Bachelor’s Thesis

Studies of peripheral tolerance in AIRE deficient mice

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1. Abstract
Autoimmune Polyendocrine Syndrome Type 1 (APS I) is a monogenic autosomal recessive autoimmune disorder which is the result of mutations in the autoimmune regulator (AIRE) gene. Symptoms of the disease include circulation of multiple organ specific autoantibodies, which leads to the breakdown of several tissues, including the adrenal cortex and the parathyroid glands. The patients also develop a number of non-endocrine disorders. This study has investigated the peripheral tolerance mechanisms controlled by the AIRE gene in Aire deficient mice, an animal model of the disease. The B cell Activating Factor (BAFF), which is a cytokine involved in B cell survival and growth, is elevated in Aire<sup>−/−</sup> mice, resulting in an increased release of autoantibodies and B cell proliferation. Therefore the BAFF level differences between TCR<sup>−/−</sup> and B6 mice was studied, and the results showed significantly higher levels of BAFF in TCR<sup>−/−</sup> mice. This is not in accordance with earlier studies. ICOS and ICOSL are involved in the activation of follicular T helper cells. The expression of ICOSL on different subpopulations of DC from mice was studied to evaluate the possible influence of AIRE expression on the T cells in the spleen. The results showed that ICOSL is significantly higher expressed in peripheral 33D1+ DCs in Aire<sup>−/−</sup> mice, showing that AIRE has a role in the over-activation of the follicular T helper cells, which can lead to autoantibody production and inflammation. These results show that AIRE is involved in peripheral tolerance.

Keywords: Autoimmunity, Peripheral tolerance, AIRE, APS I, DC, BAFF, ICOSL

2. List of Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AIRE</td>
<td>Autoimmune Regulator</td>
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<tr>
<td>APC</td>
<td>Anti-nuclear Antibodies</td>
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<td>APRIL</td>
<td>A proliferation-inducing ligand</td>
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<td>APS I</td>
<td>Autoimmune Polyendocrine Syndrome Type 1</td>
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<td>Syndrome Type 1</td>
<td></td>
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<tr>
<td>BAFF</td>
<td>B cell Activating Factor</td>
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<td>APRIL</td>
<td>A proliferation-inducing ligand</td>
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<tr>
<td>cDC</td>
<td>Conventional Dendritic Cell</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>cTEC</td>
<td>Cortical Thymic Epithelial Cell</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>FOB</td>
<td>Follicular B Cell</td>
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<tr>
<td>Hz</td>
<td>Heterozygot</td>
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<tr>
<td>ICOS-L</td>
<td>Inducible Costimulator-Ligand</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
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<tr>
<td>KO</td>
<td>Knock Out</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>mTEC</td>
<td>Medullary Thymic Epithelial Cell</td>
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<td>MZB</td>
<td>Marginal Zone B Cell</td>
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<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
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<tr>
<td>NKT</td>
<td>Natural Killer T Cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PIAS1</td>
<td>Protein Inhibitor of Phosphorylated STAT1</td>
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<tr>
<td>TACI</td>
<td>Transmembrane Activator and Calcium-Modulator and Cyclophilin Ligand Interactor</td>
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<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>tDC</td>
<td>Thymic Dendritic Cell</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>Treg</td>
<td>T regulatory Cell</td>
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<tr>
<td>Wt</td>
<td>Wild Type</td>
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<tr>
<td>QRT-PCR</td>
<td>Quantitative Real Time PCR</td>
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3. Introduction

3.1 Self-tolerance
The immune system is able to recognize and eliminate a wide variety of pathogens and tumor cells without harming any self-constituents. This ability to distinguish the self from the non-self is called self-tolerance and is divided into the central- and peripheral tolerance. The mechanisms that lie behind the development and education of the B cells in the bone marrow and the T cells in the thymus is referred to as the central tolerance, and the mechanisms in the periphery that are able to recognize danger signals to self are referred to as the peripheral tolerance (Abbas et al., 2004). Peripheral tolerance mechanisms include anergy (no response due to lack of co-stimulators), deletion by apoptosis and the actions of regulatory T cells (Treg), which all handles threatening self-reactive T cells that have escaped from the thymic control and been released into the circulation (Bouneaud et al., 2000 and Abbas et al., 2004).

T cells are both developed and educated in the thymus where they experience two phases of selection: positive and negative (Palmer, 2003). Education in self-tolerance is executed by the epithelial cells of the medulla (mTEC’s) and the thymic dendritic cells (tDC’s) and takes place during the negative selection phase. Defective processes during the negative selection phase lead to autoreactive T cells that sometimes escape the thymic control and enter the circulation. The peripheral tolerance handles these self-reactive T cells to minimize the chances of autoimmunity. B cells undergo similar steps of selectivity in the bone marrow. Occurrence of any failure regarding the mechanisms in self-tolerance may however lead to autoimmunity and can cause the immune system to attack the self.

The autoimmune diseases are increasing and today disorders such as diabetes type 1 and multiple sclerosis affect 3-5% of the population (Jacobson et al., 1997). Although extensive research has been done about environmental and genetic factors that might lead to autoimmunity and despite many advances in the field, the mechanisms are still unclear due to their complex nature, and no cures have been found. Some helping therapies do exist, such as hormonal replacement therapy or immunosuppressive therapy, but they do not target the real problem and might have side effects such as an increased sensitivity to infections (Feldmann and Steinman, 2005).

3.2 AIRE in Autoimmune Polyendocrine Syndrome type 1
The autoimmune regulator gene (AIRE) is situated on the human chromosome 21q22.3 (Nagamine et al., 1997) and plays a major role in the regulation of the self-antigen expression in thymus (Liston et al., 2003; Zuklys et al., 2000). AIRE is a transcriptional regulator and is expressed mainly by the peripheral dendritic cells (DCs) and by the epithelial cells of the medulla (mTEC’s) in thymus, but the expression of AIRE has been found in several tissues involved in autoimmunity such as the spleen, lymph nodes and the bone marrow (Halonen et al., 2001).

Any mutation in the AIRE gene causes the autoimmune disorder Autoimmune Polyendocrine Syndrome type 1 (APS I), which is a monogenic progressive disorder where patients suffer from both endocrine and non-endocrine manifestations. A well known characteristic of APS I is the circulation of multiple organ specific autoantibodies, which leads to the breakdown of several tissues, including the adrenal cortex (Addisons disease) and the parathyroid glands (hypoparathyroidism). The patients also develop a number of non-endocrine disorders, where the most frequent is the fungal infection candidiasis. Together these three manifestations make up the diagnostic triad for APS I (Ahonen et al., 1990).

APS I is because of its monogenic recessive inheritance a rare disease, and it is most common where genetic populations are isolated. The more commonly affected are the Finnish
Mutations in the AIRE gene lead to a genotype with APS I with 100% accuracy, but the phenotype–genotype relationship is not fully understood and therefore phenotype cannot be predicted in advance (Wang et al., 1998; Aaltonen and Bjorses, 1999). The full mechanism of this disease is still unknown.

3.3 AIRE deficient mice
AIRE deficient Mice (Aire<sup>-/-</sup>) work as a model for studies concerning the APS I disorder because they share similar features with the APS I patients (Ramsey et al., 2002). The mouse AIRE gene also encodes a 552 amino acid protein that resembles the human homolog with 71% (Halonen et al., 2001). These common features are lymphocytic infiltrates, an increased production of circulating autoantibodies targeting multiple organs and low fertility. In addition to these responses however, mice also develop marginal zone B cell (MZB) lymphoma (Hässler et al, 2006), an increase of peripheral APC (antigen presenting cell) mediated T cell activation (Ramsey et al, 2006), and B cell liver infiltrates in the spleen due to proliferation. The target organ in AIRE deficient mice is the liver, whilst in APS I patients the adrenal cortex is the most common target for autoantibodies (Ramsey et al., 2002). The mice also show a much milder phenotype than the APS I patients, and therefore do not develop a characteristic autoimmune disease (Kuroda et al., 2005). Due to the differences, there are some people questioning whether mice can be used as a model for APS I.

3.4 B Cell Activating Factor (BAFF)
Studies showing elevated levels of the B cell activating factor (BAFF) have been made in both human APS I patients and AIRE deficient mice (Lindh et al., 2008). BAFF is a cytokine from the TNF (tumor necrosis factor) family that is crucial for peripheral B lymphocyte survival and growth (Batten et al., 2000). BAFF is excreted primarily from the dendritic cells and stroma cells but also from monocytes, neutrophils, T lymphocytes and macrophages in the spleen, lymphnodes and bone marrow (Schneider et al., 1999; Gorelik et al., 2003). B cells are not induced to apoptosis in the presence of BAFF and remain in the circulation. Overexpression of BAFF can therefore lead to B cell proliferation, MZB lymphoma and an increased production of autoantibodies, which in turn might lead to the breakdown of organs targeted by the autoantibodies (Pers et al., 2005; Thien et al., 2004). Elevated BAFF production is therefore a contributing factor to elevated immune responses.

Another member of the TNF family is the proliferation-inducing ligand (APRIL). APRIL is a cytokine with similar effects as BAFF, but the function and expression of APRIL is not as well defined.

APRIL and BAFF however share the same receptor – TACI, and therefore it can be assumed that the function may be similar to that of BAFF.

The cytokine IFN-γ stimulate the dendritic cells to produce BAFF. IFN-γ is released by the natural killer T cells (NKT) and by the natural killer cells (NK) in the spleen. The NK and the NKT cells are as well as T cells originally from the thymus, but the development differs, and the NK and the NKT cells are active in the periphery (Carnaud et al., 1999). To be able to reach the dendritic cell core and create a response with BAFF production, IFN-γ need to go through the STAT I signalling pathway in the DC. The STAT I signalling pathway control the BAFF levels and only release it when necessary (See picture of the STAT I signalling pathway in Appendix 1).
(Illmarinen et al., 2007). However, if there is any mutation in the AIRE gene making it non-functional, PIAS cannot keep up blocking the transcriptional pathway alone and the transcription of BAFF begins. The increased production of BAFF when stimulated with IFN- \( \gamma \) leads to the increased release of autoantibodies from the Marginal Zone B Cells in the spleen, and also leads to a proliferation of B cells (Lindh et al., 2008).

### 3.5 Dendritic Cells

Most studies of AIRE deficiency have concentrated on the central tolerance, where for example the AIRE expression in mTECs has been shown to play an important role in the deletion of autoreactive T cells (Liston et al., 2003; Anderson et al., 2002). However, the expression of AIRE in the dendritic cells located in the periphery is not yet fully evaluated. Furthermore, most recent studies have not taken into account that different subtypes of dendritic cells have different functions and are situated in different organs (Lindmark et al., unpublished data).

There are two major groups of dendritic cells: non-lymphoid migratory DCs and lymphoid resident DCs (Merad and Manz, 2009). The non-lymphoid migratory DCs are able to migrate through lymphatics and function to maintain and induce specific responses in the periphery (Banchereau and Steinman, 1998; Steinman et al., 2003), whilst the lymphoid resident DCs function lies in lymphoid defence in the central tolerance, where they handle infections with the secretion of cytokines and direct induction T cell response (Liu, 2005). The lymphoid resident DCs are further divided into two different subpopulations; the CD8+ DC (surface markers CD8a and DEC205) which is located in the T cell zone of the spleen, and the 33D1+ (surface markers 33D1 and CD4) which is located in the Marginal Zone of the spleen as well as in the red pulp (Dudziak et al., 2007). Another subpopulation can also be found in the spleen, known as double negative (DN) DCs (Vremec et al., 2000). It has been shown in recent studies that the 33D1+ DCs express significantly higher amount of AIRE than the other two DC subpopulations in the spleen (Lindmark et al., unpublished data).

The inducible co-stimulator (ICOS) and its ligand ICOSL are involved in the activation of follicular T helper cells and they also regulate B cell responses (Dong et al, 2001; Rottman et al, 2001; Kopf et al, 2000). The ICOS receptor is located on the follicular T helper cell surface in the spleen and it is a known fact that ICOS must come in direct contact with the ICOSL situated on the DC surface (Wassink et al., 2004) for a working immune response in the spleen.

### 3.6 Aim of the study

The aim of this study is to investigate peripheral tolerance in AIRE deficient mice. The aims are in detail:

- To culture adrenal cortical tumor cells and make them apoptotic for injection in mice for future autoantibody studies.
- To study the expression of ICOSL on different DC subpopulations in order to investigate the mechanisms behind the over-activation of peripheral T cells seen in Aire\(^{-}\)/mice.
- To investigate the expression of APRIL on different DC subpopulations to see whether the B cells could get over-active and proliferate due to APRIL.
- To measure the concentration of BAFF in two types of AIRE deficient mice (B6 and TCR) to investigate if elevated levels of BAFF is T cell dependent.
3.7 Hypothesis
B6 Aire⁻ mice lack the AIRE gene, which together with PIAS1 work as inhibitors of the STAT I signalling pathway. With no AIRE blocking the pathway, it has been shown in recent studies on immunized B6 Aire⁻ that the DCs will get excess IFN-γ stimulation, resulting in an overexpression of BAFF (Lindh et al., 2008). Because these events take place in the periphery, the hypothesis is that AIRE regulate over-activated cells in the periphery. To show this, the involvement of the central tolerance will be deleted by using TCR⁻ mice that lack T cells and NKT cells.

The ICOSL expression will probably differ between different DC subpopulations, because it has been shown in earlier studies that T cells in the spleen proliferate more in Aire⁻ than in wt mice (Ramsey et al, 2006). Therefore it is likely that Aire is involved in regulating this response. Since Aire is expressed by the 33D1+ DCs in the spleen (Lindmark et al., unpublished data) we hypothesize that this subpopulation is expressing the ICOSL leading to over-activation of T cells in the spleen.

4. Materials and Methods

4.1 Mice
The AIRE deficient mice used in this study were originally purchased from The Jackson Laboratories (Bar Harbour, ME). The mice were eight to ten weeks old and from the B6 strain: B6 Aire mice and B6 TCR mice. B6 Aire⁻ mice are AIRE knockout mice, and TCR⁻ are double knockout mice, lacking the AIRE gene and the alfa-beta T cell receptors and the gamma-delta T cell receptors. In addition, the TCR mice also lack NKT cells.

Breeding and maintenance took place under pathogen free conditions at the MTC animal facility at the Karolinska Institute.

4.2 DNA isolation and Polymerase Chain Reaction
DNA was extracted from frozen mouse tissue from the tail or ear, with Dneasy ® Blood & Tissue Kit (Qiagen).

Extracted DNA was then used for AIRE gene amplification through Polymerase Chain Reaction. The reaction was carried out with The GeneAmp® PCR (System 2400) using the screening programme: preincubation 95 °C 5 min, (93 °C 20 s, 68 °C 2 min x 30 cycles), 68 °C 10 min, 10 °C ∞ and primers for mouse AIRE:
Neo 1 (5´- CTG AGC CCA GAA AGC GAA GGA GCA AAG CTG – 3´) and AireScreen (forward 5´ ACCAATCTCCGCTGCAAATCC-3´, reverse 5´ TATGTAGAACAGGGTGCACTAGC-3´)

4.3 Genotyping
Amplified DNA obtained from PCR reactions were run on 2% UltraPure™Agaros (Invitrogen) gel with SYBR® Safe DNA gel stain (10.000X concentrate in DMSO, Invitrogen) diluted with DNA Typing Grade 50X TAE buffer (Gibco BRL, Life technologies) to be able to separate the mice genotypes Hz, wt and ko.

The electrophoresis apparatus was set to 95 Volt and run for 20 minutes. Analysis and documentation was performed with a gel documentation camera and Quantity One Software (Bio-Rad).

Wt and ko mice were used for further studies and Hz mice were used for breeding purposes. Breeding is necessary due to that transgenic mice are infertile and two homozygotes must be crossed to create AIRE⁻ mice. Controls: Hz, wt, ko and H20
4.4 Quantitative real time PCR
RNA isolated from sorted dendritic cell populations from mice spleens was obtained from earlier experiments and RNA concentrations were measured with NanoDrop® ND-1000 (Thermo Scientific, Wilmington, Delaware, USA). The dendritic cell populations used were DN, CD8+ and 33D1+. Reverse transcriptase reaction was performed by using the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, USA) according to manufacturer’s instructions. Reaction thermal cycles used: 25 °C 5 min, 42 °C 30 min, 85 °C 5 min.

The Quantitative Real Time PCR was performed with iCyclerIQ (Bio-Rad) using 2X IQ™ SYBR® Green Supermix (Bio-Rad) and specific primers for ICOSL (forward – 5’ AGCTCCATGTTTCTAGCGGGTTC - 3’, reverse – 5’ ACCATTGCACCGACTTCAGTCTC - 3’), APRIL (forward 5’ - GCTCATGCCCAGCTCATC - 3’, reverse 5’ - CCAGGTGCAGGACAGAGTGC - 3’) and the housekeeping gene β-actin (forward 5’GACGACATGGAGAAGATCTGG -3’, reverse 5’TGTGGTGGAAGCTGTAGC- 3’). Cycle thresholds and melt curves were acquired with iCycler IQ™ Optical System Software (Bio-Rad).

ICOS L and APRIL expression values were normalized by setting them in relation to the housekeeping gene β-actin. The β-actin reference was set to one, and ΔCt values of AIRE−/− mice were compared to ΔCt of Wt, with the Delta- Delta CT (2−ΔΔCt) method.

4.5 Enzyme-linked Immunosorbent Assay (ELISA)
Serum from TCR wt, TCR Aire−/−, B6 wt and B6 Aire−/− mice were used for detection of BAFF levels. The Quantikine ® Mouse BAFF/BLys/TNFSF13B immunoassay kit was used for the quantitative determination of mouse BAFF concentrations. Serum from TCR wt and TCR Aire−/− was diluted 1:25 with the Calibrator Diluent RD6-12 and serum from TCR wt, TCR Aire−/−, B6 wt and B6 Aire−/− were diluted 1:100 in two different test runs and pipetted onto the 96 well polystyrene microplate from the Quantikine kit, precoated with a monoclonal antibody specific for mouse BAFF/Blys. No duplicates were used. Controls: Mouse BAFF/BLyS with known concentration and an empty well that only contained RD6-12.

The optical densities of the wells were determined within 30 minutes by using a microplate reader set to 450 nm, and a wavelength correction set to 550nm.

The optical density results were transported to Excel where a standard curve was generated for each experimental set up and correction and pg/mL values were calculated. Data were analyzed and documented by using GraphPad Prism 5.

4.6 Cell culture
Adrenocortical mouse tumor cells: Y-1 cells (ATCC-CCL-79) were cultured in ATCC-formulated F-12K Medium (Kaighn’s Modification of Ham’s F-12 Medium) containing Fetal bovine serum 2.5 % and Horse serum 15% (ATCC, Manassas, VA, USA) for approximately a month in 37 °C. Medium renewal was done twice weekly by using cold PBS as wash buffer and 0.05% Trypsin – EDTA (IX, From Invitrogen) for removing the cells from the plate surface. The cells were centrifuged in 300 RCF for 7 minutes, resuspended in new complete F-12 K Medium and pipetted to a new plate according to the ATCC-CCL-79 protocol.

4.7 Fluorescence-activated Cell Sorting (FACS)
The adrenocortical mouse tumor cells were washed with cold PBS, and then incubated in 0.05% Trypsin – EDTA (IX, From Invitrogen) for 15 min for the cells to detach from the plate. Cells were divided into 8 samples containing 1x10⁶ cells with different stimulation of Streptozotocin (Sigma-Aldrich); 50 µg ml⁻¹, 150 µg ml⁻¹, 250 µg ml⁻¹, 350 µg ml⁻¹, 450 µg ml⁻¹, 550 µg ml⁻¹, 650 µg ml⁻¹ and 0 µg ml⁻¹ (control). The samples were diluted with F-12K
Medium containing fetal bovine serum 2.5 % and horse serum 15% (ATCC, Manassas, VA, USA). Cells were then cultured for 19 hours on a 24-well plate, followed by a repeated procedure with PBS wash and 0.05% Trypsin – EDTA treatment. The cells were counted on fast read slides with Trypan blue 0.4% (T8154, Sigma-Aldrich) and the concentration was adjusted to 1x10⁶ cells/ml with Binding buffer solution according to the General Annexin V Staining procedure from BD Biosciences.

The cells were stained with Annexin V (conjugated to Fluorescein Isothiocyanate, FITC) and Propidium Iodid (PI) by using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, San Diego, USA). Fluorescence-activated cell sorting (FACS) was used to sort out the apoptotic cells. Cell events were collected at 488 nm with FACS Aria and analyzed with FACSDiva Software (BD Biosciences, San Diego, USA).

Controls used: unstained cells, cells stained only with Annexin V and cells stained only with PI.

4.8 Statistical analysis
Data were analysed with the nonparametric Mann Whitney U test. A Nonparametric test was used due to small sample sizes and possible deviations from normal distributions.

5. Results

5.1 No elevated BAFF expression in TCR Aire−/− mice
In order to rule out that the elevated levels of BAFF seen in AIRE deficient mice are an effect of the faulty negative selection of T cells in the thymus, the levels of BAFF in Aire−/− mice was measured, in the absence of functional T cells (TCR−/− Aire−/− mice).

ELISA results when comparing BAFF levels in TCR wt and TCR Aire−/− show no significant difference (P > 0.05) between wt and Aire−/− mice (Figure 1.). The bars represent the mean values and standard error of the mean (SEM.) P<0.05 was considered statistically significant.
5.2 TCR mice show higher levels of BAFF than B6 mice
The results from ELISA when comparing the data from BAFF concentrations in B6 Aire mice and TCR mice display significant difference ($P = 0.0022$), where the TCR mice display significantly higher levels of BAFF (Figure 2.). There are no significant differences ($P > 0.05$) between the wt and the Aire$^{-/-}$ within either of the mice genotypes.

Figure 1.BAFF levels in TCR wt and TCR Aire$^{-/-}$ mice. There is no significant difference ($P > 0.05$, Mann-Whitney U-test) when comparing TCR wt and TCR Aire$^{-/-}$ mice. Each square represents an individual mouse.

Figure 2.BAFF levels in TCR wt/Aire$^{-/-}$ and B6 wt/Aire$^{-/-}$ mice. There are significant differences ($P = 0.0022$, Mann-Whitney U-test) when comparing B6 wt and Aire$^{-/-}$ with TCR mice, but no significant differences ($P > 0.05$, Mann-Whitney U-test) within the genotypes. Each square represents an individual mouse.
5.3 AIRE is involved in peripheral tolerance

The ICOS receptor located on the T cell surface need to be engaged by ICOSL in order for the T cells to be activated. Earlier studies have shown that Aire deficient mice display an APC mediated T cell activation in the spleen (Ramsey et al., 2006), and therefore the expression of ICOSL on different DC subpopulations from the spleen was investigated.

ICOSL expression in the dendritic cell subpopulations DN, CD8+ and 33D1+ from the spleen is shown in Figure 3. The lymphoid resident DCs 33D1+ show significantly higher expression of ICOSL than the other two DC subpopulations, CD8+ and DN. These results show that ICOSL is expressed more in 33D1+ mice where AIRE is deficient, than in the CD8+ and DN mice where expression of AIRE is not deficient. The results show that AIRE has a role in peripheral tolerance.

![Figure 3. ICOSL expression in the dendritic cell subpopulations DN, CD8+ and 33D1+ from the spleen. Bars represent fold change (change from the reference set to one) of ICOSL compared to reference ΔCt. The change was normalized against the expression of β-actin.](image)

5.4 APRIL expression

No detection of APRIL expression was found, but the housekeeping gene β-actin could be detected in normal levels.

5.5 FACS results

No sample events or controls could be detected with FACS.

6. Discussion

This project aimed to investigate the peripheral tolerance in AIRE deficient mice. AIRE is expressed mainly by the peripheral dendritic cells (DCs) and by the epithelial cells of the medulla (mTEC’s) in the thymus (Halonen et al., 2001). It has also been shown that the levels of BAFF produced by the dendritic cells are elevated and over-activate the marginal zone B cells in the spleen when AIRE is deficient (Lindh et al., 2008). Because these events take place in the spleen, the hypothesis was that AIRE regulate over-activated cells in the
periphery which has nothing do to with central tolerance. To show this, the involvement of the central tolerance was deleted by the use of TCR\(^{+/−}\) mice. Because of the lack of T cell receptors, no functional T cells are present in the system and should therefore not be able to create an immune response that could elevate the BAFF levels. If the hypothesis had been correct, the TCR\(^{+/−}\) mice would have been able to produce elevated levels of BAFF without the aid of T cells and would have shown higher levels of BAFF than the TCR wt that is not AIRE deficient. However, when the expression of BAFF in TCR wt was compared to the expression of BAFF in TCR AIRE\(^{+/−}\) mice, the results showed no significant difference in BAFF expression. The reason why no elevated levels of BAFF could be detected in TCR\(^{+/−}\) mice could, however, be due to the lack of NKT cells which are the producers of IFN-\(γ\). NKT cells may be necessary to elevate the BAFF levels, and it could be of future interest to examine the IFN-\(γ\) influence from NKT cells on TCR\(^{+/−}\) mice. Theoretically this could be done by an injection of NKT cells into TCR\(^{+/−}\) AIRE\(^{+/−}\) mice to see if the BAFF levels increase. Because of the lack of T cell receptors, it will be obvious whether the T cells are involved or not in the elevated BAFF response. A direct injection of IFN-\(γ\) into the mice could also possibly be tested.

Because no differences in BAFF levels between TCR wt and TCR AIRE\(^{+/−}\) were found, TCR wt and AIRE\(^{+/−}\) mice were compared with B6 wt and AIRE\(^{+/−}\) mice. The comparison was made to investigate if the BAFF levels in TCR wt/AIRE\(^{+/−}\) were different from the B6 AIRE\(^{+/−}\) mice. The B6 AIRE\(^{+/−}\) mice were expected to have significantly higher levels of BAFF than TCR wt, TCR AIRE\(^{+/−}\) and the B6 wt. This would have shown that the B6 AIRE\(^{+/−}\) mice have elevated levels of BAFF due to the higher IFN-\(γ\) stimulation when AIRE is deficient, whilst TCR wt/AIRE\(^{+/−}\) should have shown lower levels due to the lack of the IFN-\(γ\) stimulation from NKT cells. The B6 wt mice should also have shown lower levels because they have a functional AIRE gene. However, the results showed the opposite. There were significantly higher levels of BAFF in both of the TCR mice than in the B6 mice, but no significant difference between TCR wt and TCR AIRE\(^{+/−}\) or B6 wt and B6 AIRE\(^{+/−}\). This result could be due to that earlier studies of B6 wt and B6 AIRE\(^{+/−}\) mice have been done with immunized mice. However, the difference between immunized B6 mice and non-immunized B6 mice has been examined, with no significant difference detected (Lindh et al., 2008). Therefore the B6 AIRE\(^{+/−}\) would have had to show higher levels of BAFF to be in accordance with earlier studies. Studies investigating differences between immunized and non-immunized TCR mice have not been done. Further studies would therefore be to examine if there is any differences in BAFF levels between immunized and non-immunised TCR wt and AIRE\(^{+/−}\) mice with B6 mice, to investigate possible differences and exclude possible methodological errors. The results could also be due to that T cells actually do affect the BAFF levels. However, there are yet no theories suggesting how this mechanism might work.

It has been shown in recent studies that the 33D1+ DCs express significantly higher amounts of AIRE, than the other two DC subpopulations in the spleen, CD8+ and DN (Lindmark et al., unpublished data). Earlier studies have also shown that Aire deficient mice do not show a good immune response in the spleen, which could be due to the contact between ICOS and ICOSL which is needed for a good immune response. Therefore the contact between the ICOS receptor on the follicular T helper cell and the ICOSL on the dendritic cells was studied. It is clear from the results when measuring ICOSL expression in DC subpopulations 33D1+,CD8+ and DN from the spleen that the lymphoid resident DCs 33D1+ (that also do express AIRE) express significantly higher levels of ICOSL than the 33D1+ wt and the other two DC subpopulations studied. Earlier studies have shown that T cells in the spleen proliferate more in Aire\(^{+/−}\) mice than in wt mice (Ramsey et al., 2006), and the elevated activation of ICOS due to the elevated levels of ICOSL could be the explanation of this proliferation. These results serves as a possible confirmation of that AIRE is actually
involved in peripheral tolerance due to the fact that these events were found in the spleen and not the bone marrow or thymus which are parts of the central tolerance.

The expression of APRIL in dendritic cells were also evaluated in the three DC subpopulations, to see whether the B cells could get over-active and proliferate due to APRIL. This study is of great interest due to the fact that both APRIL and BAFF share the same receptor (TACI). APRILs function is still unknown and because of the receptor, it can be assumed that they might have some function in common. The expression of the housekeeping gene β-actin could be detected in normal levels, but no levels of APRIL expression could be found. This could be due to that APRIL need to be stimulated with something else than IFN-γ. Is has recently been found that human monocyte-derived DCs can be stimulated with Poly I:C (Polyriboinosinic Polyribocytidylic Acid), an immunostimulant, to create an APRIL response (Hardenberg et al., 2007). The stimulant Poly I:C could also in future studies therefore be tested on mice DC to see whether the APRIL expression could be measured.

For future studies concerning the mouse response to adrenocortical tumour cells, adrenocortical tumor cells were cultured. The cells were supposed to be made apoptotic with the streptozotocin treatment. When the apoptotic cells are injected in AIRE deficient mice in future experiments, the adrenocortical cells will attack the adrenal cortex of the mouse, which is a common organ target for the autoantibodies in APS I patients (Ahonen et al., 1990). If the mice show analogous autoantibodies against the adrenal cortex to the human patient’s autoantibodies, the differences between mice and patients responses could be due to environmental differences. Studies in this area could be of great importance in order to answer the questions whether mice can be used as a model of APS I or not, as they have a different response to mutations in AIRE.

The adrenocortical tumor cells could not be visualized with FACS. This could be due to very adhesive cells that would not resolve in the binding buffer used before FACS measurement, or that the samples were diluted too much. Less diluted samples and other staining protocols could be tested and hopefully yield more events.

APS I is a complex progressive disease, but because of its monogenic behavior it is of great interest to study and understand the mechanisms involved. There are yet no cures against autoimmune diseases which make these studies of great interest. Progress in this area might lead to improved therapies.

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8. References


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Appendix 1.

STAT I Signaling pathway

NK  NKT

IFN-γ

DC

BAFF

MZB

Y

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