnish APS1 patients [8] from whom sera were available (initially taken at ages 3.3 to 57 y [mean 19.6]). We tested 64 patients who had been *AIRE*-genotyped (see Table 1) and another 13 "untyped" but clinically typical APS1 patients. From 51 of the *AIRE*-genotyped patients, we tested 1–5 further samples dating from 0.7 to 33 y later (mean 16). The diagnosis of APS1 was based on the classical clinical criteria (presence of at least two of CMC, HP, and AD). The patients had from one to eight (median four) autoimmune manifestations, with onset from 0.7 to 20 y later (mean 6.7). Almost all had had oral or nail candidiasis or both, since the age of 0.2 to 30 y. Their endocrine disorders are summarised in Table 2; in addition, 22 had alopecia (since ages 3.8–30 y), 16 had keratoconjunctivitis (1.3–16), 16 had vitiligo (0.7– 45), six had had a period of vasculitis, and two were growth-hormone-deficient.

We also studied 17 genotyped APS1 or APS1-like patients from Norway (16 of Norwegian and one of Pakistani origin) [22]. The majority had at least two of the APS1 triad (see Table 2 for endocrine disorders and Table 1 for CMC), although one male aged 35 y had only AD ([22]; see footnote "e" of Table 1). Two other informative brothers in this cohort (see footnote "b" of Table 1) have not yet developed AD by ages 10 and 13 y [23].

As healthy and disease controls, we tested sera from ten *AIRE*-heterozygous unaffected first-degree relatives (six from Finland with R257X allele and four from Norway—two with R257X, one with 1242^1243insA, and one with 967/979del13 alleles) and from patients with APS2 ($n = 9$) and also with sporadic AD ($n = 11$), HP ($n = 2$), or CMC ($n = 3$)—all without

Table 1. Anti–Type I IFN Antibodies, *AIRE* Genotypes, and Candidiasis in APS1 Cases from Finland and Norway

Genotype	Number of Cases	Presence of Anti–Type I		Candidiasis
		IFN-α and IFN-ω	IFN-β	
R257X:R257X	40[a]	++	−	+
R257X:R257X	11	++	+	+
R257X:X546C	3	++	−	+
R257X:C311Y	2	++	−	+
R257X:A1758T	1	++	+	+
R257X:967/979del13	3[b]	++	−	+
R257X:967/979del13	1	++	+	+
R257X:1163^1164insA	1	++	−	+
R257X:1242^1243insA	1[c]	++	−	+
R257X:1242^1243insA	2[c]	++	−	−
R257X:K83E[d]	1	++	−	−
R257X:Not detected	1	++	−	+
967/979del13:967/979del13	4[c]	++	−	+
967/979del13:967/979del13	1[c,e]	++	−	−
967/979del13:967/979del13	3[c]	++	+	+
967/979del13:967/979del13	1[c]	++	+	−
967/979del13:1244^1245insC	1[c]	++	−	+

In the anti-IFN columns a plus sign signifies a neutralising titre greater than 40; for IFN-α and IFN-ω, nearly all were far higher, and thus are designated with double plus signs.
[a]There were 39 patients from Finland and one from Norway.
[b]There was one patient from Finland and two from Norway, who were the informative Norwegian brothers (see "Correlating Anti–Type I IFN Antibodies with Clinical Components of APS1" in Results); by age 10 y, one has HP, CMC, keratitis, and alopecia, and, by 13, the other has only HP and CMC.
[c]All these patients were from Norway; unlettered patients are all from Finland.
[d]This patient developed ungual candidiasis at age 7 y, but has never shown oral candidiasis.
[e]The unusual Norwegian patient with AD alone.
Anti–type I IFN autoantibody results were also very similar in one typical APS1 patient not yet mentioned; she was a Caucasian female currently domiciled in New Zealand, who is genotyped as an R257X/exon 8–deleted compound heterozygote. Her neutralising antibody titres ranged from 500,000 against IFN-α8 and IFN-α16 and 120,000 against IFN-ω to 550 against IFN-β. She was the APS1 buffy coat donor in Figure 4.
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APS1. Six patients with sporadic AD and eight patients with APS2 were found negative for the two most common APS1 *AIRE* mutations, R257X and 967/979del13.

The patients with MG and thymoma have been reported on in detail previously [10,11]; we summarise their results here purely for comparison. All samples were taken with informed consent and local ethical committee approval; the samples were coded and stored at −20 °C and were diluted in the media appropriate for cytokine-specific immunoassays and bioassays (see below).

IFNs and Other Cytokines

IFNs and other cytokines, generously donated by the stated manufacturers, were used in autoantibody-binding ELISA and/or antiviral interferon neutralisation assay (AVINA) specific for the following: recombinant human IFN-α2a (Hoffmann-La Roche, Basel, Switzerland); other IFN-α subtypes (BPL Laboratories, Piscataway, New Jersey, United States); IFN-β (Biogen, Boston, Massachusetts, United States); IFN-ω (Bender and Co., Vienna, Austria); IFN-γ (Roussel-Uclaf, Romainville, France); granulocyte-macrophage colony-stimulating factor (Immunex, Seattle, Washington, United States); IFN-λ1 [IL-29], IFN-λ2 [IL-28A], and IL-4 (R&D Systems Minneapolis, Minnesota, United States); IL-10 (Schering-Plough, Kenilworth, New Jersey, United States); IL-12 (Hoffman-La Roche); and TNF-α (Genentech, South San Francisco, California, United States).

Binding ELISA for the Detection of Anti-IFN and Anti-Cytokine Autoantibodies

Microtitre wells (Dynatech, Orlando, Florida, United States) were coated with IFN or cytokine solutions at 2 μg protein/ml (PBS [pH 7.0]), 0.1 ml per well, for 2 h at 22 °C. Wells were blocked with 3.0% HSA in PBS for 0.5 h at 22 °C or overnight at 4 °C, and serially diluted sera were added for 2 h at 22 °C, before washing and development with anti-human IgG (γ-chain-specific)–alkaline phosphatase conjugate (Sigma Chemical, St. Louis, Missouri, United States; 0.1 ml/well of 1:1,000 dilution at 22 °C for 2 h), further washing, addition of p-nitrophenyl phosphate substrate for 0.5 h at 22 °C, and then of 3M sodium hydroxide (0.05 ml/well); absorbances were read at 405 nm. We considered as positive any values greater than two standard deviations above the mean of controls.

AVINA

For type I IFNs, we pretreated the human glioblastoma cell line 2D9 (generously provided by Dr. W. Däubener; [24]) with dilute IFN preparations (ten laboratory units [LU] per millilitre) that had been pre-incubated for 2 h with serial dilutions of test sera [25,26]. For IFN-λs, instead of 2D9, we used the human glioblastoma cell line LN319 (generously provided by Dr. A.-C. Diserens [27]), which responds well to their antiviral activity [28]. The cells were then challenged with encephalomyocarditis virus for 24 h, stained with 0.05% amido blue black, fixed with 4% formaldehyde in acetic acid buffer, and stain-eluted with 0.15 ml of 0.05 M sodium hydroxide solution before absorbance was read at 620 nm. The neutralising antibody titre [29] was the dilution of serum that reduces 10 LU/ml of IFN to 1 LU/ml (the normal end point of antiviral assays [25]). The cut-off for positivity was a titre of 40, which numerous healthy controls never exceeded. We routinely included IFN-α1 (alias IFN-α13); its low specific activity consistently results in ~10-fold lower neutralising antibody titres than for any other IFN-α subtype. While these titres generally correlate well with those for IFN-α2, they have been omitted to avoid creating a misleading impression.

Immunohistology

Paraffin sections of tonsils or normal child thymus were dewaxed, heated under pressure for 2 min in Tris:EDTA buffer (pH 9.0), and washed in PBS. They were then stained for 1 h at 20 °C with the anti-MxA monoclonal antibody M143 (1:200 [30]), and then with sheep anti-IFN-α (1:1,000 [31,32]) for 30 min before washing and incubation with donkey anti-mouse IgG-Alexa 594 (1:600) and anti-sheep IgG-Alexa 488 (1:400; both from Molecular Probes, Invitrogen, Carlsbad, California, United States), before washing and mounting.

Generation of Monocyte-Derived DCs

Buffy coats were collected from an APS1 patient with an R257X mutation in one *AIRE* allele and 967/979del13 in the other, and from two healthy blood donors; the buffy coats were washed and then cryopreserved in liquid nitrogen. Cells were then thawed and cultured in RPMI 1640/10% fetal calf serum growth medium for 72 h at 37 °C in a 5% carbon dioxide incubator. Monocytes were enriched with monoclonal anti-CD14-antibody-coated microbeads (Miltenyi Biotec,

Table 2. Binding and Neutralising Anti-IFN Autoantibodies in APS1 Patients with *AIRE* Mutations, in Their Unaffected Heterozygous Relatives, and in Patients with MG/Thymoma (as a "Positive" Control Group), with APS2, or with Sporadic Diseases from the APS1 Triad

Patients/ Controls		ELISA versus IFN-α2	Neut versus IFN-α2	ELISA versus IFN-ω	Neut versus IFN-ω	Neut versus IFN-β	Neut versus IFN-λ1	Autoimmune Features of APS1					
								HP	AD	Gonadal	Pernicious Anaemia	Diabetes mellitus	Thyroid Disease
APS1: Finnish[a]		60/60	60/60	60/60	60/60	13/60	11/60	51/60	51/60	24/60	13/60	11/60	8/60
	Median titre		129,000		35,500	450	250						
	(Range)		(320–2,000,000)		(3,000–2,560,000)	(120–8,000)	(40–3,500)						
APS1: Norwegian[a]		16/16	16/16	16/16	16/16	4/16	0/16[b]	13/16	12/16	3/16	0/16	0/16	0/16
	Median titre		140,000		75,000	85							
	(Range)		(450–512,000)		(2,500–600,000)	(60–900)							
Heterozygotes		0/10	0/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
	Median titre						835[c]						
	(Range)						(390–1,280)[c]						
MG/Thymoma[d]		27/35	27/35	22/35	22/35	8/35	N.D.	0/27	0/27	0/27	0/27	0/27	1/27
	Median titre		25,000		5,850	150							
	(Range)		(128–320,000)		(240–200,000)	(120–512)							
APS2		0/9	0/9	0/9	0/9	0/9	N.D.	0/9	9/9	0/9	0/9	2/9	8/9
Non-syndromic	AD	0/11	0/11	0/11	0/11	0/11	N.D.	0/11	11/11	0/11	0/11	0/11	0/11
	HP	0/2	0/2	0/2	0/2	0/2	N.D.	2/2	0/2	0/2	0/2	0/2	0/2
	CMC	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a]Test results apply to the most recently available samples from all APS1 patients with *AIRE* mutations in both alleles.
[b]Modest levels of binding antibodies to IFN-λ1 were recorded in 2/14 patients.
[c]The results are from the two positive samples.
[d]Before immunosuppressive therapy. These patients all had MG; one also had alopecia and another had had thyroid nodules.
N.D., not done; neut, neutralising antibody titres of sera measured in AVINAs.
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Bergisch Gladbach, Germany), and cultured for seven further days with IL-4 (1,000 U/ml); granulocyte-macrophage colony-stimulating factor (800 U/ml) was added on days 0, 3, and 6 to yield "immature DCs". On day 7, some of the DCs were matured for 3 d with monocyte-conditioned medium prepared as previously described [33].

Quantitative Real-Time PCR

RNA was isolated using TRIzol (Life Technologies, Gaithersburg, Maryland, United States) and reverse-transcribed to cDNA using the First-Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario, Canada). Quantitative real-time PCR (RT-PCR) was performed with the ABI Prism 7000 SDS instrument (Applied Biosystems, Foster City, California, United States) using qPCR SYBR Green Core Kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions, except that a 2 mM magnesium chloride concentration was used. The amplification program included an initial denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min, for 50 cycles. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification subjected to melting curve analysis. As an internal reference for quantification experiments, we performed RT-PCR reactions with the house-keeping genes *GAPDH* and *HPRT*. The primers were as follows: *GAPDH* forward, tccaccaccctgttgctgtag; *GAPDH* reverse, gaccacagtccatgccatcact; *HPRT* exon 6, gactttgctttccttggtcagg; *HPRT* exon 7, agtctggcttatatccaacacttcg; *IFN*-ω forward, gaggtacttccagggaatccg; *IFN*-ω reverse, catttcaagatgagcccaggtc; *IFN*-α*2* forward, cctgatgaaggactccatt; *IFN*-α*2* reverse, aaaaaggtgagctggcatacg; *IFN*-α*8* forward, gtgatagagtctcccctgatgtac; and *IFN*-α*8* reverse, cttcaatctttttgcaagttga [34].

Results

Autoantibodies against IFN-α and IFN-ω

We first screened for anti-IFN autoantibodies in 60 typical Finnish APS1 patients with both *AIRE* alleles mutated, and then in 16 such patients from Norway (Table 2). Both binding and neutralising autoantibodies against IFN-α2 and IFN-ω were readily detected in the last samples from all 76 patients, regardless of their exact clinical phenotype. These autoantibodies were clearly IgG, as a specific anti-human γ-chain conjugate was used to quantify them in the ELISA. In AVINAs, titres against IFN-α2 and IFN-ω were greater than 5,000 in nearly all samples. They were generally 3- to 6-fold higher than in MG/thymoma cases (Table 2). Since the ten unaffected heterozygous first-degree relatives of APS1 patients were uniformly negative in both assays (Table 2), this response evidently segregated like a recessive trait. Moreover, it appears to be APS1-restricted (see below). In our previous study [11], no neutralising autoantibodies to either IFN-α2 or IFN-ω were detected in sera from 70 healthy controls.

These findings raise numerous questions about this anti-IFN response in APS1 patients that we now address; they concern its kinetics, its specificity for different IFN-α subtypes, other IFNs, and other cytokines, its specificity for APS1 versus its component diseases, its correlation with the various *AIRE* genotypes, and the mechanisms of autoimmunisation.

Kinetics of anti-IFN-α and anti-IFN-ω autoantibodies. Serial samples were available from 51 Finnish patients (2–6 from each). Neutralising titres against IFN-ω were already high in the first samples from 50 of these (Figure 1A), and tended to be higher in the younger patients (though not significantly). We have no explanation for their later appearance in the remaining patient (patient A; Table 3).

Titres were even higher against IFN-α2 than IFN-ω (1.6-fold on average) in 47 of these 51 patients, extending up to 4×10^6 in one 3.3-y-old patient (Figure 1A); in another three of these 51 patients (patients B–D), titres were initially high against IFN-ω (>10,000; Table 3; see below), even when they were low or undetectable against IFN-α2. Notably, in all patients tested, neutralising antibody titres remained high for all subsequent time points up to 30 y thereafter (Figure 1A).

Preferences for IFN-α subtypes and IFN-ω. When we tested against a panel of ten IFN-α subtypes, sequential samples from 30/30 randomly chosen Finnish patients strongly neutralised all of the IFN-αs as well as IFN-ω (Figure 2A and 2B show a typical example). They also neutralised "leukocyte IFN preparations" produced by virally infected human peripheral blood mononuclear cells in bulk (unpublished data); these heterogeneous mixtures of IFN-α subtypes plus IFN-ω are representative of the IFNs produced during viral infections of leukocytes [35]. Together, these results argue strongly that the antibodies are recognising the native molecules.

In general, neutralisation profiles (and titres) were consistent between the first and last available samples from each patient (Figure 2A and 2B). However, there was an initial preference for IFN-ω (and/or IFN-α8) in three of the 51 patients (patients B–D; Figure 1A; Table 3) that broadened over the following ~25 y to affect other IFN-α subtypes in patient D (Figure 2E and 2F) and particularly IFN-α8 in patient B (Table 3; Figure 2C and 2D). We can see no obvious clinical explanation for the markedly higher titres in these later samples (Table 3).

Correlations with *AIRE* mutations and human leukocyte antigen genotypes. All 60 genotyped APS1 Finnish patients with mutations in both *AIRE* alleles had at least one R257X allele—which is the predominant Finnish mutation [4,8]—whereas 12 of the 16 patients from Norway had at least one 967/979del13 mutation (Table 1). All the homozygotes—and all the compound heterozygotes—had high titres against IFN-α2 and IFN-ω (Table 1). There may be other defects in the AIRE or parallel pathways, as occasionally clinically typical APS1 patients have only one identified *AIRE* mutation or none at all [8]. Titres were high in the one patient with only one identified *AIRE* mutation (Table 1; "x" in Figure 1B) and even in two of the four individuals with no detected *AIRE* mutations ("y" and "z" in Figure 1B); the other two were completely negative against all IFNs tested, but neither had the full APS1 triad by age 39 (patients E and F; see footnote of Table 3) and one (patient F) is the only affected member of a sibship of 14. Titres were also high in a further 13 "untyped" Finnish APS1 patients (included in Figure 1B).

Both anti-IFN titres and HLA-DR and -DQ types were available for 61 Finnish APS1 patients. HLA-DR15 was somewhat over-represented among the cases with very high titres (>256,000), though not significantly so after correction for multiple comparisons.

Correlating anti–type I IFN antibodies with clinical components of APS1. Some patients were strongly positive for anti-IFN antibodies despite never manifesting the full

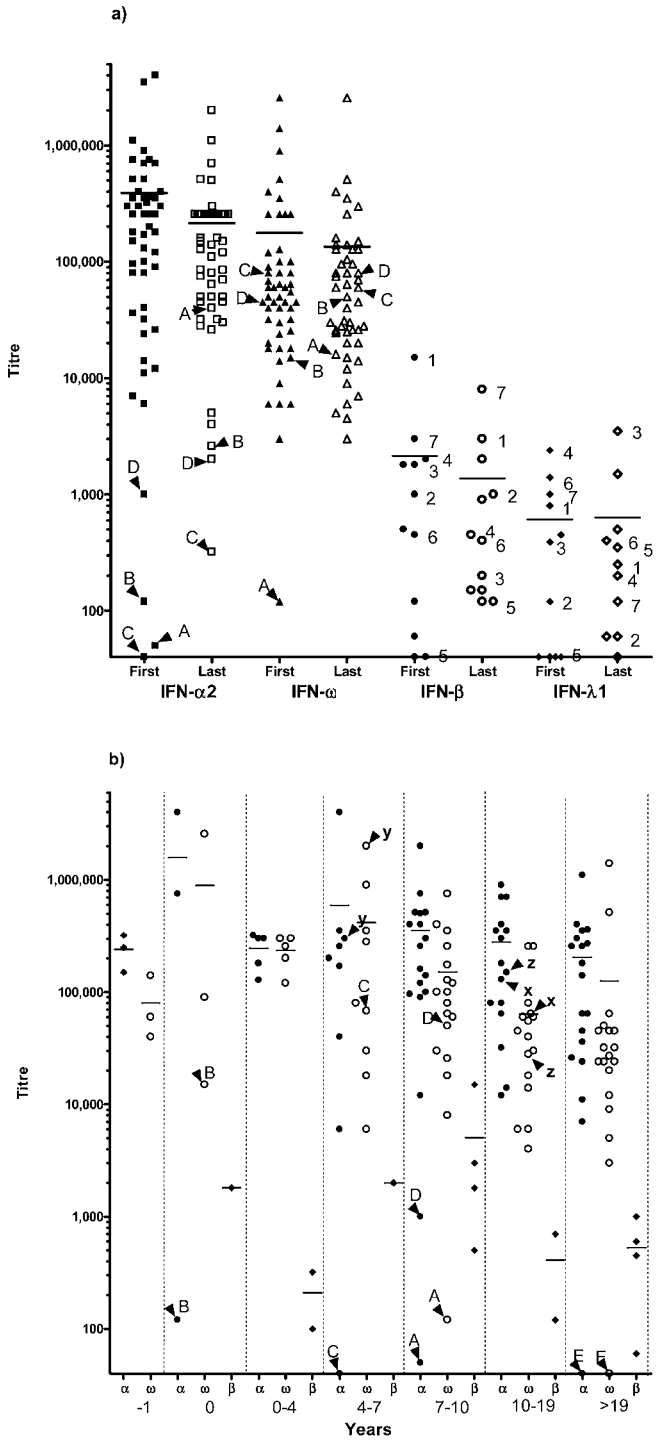

Figure 1. Anti-IFN Neutralisation Titres in APS1 Patients at Different Sampling Times

(A) Anti-IFN neutralisation titres in the first and last available serum samples from 51 *AIRE*-genotyped Finnish patients. We use arrowheads to mark the unusual APS1 patients A–D (detailed in Table 3), and numerals for patients whose samples neutralised both IFN-β and IFN-λ1. Bars indicate geometric mean titres. From first to last available samples (average interval = 16 y), titres were substantially increased, unchanged, or decreased in 19%, 43%, and 38% patients, respectively.

(B) Anti-IFN neutralisation titres in Finnish APS1 patients against IFN-α2, IFN-ω, and IFN-β in the first available sera in relation to the time of diagnosis of CMC. Results are grouped for patients sampled 1 yr prior to diagnosis of CMC (–1), at diagnosis (0), or within the indicated number of years thereafter (0–4, 4–7, 7–10, 10–19, or >19 y). This figure excludes four otherwise typical APS1 patients who never developed overt CMC but who also had high titres against IFN-ω (see text); it includes 63 *AIRE*-genotyped and 11 untyped patients. For IFN-β, only the positive patients are shown (for clarity). Arrowheads mark unusual APS1 patients: patients A–E (detailed in Table 3); patient x, who currently has only one detectable *AIRE* mutation; patients y and z, who are two of the three Finnish patients with no detectable *AIRE* mutations; and patient E, who meets the criteria for APS1 (Table 3), but for whom no neutralising autoantibodies or *AIRE* mutations have yet been found.

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APS1 triad of clinical features (AD, HP, and CMC; Table 2), including one Norwegian patient who had only AD (see footnote "e" of Table 1). Similarly, titres were already high against IFN-α2 and IFN-ω at ages 3 and 7 y in two highly informative Norwegian brothers with APS1 but no AD (see footnote "b" of Table 1). At that time, both were negative for all ten of the autoantibodies most commonly associated with APS1 [23]; only in their next samples (~5 y later) did we detect any of these (and then only against 21-hydroxylase [in both brothers] and against aromatic L-amino-acid decarboxylase [in the elder]; E. Husebye, unpublished data). A few other patients were first sampled—and already had high anti-IFN titres—before they developed the full APS1 triad. For example, AD began 6 and 21 y later in two Finnish patients, and HP began 2.7 and 12 y later in two others; again, the relevant endocrine autoantibodies probably appeared after a considerable delay (they are usually found only shortly before the AD [36]).

Moreover, the anti-IFN-α and anti-IFN-ω antibodies were clearly not an effect of CMC; in all the informative cases, the antibodies antedated or coincided with the diagnosis of CMC (Figure 1B), and they also reached high titres in the five patients who never developed CMC overtly (one was a K85E compound heterozygote; see footnote "d" of Table 1).

These antibodies are apparently restricted to APS1. They did not reach significant levels (>1/40) in patients with APS2 ($n = 9$), who show characteristic adult-onset AD, thyroid disease, and/or insulin-dependent diabetes without CMC or HP [37], nor in patients with sporadic AD ($n = 11$), HP ($n = 2$), or CMC ($n = 3$), all without APS1 (Table 2).

Autoantibodies against Other Interferons and Cytokines

Surprisingly, other infections are rarely problematic in APS1 [8], but we nevertheless screened against other cytokines.

IFN-β. As in patients with both MG and thymoma [10,11], we detected antibodies against the more distantly related type I IFN, IFN-β (30% homology with IFN-α), much less frequently than against either IFN-α or IFN-ω, and at much lower titres (Tables 1 and 2; Figure 1A); we found them mostly in patients with especially high titres against IFN-α2 and IFN-ω ($n = 15$), and usually from the first sample onwards. When positive, titres against IFN-β (in the Finnish cohort) averaged slightly higher than in MG/thymoma patients (Table 2). They were consistently negative in the ten unaffected heterozygotes, and in patients with APS2 ($n = 9$) or sporadic cases of AD alone ($n = 11$), CMC alone ($n = 3$), or HP alone ($n = 2$) (Table 2).

IFN-λs. These recently identified "type III" IFNs are ~15% homologous to the type I IFNs [17,18]. As yet, there have been no reports of autoantibodies to them; indeed, we found none in the Norwegian cohort. However, we saw clear neutralisation of IFN-λ1 in about two-thirds of the Finnish APS1 sera

Table 3. Autoantibodies against IFN-α2, IFN-α8, IFN-ω, and IL-12 and Autoimmune Components in Five "Unusual" Finnish APS1 Patients

Patient (All Female)	*AIRE* Genotype	Ages at Sampling		Neutralising Titre versus						Anti-IL-12 (Optical Density in ELISA)		Onset Age (in Years) of APS1 Feature[a]					
				IFN-α2		IFN-α8		IFN-ω									
		Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	HP	AD	Ovaries	Pernicious Anaemia		Asplenia
A	R257X:R257X	9.5	36.6	50	64,000	<40	50,000	120	16,000	1.15	0.06	3.6	8.7	17	—		(33)
B	R257X:R257X	4.4	29.6	120	2,600	320	9,000	15,000	50,000	0.19	0.16	4.2	25	26	15.5		—
C[b]	R257X:C311Y	9.7	38.6	<40	320	12,000	20,000	80,000	64,000	0.12	0.15	4.9	10	(10)	—		(36)
D[b]	R257X:C311Y	15.9	42.7	1,000	2,000	12,000	10,000	45,000	80,000	0.21	0.05	—	16	33	—		(40)
E	No mutations found	33.7	39.8	<40	<40	<40	<40	<40	<40	0.14	0.14	14.6	—	—	31		—

There was no obvious clinical correlate for the rise in titres in patient A. Patient E also developed obesity at age 14, hypertension at age 20, hypothyroidism at age 21, and type 2 diabetes at age 39. Norwegian patient F, with no detectable *AIRE* mutations (and no sign of HP by age 43), similarly had no detectable antibodies to type I IFNs. Both patients E and F had CMC.

[a]Numbers in parentheses indicate age when the sign was first noticed.

[b]These patients are sisters.

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with anti-IFN-β antibodies, but in very few of the others (Table 2; numerals in Figure 1A); the 11 positive patients were all *AIRE* R257X homozygotes. These autoantibodies were not always constant, either disappearing ($n = 1$) or appearing de novo ($n = 3$) several years after anti-IFN-α or anti-IFN-ω autoantibodies were first detected. These results argue against cross-neutralisation of IFN-β and IFN-λ1 by the same antibodies. So do the independent fluctuations in titres against IFN-β and IFN-λ1 in patients 3 and 7 (Figure 1A). Neutralisation of IFN-λ2 was found only in the samples positive against IFN-λ1, and generally at lower titres (A. Meager, unpublished data).

Notably, two of the ten unaffected heterozygotes also had neutralising antibodies against IFN-λ1 (at ages 32 and 39 y; Table 2) but not detectably against IFN-λ2. However, we detected none of these antibodies in sera from the patients with sporadic CMC alone ($n = 3$) or HP alone ($n = 2$) (Table 2) or from 18 healthy controls. In MG/thymoma patients, these autoantibodies were rare and very low in titre, even in sera positive against IFN-β (unpublished data).

Other cytokines. We tested against other cytokines only in the 77 Finnish sera. Against IFN-γ, only "late" sera from two R257X homozygous patients (one from ~28 and 37 y and the other ~17 y after onset) gave moderate to high ELISA signals against IFN-γ—which we have never seen in MG/thymoma cases [10,11]—but they showed no detectable neutralising activity; both were strongly positive at diagnosis against IFN-α2 and IFN-ω.

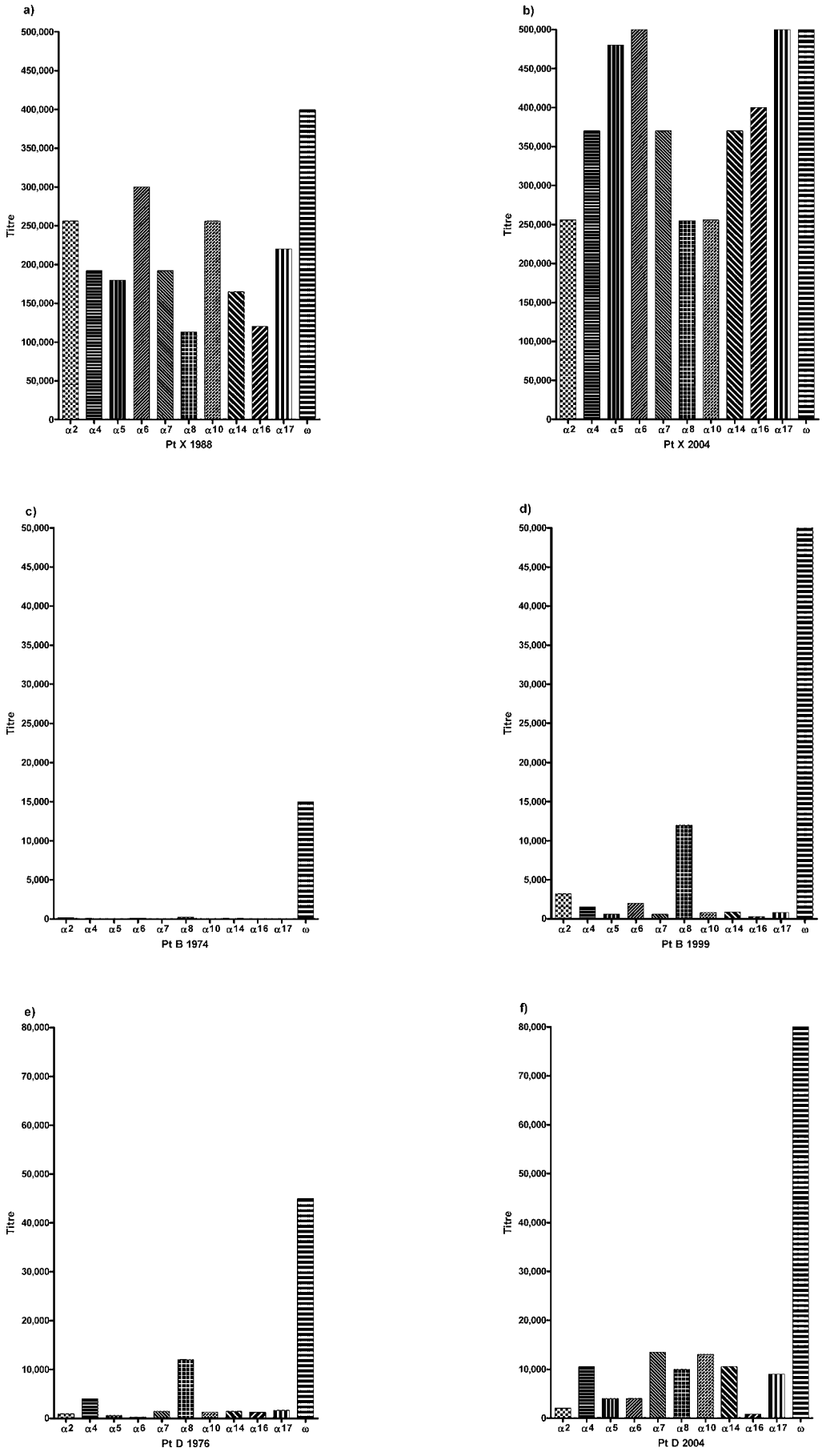
Against IL-12, we also found moderate levels of binding autoantibodies in early sera from only five R257X homozygous patients. Only in one of patient did the anti-IL-12 antibodies precede the anti-IFN antibodies (patient A; Table 3), and only in one other did they persist (for ~6 y).

Against IL-10, granulocyte-macrophage colony-stimulating factor, and tumour necrosis factor–α, we found no significant binding in ELISAs, even though IL-10 is ~12% homologous to the IFN-λs [17,18]. In numerous sera from many infectious, autoimmune, and neoplastic disorders, we have very rarely found neutralising activity without significant binding (in ELISAs), whereas the reverse is more common [11].

Potentially Autoimmunising Cell Types

In normal thymus. The similar anti-IFN-α autoantibodies in APS1 and thymoma-associated MG hint at a thymic origin for these responses. To pursue that, we next focussed on the expression and secretion of IFN-α in sections of normal thymus. Essentially all the IFN-α^+ cells appeared to be $CD68^+$ APCs; we saw no significant IFN-α labelling in cytokeratin$^+$ cells (not shown). Next, we double-stained for the MxA protein that is up-regulated in cells responding to type I and III IFNs [30]. In the cortex, as expected [31], scattered macrophages ($CD68^+$) were strongly IFN-α^+, but we saw almost no MxA labelling (Figure 3A). By contrast, in the medulla—where *AIRE* is expressed most strongly [4]—there were numerous IFN-α^+ cells (Figure 3A), and MxA^+ cells were even more abundant—which is strong evidence of local secretion of type I and III IFNs. Many of the medullary IFN-α^+ cells were macrophages, and some were almost certainly DCs.

In reactive lymph-node-like tissue. Another striking feature of the anti-IFN autoantibodies in both APS1 and thymoma/MG patients is their prolonged persistence. We uncovered a possible explanation while staining "normal" tonsils (where


a)
Titre
500,000
450,000
400,000
350,000
300,000
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α2 α4 α5 α6 α7 α8 α10 α14 α16 α17 ω
Pt X 1988
b)
Titre
500,000
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Pt X 2004
c)
Titre
50,000
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d)
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e)
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80,000
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α2 α4 α5 α6 α7 α8 α10 α14 α16 α17 ω
Pt D 2004

Figure 2. Typical and Unusual Neutralisation Profiles against IFN-α Subtypes and IFN-ω
Patient (Pt) X has a typical profile (A and B); patients B (C and D) and D (E and F) have unusual profiles (also detailed in Table 3).
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others have described MxA labelling in extra-follicular areas [38]). The most strongly stained IFN-α^+ cells proved to be $CD68^+$ macrophage-like cells in the germinal centres. Notably, these structures consistently also showed strong to intermediate labelling for MxA (Figure 3B); the reticular pattern of the labelling strongly suggests expression in follicular dendritic cells, and hence suggests that these cells were responding to secreted IFNs. Normally, these highly specialised APCs play a key role in antibody diversification by retaining and displaying native antigens that select and stimulate germinal centre B cells as their antibodies mutate [39]. Thus, any native IFNs being presented there would be ideally situated to perpetuate autoantibody responses against them once initiated (e.g., in a thymoma [20,21]).

In APCs cultured from blood. The above findings imply that the strongest IFN-α production is by APCs, even in the thymus. Since no APS1 thymic cells were available, we compared IFN expression (assessed by RT-PCR) by monocytes and immature and mature DCs cultured from blood from a compound heterozygous APS1 patient (with high anti-IFN-α2 and anti-IFN-ω titres). For IFN-α2 and IFN-α8, the expression levels were consistently higher in the immature DCs from this APS1 patient than from either of two healthy controls (Figure 4A). However, the APS1/control ratios were more variable for the $CD14^+$ monocytes (Figure 4B) and mature DCs, and also for IFN-ω in all three cell populations (Table S1).

Discussion

In two cohorts of Nordic APS1 patients, we have now shown almost 100% prevalences of very high titre IgG neutralising autoantibodies against most IFN-α subtypes and against the related, but antigenically distinct, IFN-ω. However they may be provoked, these precocious, persistent, and highly selective responses against this narrow range of cytokines appear to be APS1-specific and to segregate like a Mendelian recessive trait, regardless of the exact *AIRE* mutations. Their 100% "penetrance" in the homozygotes and compound heterozygotes is notable because APS1 shows such great variability both clinically and serologically [4,7,8,22,23,36]. Moreover, the autoantibodies reached high levels so early that, in almost every informative case, they preceded certain of the autoimmune disorders, and also some of the corresponding endocrine autoantibodies [36]; they even antedated overt CMC, which is usually the earliest sign of APS1. Clearly, these anti-IFN antibody responses cannot be a by-product of any one of these disorders. The converse possibilities—that type I IFNs are involved in autoimmunisation or that the antibodies predispose individuals to CMC—are considered below. For clinicians, our findings together imply a valuable role for these antibodies in diagnosis.

Clinical Applications

In diagnosis. It now appears that APS1 is even more variable than previously realised; one or more of the characteristic "APS1 triad" (CMC, HP, and AD) may be missing (e.g., Tables 1 and 2)—even in patients with both *AIRE* alleles mutated [22,23]. Since the anti-IFN antibodies show such high prevalences and APS1 specificity, and appear so early and persist so long, they should be valuable for diagnosis of such "incomplete cases" (e.g., the Norwegian brothers discussed in Results), as well as of prodromal APS1 before the appearance of its full clinical picture or of the

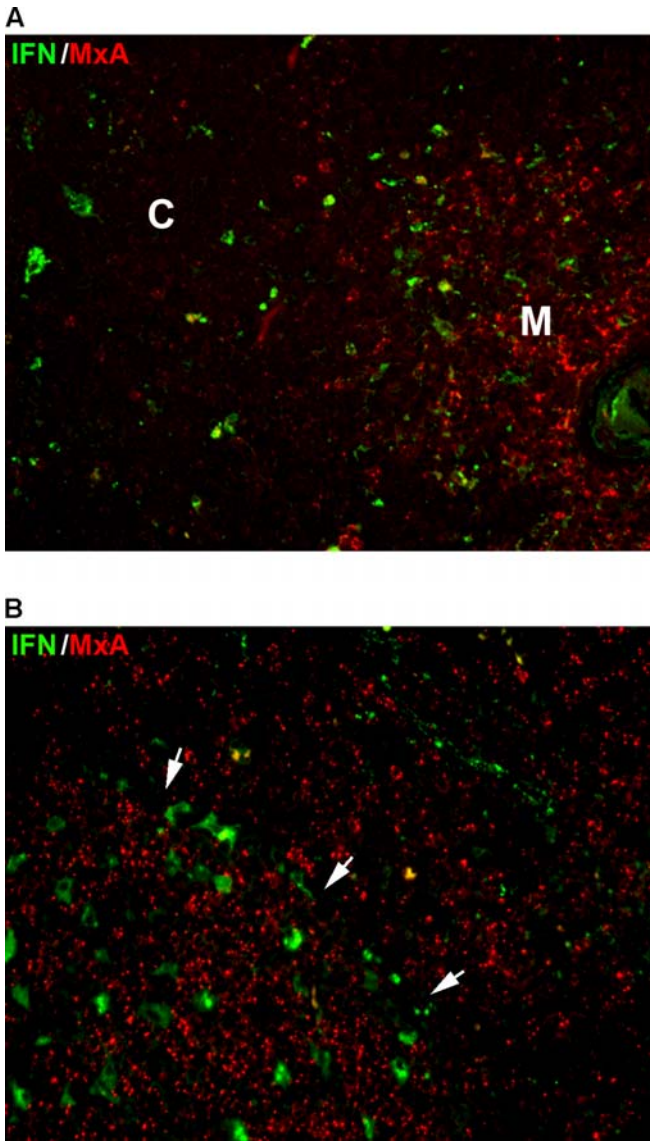

Figure 3. Paraffin Sections of Normal Child Thymus or Reactive Tonsil Double-Labelled for IFN-α and MxA
(A) In the thymic cortex (C) to the left, there are scattered IFN-α^+ cells (green) with almost no nearby MxA^+ cells (red), but MxA^+ cells are much more abundant in the medulla (M) to the right, especially around the Hassall's corpuscle at the edge. Results were similar in two other child thymi, though one showed fewer IFN-α^+ cells in the cortex. The very dense packing precludes precise phenotyping of medullary IFN-α^+ cells in paraffin sections.
(B) Similar staining in a reactive tonsil shows a germinal centre (to the left of the arrows) with several IFN-α^+ cells and punctate labelling for MxA. Throughout the adjacent T cell area (right), MxA is expressed strongly by a variety of cell types, as already reported [38], whereas IFN-α^+ cells are concentrated along a small blood vessel.
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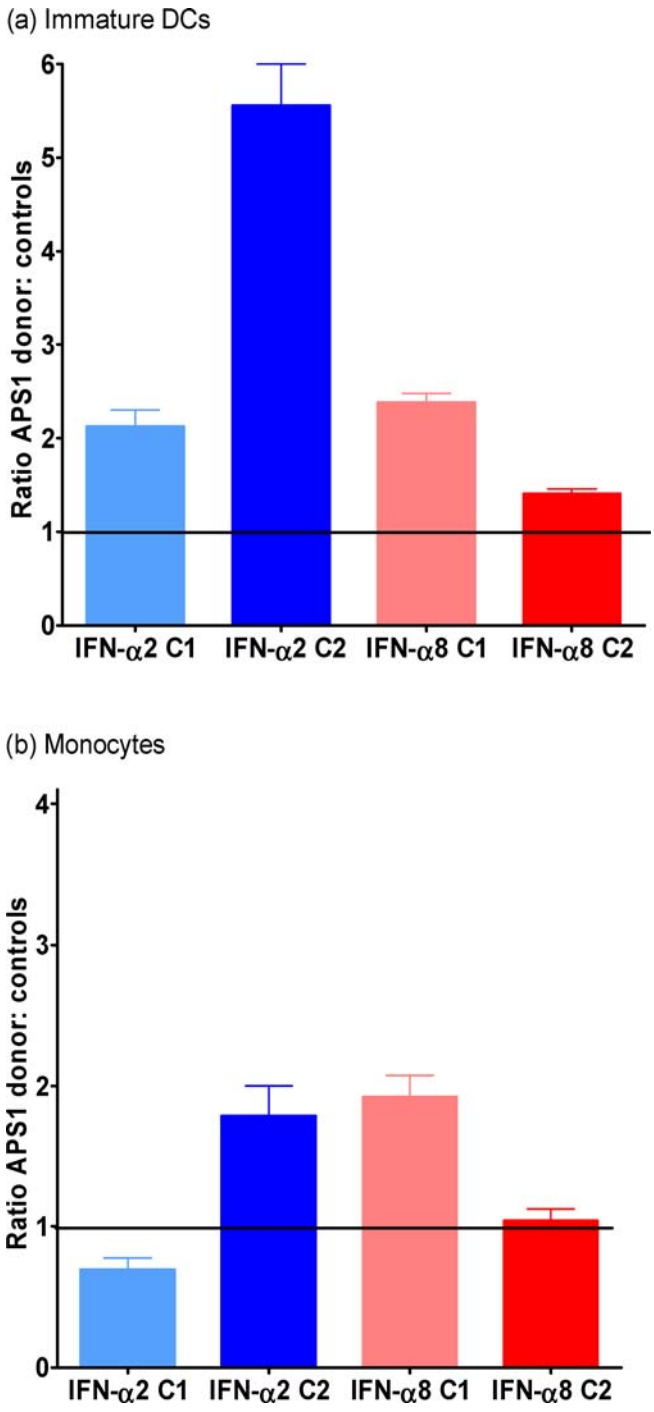

Figure 4. Expression of IFN-α2 and IFN-α8 by Immature DCs and Monocytes Cultured from an APS1 Patient Relative to Two Healthy Blood Donors

Expression by immature DCs (A) and monocytes (B). The APS1 donor has typical clinical features of APS1 and high anti–type I IFN titres (see footnote of Table 1). RT-PCR reactions were performed in parallel with *HPRT* as a "house-keeping" gene; *GADPH* gave very similar results when tested in one of the healthy donors, C1 (for IFN-α8). The relative gene (mRNA) expression levels were calculated using the comparative Ct (ΔΔCt) method (according to Applied Biosystems) to yield $2^{-\Delta\Delta Ct}$, where Ct represents the threshold cycle. Every sample was run at least three times in three parallel reactions. Results are depicted in histogram format as ratios of the expression levels in cells from the APS1 donor (which have been given a nominal value of 1.0) to those in the cells from the controls, C1 and C2. The bars represent the standard error of the mean of each ratio.
DOI: 10.1371/journal.pmed.0030289.g004

typical organ-specific autoantibodies. Thus, our data suggest that assays against IFN-α2 and IFN-ω may become the antibody tests of choice, since they were so clearly positive even in patients lacking the other antibodies associated with APS1 [23]. In addition, they were clearly negative in two further clinically doubtful cases with no *AIRE* mutations detected (patients E and F see footnote of Table 3), and in unaffected parents or siblings.

In prognosis. Antibodies against IFN-α2 and IFN-ω might also prove valuable prognostically, since titres clearly can be extremely high even in early childhood. How long or how frequently they precede the onset of APS1 signs is an intriguing question that others may already be able to address.

Infections in APS1. In theory, the anti–type I IFN antibodies might predispose to candidiasis. So might the anti-IL-12 antibodies that we saw early and transiently in a few patients. However, the *AIRE* mutations probably do so more strongly, since this infection is rare in MG/thymoma patients (despite their frequent additional anti-IL-12 antibodies) [11]. If these mutations predispose via parallel pathways to CMC and anti-IFN responses, the latter might hold clues to some even more crucial common defect upstream. If the anti-IFN autoantibodies do contribute to the candidiasis, then cautious treatment with IFN-γ might be considered for intractable cases (with no anti-IFN-γ antibodies); use of IFN-β might carry some risk of autoimmune complications [40]. Recently, Döffinger et al. [41] reported an extraordinary patient (aged 47) with high titre neutralising anti-IFN-γ antibodies who presented with intractable mycobacterial infections (from which he subsequently died). While on IFN-γ therapy (at age ~51), he developed an APS1-like syndrome including CMC, but no *AIRE* mutations were detected.

The general rarity of recurrent infections in APS1 is a puzzle, in view of (i) the very high neutralising titres against type I IFNs (and IL-12 in MG/thymoma patients), (ii) the role of these IFNs (and of IL-12) in polarising towards pro-inflammatory "T helper 1" T cell responses in humans [15], and (iii) the asplenia in some cases (e.g., Table 3). Any resistance of IFN-αs to neutralisation in vivo might also be relevant clinically, because treatment with anti-IFN-α antibodies has been proposed for certain autoimmune disorders such as systemic lupus erythematosus (SLE), where it is implicated in pathogenesis [42].

Neutralisation of IFN-α evidently can occur in vivo during therapy with IFN-α2; some patients clearly become resistant because they make antibodies against it—usually with much lower titres/cross-reactivity than in APS1 [43,44]. Possibly, however, neutralising antibodies might be less effective in vivo if IFNs secreted in tissues can bind to their receptors before the antibodies neutralise their activity, e.g., because IFN-producing cells are so ubiquitous and mobile or the IFNs they release act at short range. Secondly, many viruses infect mucosal surfaces, where locally produced IFNs may be poorly accessible to circulating neutralising autoantibodies (we find no serum IgA antibodies against IFN-α in MG/thymoma patients; A. Meager, unpublished data). Thirdly, patients with anti-IFN antibodies may instead be protected by IFN-β or IFN-λ—which were neutralised less frequently and mostly at much lower titres. In fact, there are occasional examples of intractable infections in both APS1 and MG/thymoma patients [8,10,11,21]; it may be relevant that one young child

with APS1 who had almost continuous respiratory infections also had neutralising titres of 500–2,000 against IFN-β by age 7–9 y. Since MG/thymomas mostly arise in adulthood, we argued [45] that such patients are well-endowed in advance with memory T helper 1 cells that can protect against familiar infections, even during subsequent immunosuppressive therapy. That seems less likely in APS1, where onset is often so much earlier, but where the IL-12→IFN-γ axis may well be compensating.

Comparing Findings in APS1 and MG/Thymoma Patients

Both the prevalences and the titres of the anti–type I IFN autoantibodies are higher in APS1 patients than in MG/thymoma patients [10,11]; they are also far higher—and much broader in IFN-α subtype cross-reactivity—than those of antibodies induced by therapy with IFN-α [43,44]. There are many provocative parallels with the autoantibodies in MG/thymoma patients [10,11]: their high titres at diagnosis against a similar range of IFN-α subtypes, their protracted persistence—despite immunosuppressive therapy for the MG ([11]; Figure 1A), and the constancy of their IFN-α subtype preferences ([11]; Figure 2).

Among the differences between these two conditions, the hitherto unrecognised autoantibodies to the IFN-λs were barely detectable in MG/thymoma patients, even those positive against IFN-β (A. Meager, unpublished data). In marked contrast, anti-IL-12 antibodies were much more common in MG/thymoma patients [10,11,45] than in patients with APS1, where their very early transient appearance in a few patients suggests that they might be more prevalent before the onset of APS1.

Clues to Autoimmunising Mechanisms

There is clearly a hierarchy of cytokines recognised in APS1, ranging from IFN-ω (100%) to IFN-α8, to IFN-α2, to IFN-β and IFN-λ1/IFN-λ2, to IL-12 and IFN-γ (<5%). Does that ranking reflect the cell types producing these cytokines, the amounts produced, the stimuli that evoke them, or their immunogenicity or antigenicity? Are the responses against them initiated in the thymus or in the periphery, where *AIRE* is also expressed [2–5]? Are type I IFNs involved in organ-specific autoimmunisation in APS1? The answers to these questions must hold valuable new clues to autoimmunising mechanisms, which are very hard to study in humans. Other clues include (i) the occurrence of anti-IFN-λ1 autoantibodies even in two of ten *AIRE* heterozygotes—which recalls the recently reported gene dosage effects on thymic deletion in $Aire^{+/-}$ mice [46]; (ii) that type I IFN therapy (e.g., for hepatitis C) sometimes precedes a variety of autoimmune disorders [40], including MG, SLE, sarcoidosis, and thyroid disease [47,48]; (iii) that aberrant IFN-α production is well known in SLE [42,49], where sporadic anti-IFN-α autoantibodies have also been noted [50], some of which prefer IFN-α8 (D. Isenberg and A. Meager, unpublished data); and (iv) that autoimmunisation apparently occurs within thymomas ([20,21]; see below).

The anti–type I IFN autoantibodies might be evoked by changes in the cells producing IFNs—which include potent APCs. For example, DCs that produce IFN-α and IFN-β are strongly implicated in autoimmunisation in psoriasis and type 1 diabetes (reviewed in [51]) as well as SLE [49]. It may be relevant that we found increased expression—particularly of IFN-α2 and IFN-8—in blood-derived immature DCs in one APS1 patient (Figure 4A). Because of the variability in expression levels (especially for IFN-ω and in mature DCs), these initial experiments demand to be extended to larger numbers of patients and controls, and above all to cells from thymus (both APCs and MECs). The higher transcript levels in the APS1 patient than the controls (Figure 4A) already suggest that *AIRE* can have inhibitory effects. In fact, these are now well recognised, and extend to many other genes, such as major histocompatibility complex class II genes, and to macrophages [52] as well as MECs [53].

Possibly, in APS1, there might be dysregulated surges in IFN secretion in vivo, e.g., in response to childhood infections such as *Varicella;* indeed, we also find modest neutralising titres against IFN-αs, IFN-ω, and IFN-λ1 (but not IFN-β or IFN-γ) in some hyperimmune anti-*Varicella* Ig preparations from healthy donors ([54]; A. Meager, unpublished data). Furthermore, type I IFNs can themselves act as pro-inflammatory "danger" signals [49] and activate APCs [55]—and thus play a very important role in linking innate and adaptive immune responses. Notably, too, a very early change in newly diabetic pancreatic islets is a striking and sometimes long-lasting up-regulation of IFN-α expression in the β cells [32,56]. In theory, that might help to autoimmunise against islet cell antigens; however, we have found no autoantibodies against IFN-α or IFN-ω in children with sporadic type I diabetes (or in adults with thyroid disease) [11].

It is tempting to look in the thymus for links between APS1 and thymomas; our evidence of autoimmunisation in these tumours [20,21] implies that expression of peripheral self-antigens there is "dangerous", possibly because of pro-inflammatory signals from dying cells [57] or natural killer cells [58] that could activate DCs and invoke innate as well as adaptive responses. Since they are clearly expressed and secreted in the normal thymic medulla (Figure 3), type I IFNs might, in theory, be involved in the normal induction of tolerance there to peripheral self-antigens, or in the early stages of autoimmunisation against them in APS1. If so, the abnormalities in this process in *AIRE*-mutant patients—or excessive cell death—might somehow lead to autoimmunisation against autoantigens such as type I IFNs that might be even more abundant in the APS1 than the normal thymus; the above hierarchy might be a valuable clue to help to identify the cell types responsible. At a later stage, the expression—and evident secretion—of IFN-α in germinal centres (key sites of B cell clonal diversification/memory generation [39]) might help to perpetuate the autoantibody response against it, and so explain its remarkably prolonged persistence. It might also contribute to the induction of IFN regulatory factor–8 there, which plays an important role in germinal centre physiology [59].

In conclusion, we demonstrate early and persistent prevalence in APS1 patients of apparently spontaneous neutralising autoantibodies to type I IFNs, responses that behave like a Mendelian trait. Such high "penetrance" is very unusual, even in inbred mice, and may have practical implications for clinicians as well as researchers. Because they are so APS1-specific and so consistent in this very variable condition, the autoantibodies against IFN-α and especially IFN-ω should be valuable diagnostically, e.g., to identify atypical or prodromal APS1 in patients with isolated candidiasis, AD, or HP [23], and perhaps also for prognosis, if they subsequently prove to predict onset time of the disease.

Finally, if the autoantibodies predispose to candidiasis, this condition might yield to treatment with IFN-γ, which was rarely recognised by APS1 sera.

Supporting Information

Table S1. Expression of IFN-α2, IFN-α8, and IFN-ω by Monocytes and Immature and Mature DCs Cultured from an APS1 Patient and Healthy Blood Donors

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Author contributions. A. Meager, P. Peterson, and N. Willcox initiated and designed the study. A. Meager, K. Visvalingam, P. Peterson, K. Möll, A. Murumägi, K. Krohn, P. Eskelin, J. Perheentupa, Y. Kadota, E. Husebye, and N. Willcox collected samples or data or did experiments for the study. K. Möll cultured monocyte-derived DCs from the APS1 patients' and healthy donors' blood, and extracted total RNA and synthesized cDNA, which was used in the quantitative RT-PCR experiment. A. Meager, P. Peterson, K. Möll, J. Perheentupa, and N. Willcox analyzed the data. P. Eskelin designed and performed mutational analyses and human leukocyte antigen genotyping of the APS1 patients. J. Perheentupa and E. Husebye enrolled Finnish and Norwegian APS1 patients, respectively. K. Krohn enrolled the APS1 patient whose peripheral blood monocytes were used by K. Möll to generate DCs. J. Perheentupa and E. Husebye are the clinicians who followed all the APS1 patients involved, collected and organized all the clinical data defining each individual's course of disease, enrolled them for the study of the immune pathology of APS1, stored all the serum samples that were analyzed in this study, participated in the analysis of the immunological versus the clinical findings, and contributed to the revision of the manuscript at several stages. P. Peterson and N. Willcox collected serum samples from patients with APS2, or sporadic CMC, HP, or AD. N. Willcox also helped to collect the samples from the MG/thymoma patients mentioned in Table 2. A. Meager and N. Willcox played a major role in drafting the text, and P. Peterson, K. Möll, K. Krohn, J. Perheentupa, and E. Husebye contributed to writing the paper.

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Editors' Summary

Background. The human body is under constant attack by viruses, bacteria, fungi, and parasites, but the immune system usually prevents these pathogens from causing disease. To be effective, the immune system has to respond rapidly to foreign antigens (bits of protein specific to pathogens) while ignoring self-antigens. If tolerance to self-antigens breaks down, autoimmunity develops, often causing disease. There are many common autoimmune diseases—type I diabetes and multiple sclerosis, for example—but because these involve defects in many genes as well as environmental factors, the details of how autoimmunity develops remain unclear. Autoimmune polyendocrinopathy syndrome type 1 (APS1), however, is caused by defects in a single gene. Patients with this rare disease characteristically have defects (or mutations) in both copies of a gene called *AIRE* (for autoimmune regulator). In normal people, the protein product of this gene helps to establish tolerance to a subset of self-antigens. People carrying *AIRE* mutations make an autoimmune response against some of their own tissues, typically the endocrine (hormone-producing) tissues that control body metabolism. A major component of this autoimmune response are "autoantibodies" (antibodies are immune molecules that normally recognize and attack foreign substances, whereas autoantibodies are directed against the body's own molecules).

Why Was This Study Done? For a diagnosis of APS1, a patient must have at least two of the following symptoms: recurrent, localized yeast infections (usually the first symptom of the disease to appear in early childhood), hypoparathyroidism (failure of the gland that controls calcium levels in the body), and Addison disease (failure of the steroid-producing adrenal glands, which help the body respond to stress). The researchers who did this study had previously noticed that these yeast infections and autoimmunity (usually against muscle) can also occur in patients with tumors of the thymus (thymomas). The thymus is the organ that generates immune cells called T cells. Generation of the T cell repertoire in the thymus involves selection of those T cells that recognize only foreign substances. T cells that can react against self-antigens are eliminated, and the *AIRE* gene is thought to be involved in this "education process." Like those with APS1, patients with thymomas make autoantibodies not only against target organs (especially muscle in their case), but also against interferon alpha (IFN-α) and interferon omega (IFN-ω), two secreted immune regulators. The researchers wanted to know if patients with APS1 also make autoantibodies against interferons, because this could provide insights into how autoimmunity develops in APS1 and other autoimmune diseases.

What Did the Researchers Do and Find? The researchers tested blood from nearly 100 APS1 patients for antibodies to IFN-α, IFN-ω, and other immunoregulatory cytokines. They found that almost all patients made large amounts of antibodies that blocked the function of IFN-α and IFN-ω; some also made lower amounts of antibodies against two related interferons, but none made blocking antibodies against unrelated interferons or other immune regulators. For many patients, serum samples were available at different times during their disease, which allowed the researchers to show that the antibodies appeared early in disease development, before the onset of yeast infections or damage to endocrine tissues, and their production continued for decades as the patient aged. Furthermore, only patients with APS1 made these antibodies—they were absent in patients with Addison disease alone, for example.

What Do These Findings Mean? The discovery that autoantibodies to IFN-α and IFN-ω are made persistently in patients with APS1 suggests ways in which autoimmunity develops in these patients. These can now be investigated further both in patients and in animal models of the disease. The discovery also has practical implications. Measurement of these autoantibodies might help some APS1 patients by allowing earlier diagnosis—and prompter treatment—than in current practice. The levels of these autoantibodies might also help to predict the time course of APS1 in individual patients, although more studies will be needed to check this out. Finally, if future studies show that interferon autoantibodies are responsible for the patients' susceptibility to yeast infections (which seems plausible), treatment with IFN-γ, an interferon to which APS1 patients do not make autoantibodies, might provide an alternative way to deal with this problem.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0030289.
- MedlinePlus pages on autoimmune diseases
- Online Mendelian Inheritance in Man page on APS1
- Links to patient information on APS1 from the Stanford Health Library
- Wikipedia page on autoendocrine polyendocrinopathy (note: Wikipedia is a free online encyclopedia that anyone can edit)
- Information on autoimmunity from the American Autoimmune Related Diseases Association