Study of B Cell Factors in Autoimmune Polyendocrine Syndrome Type 1

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS **2**

SELECTED ABBREVIATIONS **5**

ABSTRACT **6**

1. INTRODUCTION **7**

1.1 THE IMMUNE SYSTEM 7
1.1.1 The Adaptive Immune System *7*
1.1.2 T cells *8*
1.1.3 The Autoimmune Regulator Gene *10*
1.1.4 B cells *10*
1.2 IMMUNOLOGICAL TOLERANCE 12
1.2.1 Autoimmunity *13*
1.3 AUTOIMMUNE POLYENDOCRINE SYNDROME TYPE 1 (APS-1) 13
1.3.2 B cells in APS-1 .... *14*
1.4 IMPORTANT FACTORS FOR B CELL FUNCTION 15
1.4.1 Phenotypic Surface Markers *15*
1.4.2 Interleukin 10, 27 and 35 .... *17*
1.4.3 B cell Activating Factor, A Proliferation-Inducing Ligand and Their Receptors *17*
1.5 AIM AND OBJECTIVES OF THE STUDY 19

2. MATERIALS **20**

2.1 CHEMICALS 20
2.2 COMMERCIAL KITS 21
2.3 PRIMERS AND ANTIBODIES 22
2.4 INSTRUMENTS AND EQUIPMENT 23
2.5 COMPUTER SOFTWARE 23

3. METHODS **24**

3.1 ETHICAL CONSIDERATIONS AND THE ROAS REGISTRY 24
3.2 SANGER SEQUENCING 25
3.3 ISOLATION OF TOTAL RNA FROM HUMAN WHOLE BLOOD 26
3.4 cDNA SYNTHESIS AND qPCR REACTION 26
3.4.1 cDNA Synthesis *26*
3.4.2 qPCR Reaction *27*
3.4.3 Calculating the $2^{-\Delta\Delta Ct}$-Value *27*
3.5 SANDWICH ELISA 27
3.5.1 DuoSet ELISA, Measuring Levels of EBI3, TACI, APRIL *28*
3.5.2 Quantikine ELISA, Measuring Levels of BAFF, BAFF-R, IL-10 and BCMA *28*
3.5.3 Making and Interpreting the Standard Curve *29*
3.6 ISOLATION OF PBMC 29
3.7 PRIMARY CELL CULTURE 29
3.7.1 Magnetic Labeling and Separation with MS Columns *29*
3.7.2 Cell Proliferation Staining *30*
3.7.3 Activating and Expanding the B Cells *30*

3.8 FLOW CYTOMETRY 30
3.9 STATISTICS 31

4. RESULTS 33

4.1 VERIFICATION OF *AIRE* MUTATIONS IN APS-1 PATIENTS 33
4.2 GENE EXPRESSION OF B CELL FACTORS 34
4.4 DETERMINING LEVELS OF B CELL FACTORS IN SERA 36
4.5 FUNCTIONAL CHARACTERIZATION OF B CELLS 37
4.5.1 Isolation, Characterization and Expansion of B cells 38
4.5.2 Characterization of B cells by Flow Cytometry 39
4.5.3 Levels of B cell Factors in Supernatant After Cell Culture 45

5. DISCUSSION 46

5.1 VERIFICATIONS OF MUTATIONS IN THE *AIRE* GENE 46
5.2 COMPARISON OF B CELL FACTORS 46
5.2.1 Comparison of B Cell Factor Concentrations in Sera versus Supernatant 46
5.2.2 Protein Expression versus Gene Expression 49
5.3 PHENOTYPICAL CHARACTERIZATION OF B CELLS 50
5.4 CONCLUSIONS 52
5.5 FUTURE PERSPECTIVES 52

6. REFERENCES 54

7. SUPPLEMENTARY DATA 60

7.1 VERIFICATION OF *AIRE* MUTATIONS IN APS-1 PATIENTS 60
7.2 RNA ISOLATION 60
7.3 CONCENTRATIONS FROM ELISA ANALYSIS WITH SERA 61
7.4 FUNCTIONAL CHARACTERIZATION OF B CELLS 67
7.4.1 Isolation of PBMC from APS-1 patients and controls 67
7.4.2 Cell Culture Plating 68
7.4.3 Raw Data from Flow Cytometry 68
7.4.4 ELISA Analysis after Cell Culture 70

SELECTED ABBREVIATIONS

AIRE	—	Autoimmune Regulator
APC	—	Antigen-Presenting Cell
APRIL	—	A Proliferation Inducing Ligand
APS-1	—	Autoimmune Polyendocrine Syndrome type 1
BAFF	—	B cell Activating Factor
BAFF-R	—	B cell Activating Factor Receptor
BCMA	—	B cell Maturation Antigen
BCR	—	B Cell Receptor
CD	—	Cluster of Differentiation
CFSE	—	Carboxyfluorescein Diacetate Succinimidyl Ester
EBI3	—	Epstein-Barr virus Induced gene 3 (subunit of IL-27 and IL-35)
ELISA	—	Enzyme-linked Immunosorbent Assay
Ig	—	Immunoglobulin
IL	—	Interleukin
MHC	—	Major Histocompatibility Complex
PBMC	—	Peripheral Blood Mononuclear Cell
SLE	—	Systemic Lupus Erythematosus
TACI	—	Transmembrane Activator and Calcium-Modulating Cyclophilin Ligand (CAML) Interactor
TCR	—	T Cell Receptor

ABSTRACT

Autoimmune polyendocrine syndrome type 1 (APS-1) is a rare, recessively inherited monogenic autoimmune disease that is characterized by mutations in the *autoimmune regulator* (*AIRE)* gene. Although APS-1 is considered a T cell mediated disease, some studies indicate that the B cells role in APS-1 can be more essential to the development of the disease than initially assumed. In-depth knowledge about B cells and their autoantibodies in APS-1 is imperative for further elucidation of their role in disease development and progression, and to establish their potential as diagnostic markers and as candidates for targeted treatment of APS-1 patients.

In this study, we first confirmed mutations in *AIRE* in a subgroup of APS-1 patients before measuring levels of B cell factors in sera from a large cohort of APS-1 patients and healthy controls. Interestingly, we confirmed B cell-activating factor (BAFF) protein levels in sera to be elevated in APS-1 patients compared to controls. A proliferation-inducing ligand (APRIL) on the other hand, revealed decreased protein levels in sera from APS-1 patients. These are two important regulators of B cell activation and survival. The gene expression of *B-cell maturation antigen* (*BCMA),* a receptor for BAFF and APRIL, further showed a decreased expression in APS-1 patients compared to controls, which may affect the differentiation into memory and plasma cells in APS-1 patients. B cells from APS-1 patients and sex- and age-matched controls were further activated *in vitro,* and their phenotype determined by flow cytometry where a decrease of memory and naïve cells was seen after seven days in culture. Optimalisation of the method and further studies on B cells in activated form are needed to draw firm conclusions, as the cells did not expand well in culture. We also measured the B cell factors in the cell medium, however no major deviations between APS-1 patients and healthy controls were found.

Taken together, this study confirms the previous described changes in sera levels of BAFF in APS-1 patients, while revealing differences in sera levels of APRIL, thus expanding our knowledge of B cells and their factors in APS-1.

1. INTRODUCTION

1.1 THE IMMUNE SYSTEM

The most fundamental physiological function of the immune system is to defend the human body against a broad range of infections. These defenses are divided into two different types of responses, the innate and the adaptive immune system. An overview of the major cells within the respective branches is given in Figure 1.1. The innate, or non-specific, immune system is the first resistance a pathogen encounters when entering host tissue[1,2]. Cells of the innate immune system recognizes foreign substances and recruits yet other immune cells to the infection site by producing signaling molecules such as cytokines and chemokines. These will furthermore activate defense mechanisms and local cellular responses to the infection site. Subsequently, this may lead to initiating of the adaptive immune response by activating antigen-presenting cells (APCs)[3], B cells, and T cells. Macrophages, B cells and dendritic cells are the main types of APCs, also called professional APCs, which are immune cells that will process and introduce an antigen to a T cell which in turn leads to T cell activation[4]. The rapid response mounted by the innate immune system is followed by activation of the adaptive arm which react slower, but have immunological memory[4].

1.1.1 The Adaptive Immune System

The most essential function of the adaptive, or specific, immune system is being able to differentiate between foreign antigens and self-antigens in order to initiate the correct responses when a pathogen enters the host. These responses are destructive, and it is therefore crucial that they appear as a response to foreign molecules and not to the hosts own molecules[5]. A key difference between the innate and the adaptive immune response is that the adaptive immune response is able to develop immunologic memory, which can remember previous encounters with specific pathogens and in this way eliminate them quicker upon the next encounter[3]. The adaptive immune system is divided into cell-mediated and humoral immunity. The cell-mediated immunity is mediated by T cells and defends the host predominantly against extracellular microbes, while the humoral immunity is mediated by B cells and defends mainly against intracellular microbes[6].

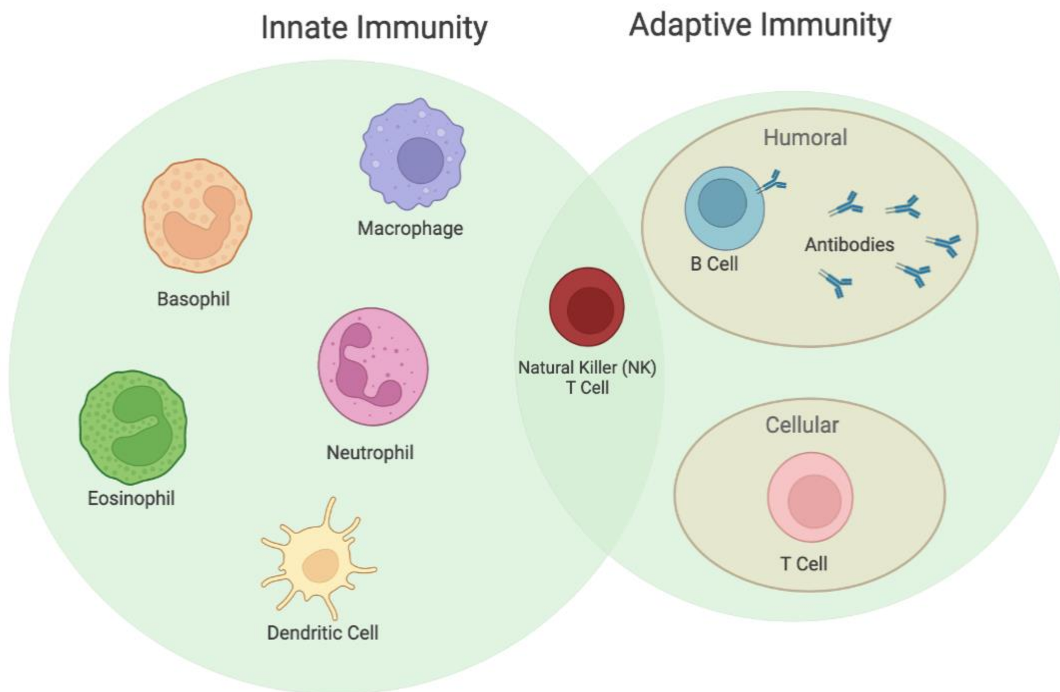


Figure 1.1 – Overview of the different cells that are involved in the innate and the adaptive immune system. Depicted are the two different types of immune responses, the innate and the adaptive, and their respective cell types. Macrophages, basophil, neutrophil, eosinophil and dendritic cells are a part of the innate immune system, while natural killer (NK) T cells are somewhat in between these two immune responses. The adaptive immune system is divided into humoral and cellular immunity which are mediated by B cells and T cells, respectively[1,6]. Figure created using BioRender.com and adapted from the Cell Signaling Technology website. https://blog.cellsignal.com/immunology-overview-how-does-our-immune-system-protect-us

1.1.2 T cells

To fully understand the activation and function of B cells, T cells role in the adaptive immune response needs to be specified. T cells originate in the bone marrow and matures in the thymus gland. Through a process called positive selection in the thymus, the cells differentiate into either $CD4^+$ helper T cells or $CD8^+$ cytotoxic (or killer) T cells. This is based on recognition by either major histocompatibility complex (MHC) class I or MHC class II, which in turn determines whether the cell differentiate into a $CD8^+$ or $CD4^+$ T cell, respectively[4,7]. If the cells fail the positive selection process by not binding to self-MHC, they undergo apoptosis and is subsequently removed by thymic cortical macrophages[8,9]. Regulatory T cells (Tregs) are also produced in the thymus through a prosses called negative selection, but they may be produced in the periphery from naïve T cells as well, while memory cells are only produced in the periphery[10]. Negative selection is a critical self-tolerance possess, where developing T cells that are capable of recognizing self-antigens induces cell death[11]. T cells recognizes pathogens with

their T-cell receptor (TCR), which recognize small fragments of the pathogens that are presented to them on MHC by APCs. Subsequently, the T cell will become activated and start to divide and obtain a variety of effector functions[5,12]. There are two main types of T cells, which are the $CD4^+$ helper T cells and the $CD8^+$ cytotoxic T cells. The latter recognizes and eliminates cancer cells and cells that are infected with pathogens, while $CD4^+$ T cells respond to the many different signaling molecules within the immune system. Hence, the $CD4^+$ T cells will affect the function of both $CD8^+$ T cell as well as B cells[5,13]. $CD4^+$ T cells express characteristic molecules on the surface, such as CD154 (also called CD40 ligand (CD40L)), and CD70 (also called CD27 ligand (CD27L)), which will interact with B cells by binding to CD40 and CD27, respectively (Figure 1.2). These bindings regulate B cell activation, proliferation, differentiation, and cell death, where the CD40/CD154 interaction is responsible for activation, proliferation and secretion of antibodies in the B cells[14,15], and subsequently the CD27/CD70 interaction will lead to B cell differentiation into plasma cells[16-18].

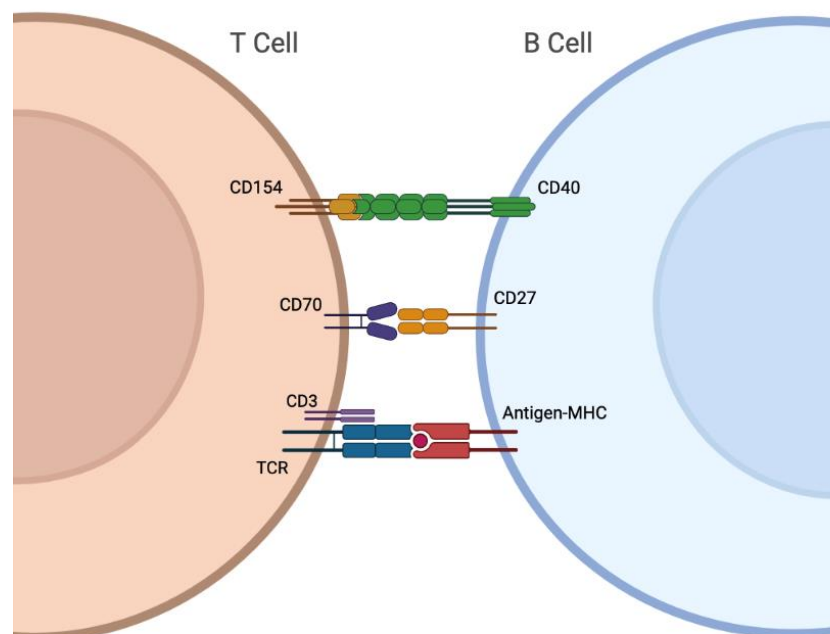


Figure 1.2 – The interactions between T and B cells. In order to activate B cells, three main interactions need to occur. The MHC (here: MHCII) will together with a peptide form the peptide:MHCII complex, which can be recognized by antigen-specific CD4+ T cells. This will subsequently lead to production of proteins that stimulates the B cells to proliferate. The CD40 and CD154 interaction induces a signal to help stimulate the B cells for proliferation and antibody secretion and plays a critical role in the regulation of the immune response[14,15]. Subsequently, the CD27 and CD70 interaction will lead to B cell differentiation into memory and plasma cells[16-18]. Figure created using BioRender.com.

1.1.3 The Autoimmune Regulator Gene

The *Autoimmune Regulator* (*AIRE*) gene is 13 kilo bases long, located on chromosome 21, and harbours 14 exons which encodes a 545 amino acid protein[19]. It is a transcription factor that is highly expressed in the thymus gland, particularly in medullary thymic epithelial cells. The AIRE protein is encoded by the *AIRE* gene and plays an essential part in negative selection of self-reactive T cells as well as in the development of T cells by regulating expression of tissue-specific antigens in the thymus. The AIRE protein's role is to form and prepare the T cells for their role in the immune system. This process is called thymic education and is important for prevention of autoimmunity by eliminating self-reactive T cells that bind to self-antigens through negative selection. A deficiency, for instance a mutation, in the *AIRE* gene, can result in failure in eliminating these self-reactive T cells, which are then released into the blood stream with the potential to cause an autoimmune reaction. This is indeed what happens in the severe disorder Autoimmune polyendocrine syndrome type 1 (APS-1)[20-22]. The knowledge of AIRE's function and details on immune tolerance was initially discovered in mouse experiments[23], but in the last decade, there has been a large focus to understand AIRE's impact on autoimmune diseases and health in humans. Since the deficiency of AIRE has been thought to mostly consider the T cell, not much research has been done on B cells in regard to APS-1. Importantly, it is known that T and B cells work together in the adaptive immune system to provide a proper immune response, and hence, the role of B cells in APS-1 needs to be researched more extensively[5].

1.1.4 B cells

The number of B cells in blood in general is lower than T cells, where the B cells account for 5-10% of all cells among all peripheral blood cells[24]. B cells originate and matures in the bone marrow, and they are responsible for production of antibodies[2]. As previously mentioned, the CD40/CD154 and the CD27/CD70 interactions between T and B cells regulate B cell activation, proliferation, differentiation and cell death[18]. As shown in the overview in Figure 1.3, a B cell becomes mature when expressing both immunoglobulin M (IgM) and IgD. The mature B cell can then move into the periphery to become activated by antigens to further differentiate into a memory B cell, residing in germinal center, or a plasma cell which produces antibodies[9,25].

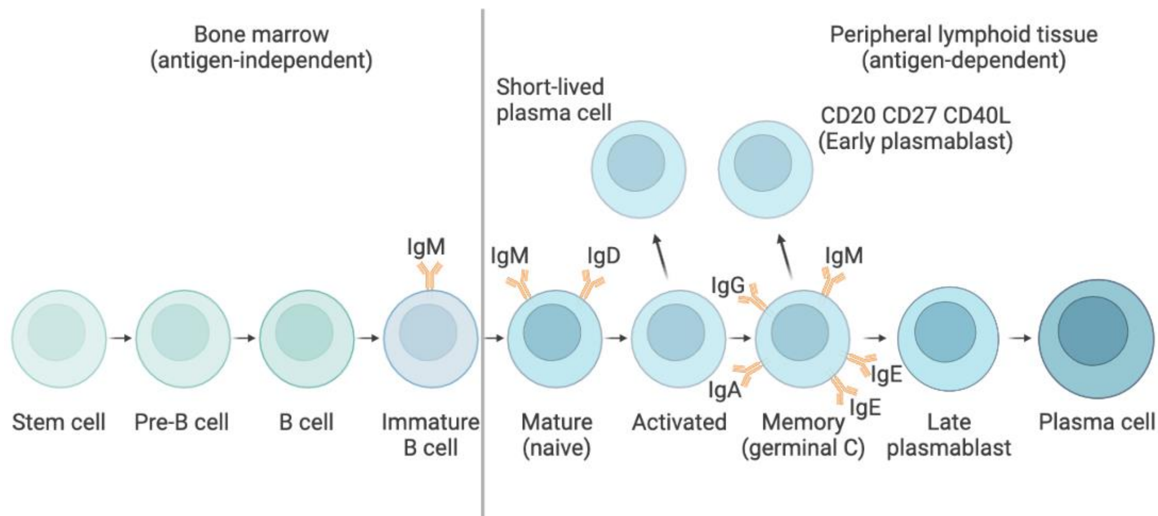


Figure 1.3 – Overview of the development process of B cells. The figure shows the different B cell development stages from stem cell in the bone marrow, to plasma cell in the peripheral lymphoid tissue. Immature B cells express IgM, however when IgD is additionally expressed, the B cell becomes mature and can move into the periphery. In the periphery it becomes activated by different antigens and goes through an isotype switch to IgG, IgA and IgE. After activation, they can differentiate into a memory B cell or plasma cell[9,25]. Figure created using BioRender.com and adapted from CusaBio website. https://www.cusabio.com/Cell-Marker/B cell.html

B cells that have not yet encountered a pathogen will harbour their antibodies in the cell membrane, using them as receptors that can recognize pathogens in their primary state. When the antibodies recognize a pathogen, the B cells will become activated and induce production of many daughter cells which recognize the same pathogen[4,6,26]. Some of the daughter cells can release antibodies which are secreted from the cell and circulate freely. They can detect and bind the pathogens, either neutralizing them or marking them for elimination by other immune cells such as $CD8^{+}$ T cells. Other daughter cells bind to the pathogen and consumes it into the cell, where it will be broken down into fragments and subsequently transported to the cell surface. The pathogens are then bound to MHCI that will further present them to T cells and TCR as mentioned previously[6,26,27].

B cells produce secreted antibodies and uses them as part of the BCR on the cell surface. There are five classes, named IgM, IgD, IgG, IgA and IgG, serving different functions and residing in different parts of the body. When B cells are activated, they undergo isotype switching (also called class switching) to produce antibodies of different isotypes, such as IgG, IgE and IgA. Isotype switching is when an activated B cell together with BCR, replace its production of IgM to generate other isotypes and it is an essential part in the development of B cell memory. This changes the function of the isotype but does not affect antigen specificity, and by repeated

exposure with the same antigen, the immune system will produce antibodies with increased affinities as a response to the antigens. This prosses is called affinity maturation[4,28,29].

The antibodies that are produced first in the humoral immune response, are always IgM. IgM has an essential role in the initial immune system defense and is the main antibody of primary responses because it can be expressed without isotype switching. IgM is made up of five monomers, forming a pentamer, however the monomeric form of IgM functions as part of the B cell receptor (BCR). The latter is also true for IgD[4,30]. IgD is expressed on the surface of B cells and may also play a role in the induction of antibody production[31]. IgG is the main blood antibody of secondary responses and plays an essential role in the humoral immune system with its ability to neutralize toxins and protect against pathogens by binding them and preventing the pathogens to replicate and enter host cells[4,32]. IgE is the antibody of allergy and antiparasitic activity. IgE is mostly bound on mast cells through receptors and are found in mucosa and beneath the skin. However, IgE is also present in blood and extracellular fluid, but in low levels. IgG and IgE are always in the monomeric form, while IgA can form dimers as well. In the blood, IgA is most commonly present as a monomer, but when they form dimers they are transported across different epithelia and into for instance lumen of the gut, where they protect the immune system by blocking entry of bacteria and neutralize viruses and toxins[4,33,34].

1.2 IMMUNOLOGICAL TOLERANCE

Immunological tolerance is defined as *" a state of indifference or non-reactivity in the immune system towards a substance that would normally be expected to excite an immunological response"*[35]. Immunological tolerance can be divided into peripheral tolerance and central tolerance. The latter refers to the mechanisms in primary lymphoid organs (bone marrow and thymus) when autoreactive cells are eliminated in their maturation phase. Peripheral tolerance occurs after the T and B cells have entered the peripheral tissue and lymph nodes acting like a safety net which inhibits immune responses against the hosts own tissue. Apoptotic cell death, anergy and suppression by Tregs are some functions of the T cell peripheral tolerance[11,36]. B cells that recognize self-antigens in the absence of specific helper T cells will not receive the appropriate signals for activation and will become anergic and die by apoptosis[37]. As mentioned earlier, the interactions between T and B cells through the binding of CD154 and CD70 to CD40 and CD27, respectively (Figure 1.2), are responsible for B cell activation, proliferation, differentiation, indicating that the B cells are dependent on T cells[14-18]. Loss of immunological

tolerance results in autoimmune disorders, such as type 1 diabetes (T1D), due to interaction with self-antigens in sub-optimal environment[36,38,39].

1.2.1 Autoimmunity

Autoimmunity is defined as *"an immune response leading to reaction with self-antigen, i.e., any molecule that is a normal body constituent of the animal mounting the response"*[40]. This occurs when the immune system attacks the body´s own healthy tissue and cells, and diseases that occur from this type of immune response is termed "autoimmune disease". Autoimmune diseases affect about approximately 8% of the general population, where about 80% of them are women[41]. These diseases are divided into two classes, systemic and organ-specific, where in systemic diseases, such as Systemic Lupus Erythematosus (SLE) or Sjögren's syndrome (SS), the immune system will attack self-antigens that are ubiquitously expressed, whereas in organ-specific diseases, such as APS-1 or T1D, it will only attack self-antigens in restricted organs. In normal circumstances, the lymphocytes that would trigger these immune responses are destroyed before they mature, but sometimes this prosses fails, e.g. by mutations in immune relevant genes, which subsequently leads to autoimmune diseases[4,42].

1.3 AUTOIMMUNE POLYENDOCRINE SYNDROME TYPE 1 (APS-1)

APS-1, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy/dysplasia (APECED) or polyglandular autoimmune (PGA) syndrome type 1, is a rare, recessively inherited monogenic autoimmune disease that is characterized by mutations in the *AIRE* gene[6,43]. This means that patients harbor mutations in both *AIRE* alleles, one from each parent, which leads to loss of function or lack of expression of *AIRE*[21,44,45]. However, dominant mutations have occasionally been identified, although this causes milder phenotypes and symptoms compared to classical APS-1[46]. The prevalence of the disease in Norway is estimated at 1 in 90,000[47], and at 1 in 130,000 in Ireland[48]. APS-1 is more common in Finland, Sardinia, and among Persian Jews living in Israel, where the prevalence is estimated at 1 in 25,000, 1 in 14,000 and 1 in 9000, respectively[49]. The three hallmarks of APS-1 are adrenocortical insufficiency (Addison´s disease), hypoparathyroidism and chronic mucocutaneous candidiasis, and the criteria for APS-1 diagnosis is at least two of the three

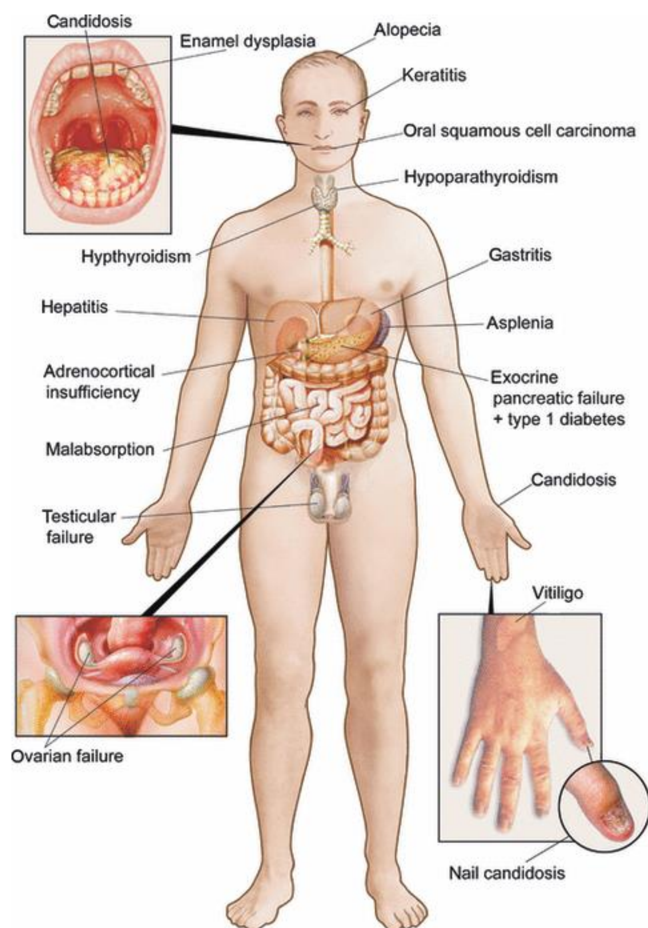


Figure 1. 4 – The various manifestations in patients suffering from Autoimmune Polyendocrine Syndrome type 1. The three main symptoms of APS-1 are adrenocortical insufficiency (Addison's disease), chronic mucocutaneous candidiasis and hypoparathyroidism. Other manifestations, such as vitiligo and alopecia, are usually also present in patients as depicted in the figure. From Husebye: https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2796.2009.02090.x

hallmark manifestations. The symptoms most commonly appear in childhood, but they may, in some cases, appear in adolescence or early adult life. This makes it difficult to diagnose because it varies greatly in phenotype and how it portrays from patient to patient[43,50]. In APS-1, the immune system causes injury to various endocrine organs, such as adrenal glands, ovaries/testes, thyroid glands and pancreas. Other symptoms like alopecia, candidiasis and vitiligo, to mention a few, may also appear (Figure 1.4). Another hallmark of the disease is the high levels of autoantibodies produced by B cells, which recognize organ-specific proteins[6,51]. To this day, treatment of APS-1 patients is still challenging due to the many different manifestations and symptoms they have. Some of treatments may include immunosuppression, hormone therapy, antifungals, and replacement therapy, however personalized treatment of the patients is lacking[43,50]. These treatment strategies have not evolved much since the 1950s. Research on APS-1 is therefore essential to obtain better treatment for the APS-1 patients, as well as implementing these findings in other more common autoimmune endocrine diseases, like T1D.

1.3.2 B cells in APS-1

The role of B cells and their autoantibodies in APS-1 are still not fully understood since APS-1 is considered a T cell mediated disease. However, some studies indicate that the B cells' role in APS-1 can be more essential to the development of the disease than initially assumed[52,53].

Studies using Rituximab as treatment, which is a monoclonal antibody that deplete B cells by binding through CD20, has shown a reduction of infiltration, inflammation and destruction of tissue in AIRE knockout mice, and also improved lung function in an APS-1 patient with lung disease[53,54]. Not enough research has been done in APS-1 on a proliferation-inducing ligand (APRIL) nor on the receptors of APRIL and B cell activating factor (BAFF, also known as BlyS). BAFF on the other hand, have been studied in other autoimmune diseases as well as in APS-1[55-58]. BAFF have shown to be a promising approach for treatment of patients suffering from autoimmune diseases[55,56]. Belimumab is an anti-BAFF monoclonal antibody which neutralize soluble BAFF and has shown to reduce the number of circulating naïve B cells in SLE patients. Other treatment such as blisibimod and tabalumab are also being investigated for treatment of SLE[59]. Atacicept is a recombinant fusion protein, that has been shown to neutralizes BAFF and APRIL activity in patients with SLE and rheumatoid arthritis by binding to them[60]. This shows that the different B cell factors may possibly be used as treatment or diagnostic markers in APS-1 as well.

1.4 IMPORTANT FACTORS FOR B CELL FUNCTION

During the differentiation process of B cells they obtain different phenotypic surface markers, such as CD19, CD20, CD24, CD27, CD38 and IgD, which can be used to separate them from other types of immune cells[61]. Targeting BAFF has shown to be a promising way to treat different autoimmune diseases in various studies[55-58], however there is a knowledge-gap of APRIL´s and BAFF´s receptors; BAFF receptor (BAFF-R, also known as BR3), transmembrane activator and calcium-modulating cyclophilin ligand interaction (TACI) and B cell maturation-antigen (BCMA) roles in autoimmune diseases. Below is an overview of these markers and their functions.

1.4.1 Phenotypic Surface Markers

CD19

CD19 is a common surface biomarker for B cells and is important for B cell activation and proliferation. CD19 is a single-pass transmembrane protein in the immunoglobulin superfamily. CD19 forms a complex with BCR and other surface molecules and is essential for establishing B cell signaling and to provide the most effective immune response[62,63].

CD20

CD20 is a surface protein for pre-B lymphocytes and mature B cells, however, when the B cells differentiate into plasma cells, the expression of CD20 is lost. CD20 is important for optimal B cell immune response and plays a role in regulation and the development of B cells into plasma cells. However, other functions of CD20 and how the expression of CD20 is regulated is still not completely clear[64,65].

CD24

CD24 is a highly glycosylated signal-transducing molecule, which plays a role in B cell selection and development in the bone marrow and is expressed on early-stage and transitional B cells, but not on mature B cells and plasma cells[66,67]. However, the increased prevalence of CD24 in the early-stage and transitional B cells compared to mature B cells, is still not completely investigated, but some studies suggests that B cell can still be subdivided based on CD24/CD38 expression[66,68,69].

CD27

CD27 is a memory marker of B cells, and it also plays a role in the pathway for B cells to plasma cells, which means that CD27 expression can be used to differentiate between memory B cells and naïve B cells. CD27 ligand (CD70) is a transmembrane protein which is expressed on both T and B cells in antigen stimulation response, and when CD27 and CD70 binds, they stimulate survival and activation of T, B and NK cells[16,70]

CD38

CD38 is a transmembrane surface protein, where the expression of CD38 will increase simultaneously with normal B cell activation[71]. CD38 has many different roles in immune cells, such as modulating cell differentiation, regulation of cell recruitment and it also plays an important role in inflammatory processes during autoimmunity. CD38 ligation with agonistic antibodies may lead to various outcomes, such as growth stimulation, activation of kinases, induction of cytokines, protection from apoptosis and phosphorylation of certain proteins[72,73]. Due to the many different functions of CD38, the biology of the molecule during autoimmune inflammation is still not fully understood[74].

IgD

IgD is a monomeric antibody isotype expressed on the surface of B cells where it functions as part of the BCR, as previously mentioned and it may also be secreted into the blood stream. Expression of high levels of IgD is usually associated with human naïve B cells[68,75]. IgD acts as an activation signal for B cells which leads to the participation in the immune defense. It is suggested that IgD has essential immunological functions, due to that it is present in different species and has been preserved[76-78].

1.4.2 Interleukin 10, 27 and 35

Interleukin 10 (IL-10, also known as cytokine synthesis inhibitory factor (CSIF)) is an anti-inflammatory cytokine, which plays a role in autoimmunity, inflammation and infection, and its main function is to limit inflammatory responses. IL-10 is produced by various different cell types, such as B cells, $CD8^+$ and $CD4^+$ T cells, NK cells, dendritic cells, monocytes and macrophages. IL-10 has various functions in regulating immune responses, such as regulating Ig class switching in B cells, antibody secretion, and survival of B cells[79].

Epstein-Barr virus induced gene 3 protein (EBI3) is a subunit of IL-27 and IL-35 and plays a role in regulating cell-mediated immune responses. Both IL-27 and IL-35 belong in the IL-12 family of cytokines. IL-27 is a heterodimeric cytokine which consists of EBI3 and p28 protein and is primarily produced by APCs, such as dendritic cells and macrophages[80,81]. IL-27 induces IgG class switching and signaling in B cells where it will promote proliferation and survival of B cells[82]. IL-35 is an inhibitory cytokine which consists of EBI3 and p35 subunit of IL-12 and is predominantly secreted by Tregs. IL-35 inhibits immune function and is involved in regulatory cell function[83,84]. IL-27 and IL-35 will hereinafter be referred to as EBI3.

1.4.3 B cell Activating Factor, A Proliferation-Inducing Ligand and Their Receptors

BAFF and APRIL are cytokines which belongs in the tumor-necrosis-factor (TNF) superfamily of growth factors, and they share a 30% sequence identity to each other. BAFF and APRIL assemble as homotrimers before binding to the receptors, as all other TNF ligands do as well[85,86] (Figure 1.5). BAFF is expressed in both secreted and membrane-bound forms as homotrimers, however, a BAFF 60-mer has been obtained in an *in vitro* study, where at a neutral or alkaline pH, the formation of the more active BAFF 60-mer will occur. At an acidic pH the BAFF 60-mer will dissociate into the less active BAFF trimers[59,87,88].

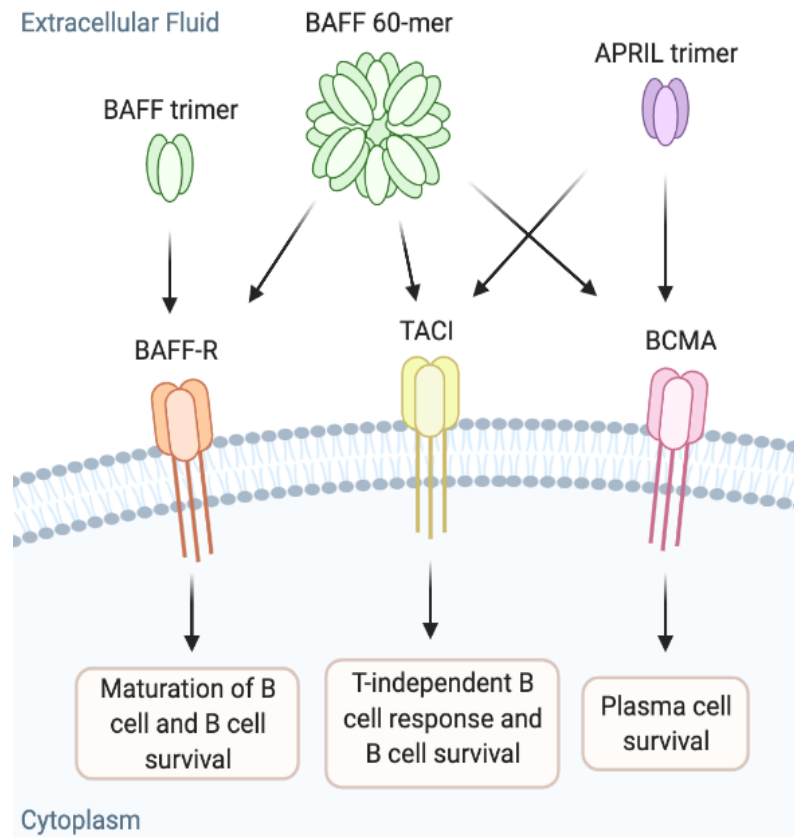


Figure 1. 5 – Overview of BAFF and APRIL with their receptors BAFF-R, TACI and BCMA. Indicated in the figure are the different binding of BAFF and APRIL to their receptor. As shown, BAFF binds to all three receptors depicted in the figure, while APRIL only binds to TACI and BCMA. All three receptors promote cell survival when their respective ligands are bound. In addition, BAFF-R promotes maturation of B cell, while TACI promotes T-independent B cell response[56,58]. Figure created using BioRender.com.

BAFF is produced by myeloid cells and plays an essential role in B cell survival, function and differentiation. It binds to three receptors as a homotrimer, which are BAFF-R, TACI and BCMA[57,89-91]. BAFF-R is expressed on B cells and on resting T cells, and the binding of BAFF to BAFF-R is exclusive. The binding between BAFF and BAFF-R has shown to play a role in the transition from immature transitional stage 1 (T1) B cell to T2 B cell, which further indicates that this binding plays a role in the maturation of B cells. This binding also leads to transcription of the anti-apoptotic factor called Bcl-2, indicating that binding of BAFF to BAFF-R is essential for B cell survival during the prosses from immature T1 B cell to mature B cell[92,93].

Some studies show that BAFF acts mainly through BAFF-R, even though it also binds to TACI and BCMA. A study done on mice lacking BAFF-R showed loss of mature B cells, while mice lacking TACI and BCMA did not have any loss of mature B cells, further indicating that the binding between BAFF and BAFF-R is essential for B cell survival[93,94]. Overexpression of

BAFF has shown to lead to autoimmune diseases in mice and some studies have also shown that patients that are suffering from autoimmune diseases, for instance SLE or SS, have elevated levels of BAFF in the sera. Therefore, BAFF can be a new possible approach for the treatment of autoimmune diseases[55-58].

While BAFF-R can only bind with BAFF, TACI and BCMA can bind with APRIL as well. APRIL is a TNF-like cytokine and is predominantly expressed in secreted form from myeloid cells. APRIL plays a role in B cell survival and proliferation[6,85] and binds BCMA and TACI with high affinity. It has been shown that BCMA have significantly higher affinity for APRIL then for BAFF[85]. BCMA is only expressed on B cells, and the binding of BAFF and APRIL to BCMA mediates plasma cell survival[6,91]. TACI is however expressed on B cells and on resting T cells, and the binding of BAFF and APRIL to TACI mediates T-independent B cell responses as well as B cell survival[6,91]. All three receptors exist in soluble forms in sera[95]. BAFF 60-mer and multimerized APRIL are able to activate TACI, while the homotrimer of APRIL and BAFF are incapable of doing so, however they are still able to bind to TACI[96,97]. By characterizing the levels of these different B cell factors, one may elucidate their function in B cells, and come closer to understanding the role of B cells in autoimmune diseases.

1.5 AIM AND OBJECTIVES OF THE STUDY

We hypothesize that APS-1 patients have inborn or acquired defects in their B cells due to the lack of AIRE. We aim to investigate key B cell factors in sera and stimulate B cells from APS-1 patients *in vitro* with the long-term goal of exploring these as suitable targets of immunotherapy, as well as investigate how the B cells respond upon general activation.

The specific aims were:

1. Verify mutations in the *AIRE* gene in a subgroup of APS-1 patients by Sanger sequencing.
2. Activate and expand B cells *in vitro* from APS-1 patients and healthy controls and investigate the phenotype by flow cytometry.
3. Investigate levels of B cell signalling molecules in
    a. Sera and whole blood from APS-1 patients and healthy controls.
    b. The medium of primary B cells expanded *in vitro* from APS-I patients and healthy controls.

2. MATERIALS

2.1 CHEMICALS

Table 2.1: Chemicals and reagents used in various experiments.

Chemical	Supplier	#Cat. Number
1x BigDye Buffer	Thermo Fisher Scientific	4336697
AB-Serum, Human	Sigma	H4522-100ML
Agarose NA	GE Healthcare	17-0554-02
AmpliSize Molecular Ruler	Bio-Rad	1708200
Betaine Solution	Sigma	B0300-1VL
CD19 MicroBeads, Human	Miltenyi Biotec	130-050-301
DEPC-Treated Water	Ambion	AM9906
Dimethyl Sulfoxide	Sigma	D8537-500ML
Ethanol 100%	Kemetyl	600068
ExoProStar	Cytiva	US77720V
Fetal Bovine Serum (FBS)	Sigma	F7524
Ficoll-Paque PLUS	Cytiva	17144003
GelRed	Biotium	41003
GeneRuler 50 bp DNA Ladder	Thermo Fisher Scientific	SM0373
Human BD Fc Block	BD Biosciences	564220
MACS BSA Stock Solution	Miltenyi Biotec	130-091-376
Phosphate Buffered Saline (PBS)	Sigma	D2650-100ML
Pre-Developed TaqMan Assay Reagents Human ACTB (20X)	Thermo Fisher Scientific	4333762F
Pre-Developed TaqMan Assay Reagents Human B2M (20X)	Thermo Fisher Scientific	4333766F
StemMACS HSC Expansion Media XF, Human	Miltenyi Biotec	130-100-463
TaqMan Gene Expression Assay (FAM)	Thermo Fisher Scientific	4331182
- APRIL TNFSF13		Hs00601664_g1
- BAFF TNFSF13B		Hs00198106_m1
- BAFF-R TNFRSF13		Hs00606874_g1
- BCMA TNFRSF1		Hs00171292_m1
- IL-10 IL-10		Hs00961622_m1
- IL-35 EBI3		Hs01057148_m1
- TACI TNFRSF13B		Hs00963364_m1
TaqMan Gene Expression Master Mix	Thermo Fisher Scientific	4369016
TBE Buffer 10X	Invitrogen	15581-044
Trypan Blue Stain 0.4%	Invitrogen	T10282
UltraComp eBeads Compensation Beads	Invitrogen	01-2222-42

2.2 COMMERCIAL KITS

Table 2.2: Different commercial kits used in various experiments.

Kit	Supplier	#Cat. Number
AmpliTaq Gold DNA Polymerase with Gold Buffer & $MgCl_2$	Thermo Fisher Scientific	4311814
B Cell Expansion kit, Human	Miltenyi Biotec	130-106-196
BD Cytofix/Cytoperm Fixation/Permeabilization Kit	BD Biosciences	554714
BigDye Terminator v1.1 Cycle Sequencing Kit	Thermo Fisher Scientific	4336774
CellTrace CFSE Cell Proliferation Kit	Thermo Fisher Scientific	C34554
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	DY008
DuoSet ELISA Human APRIL/TNFSF13	R&D Systems	DY884B
DuoSet ELISA Human EBI3	R&D Systems	DY6456-05
DuoSet ELISA Human TACI/TNFRSF13B	R&D Systems	DY174
ELISA Human BCMA/TNFRSF17	Thermo Fisher Scientific	EH41RB
High-Capacity RNA-to-cDNA Kit	Thermo Fisher Scientific	4387406
Human CD40-Ligand Multimer Kit	Miltenyi Biotec	130-098-775
Human BAFF-R ELISA Kit	Abcam	ab213839
Human IL-10 Quantikine ELISA Kit	R&D Systems	D1000B
Live/Dead Fixable Aqua Dead Cell Stain Kit	Thermo Fisher Scientific	L34965
PAXgene Blood RNA Kit	PreAnalytiX	762174
Quantikine ELISA Human BAFF/BlyS/ TNFSF13B	R&D Systems	DBLYS0B

2.3 PRIMERS AND ANTIBODIES

Table 2.3: Different primers, forward and reverse, used for Sanger sequencing.

Primer	Direction	Sequence (5´- 3´)	Supplier
AIRE Exon 1	Forward	CAA GCG AGG GGC TGC CAG TG	BioNordika
AIRE Exon 1	Reverse	GGA TCT GGA GGG GCG GGG TC	BioNordika
AIRE Exon 6	Forward	CAC CCT GGG GCC TAC ACG ACT	BioNordika
AIRE Exon 6	Reverse	GAA GAG GGG CGT CAG CAA TGG	BioNordika
AIRE Exon 7	Forward	CCA GGA ACA GCG TTG CCT C	BioNordika
AIRE Exon 7	Reverse	CGG TGC TCA TCC CTG AGT GCC	BioNordika
AIRE Exon 8	Forward	CAG GTG GTC AGG GCA GAA TTT CA	BioNordika
AIRE Exon 8	Reverse	AGG CTG GGC AGC AGG TGT G	BioNordika
AIRE Exon 10	Forward	TGC CAC AGC CTT TCC CAC TCA GT	BioNordika
AIRE Exon 10	Reverse	CCT CCC GGA GCC TTT CTC GC	BioNordika

Table 2.4: Different antibodies used for staining cells for flow cytometry.

Antibody	Fluorochrome	Isotype	Supplier	#Cat. Number
Anti CD20, Human	PE/Cyanine5	Mouse IgG2b	BioLegend	302308
Anti CD24, Human	Brilliant Violet 785	Mouse IgG2a	BioLegend	311142
Anti CD27, Human	Brilliant Violet 605	Mouse IgG1	BioLegend	302830
Anti CD38, Human	PE	Mouse IgG1	BioLegend	303506
Anti IgD, Human	APC/Cy7	Mouse IgG2a	BioLegend	348218

2.4 INSTRUMENTS AND EQUIPMENT

Table 2.5: Instruments and equipment used in various experiments.

Instruments and equipment	Application	Manufacturer
BD LSR Fortessa Flow Cytometer	Analyzing B cells	BD Biosciences
Centrifuge 5810	Isolation of RNA and PBMC	Eppendorf
Fisher AccuSpin Micro 17	Isolation of RNA	Thermo Fisher Scientific
Gel Doc EZ Imager	Gel Imaging	Bio-Rad
Gel Doc EZ System, Sample Tray	Gel Imaging	Bio-Rad
Horizontal Orbital Microplate Shaker	Incubation in ELISA assays	Heidolph
Nanodrop Spectrophotometer ND-1000	Measure RNA concentration	Thermo Scientific
SpectraMax Plus 384 Microplate Reader	Measure absorbance in ELISA assays	Molecular Devices
Thermomixer Compact	Isolation of RNA	Eppendorf
Veriti 96-Well Fast Thermal Cycler	PCR	Thermo Fisher Scientific
QuantStudio 5	qPCR	Thermo Fisher Scientific

2.5 COMPUTER SOFTWARE

Table 2.6: Computer software and web resources used for illustrations and analyzing results.

Software	Application	Manufacturer
BioRender.com	Illustrations	BioRender
FlowJo 10.8.1	Analyzing flow-data	FlowJo LLC
GraphPad Prism 9.2.0	Obtaining graphs	GraphPad Software
Image Lab 3.0	Verifying PCR product	Bio-Rad
ND-1000 V3.8.1	Measure RNA concentration	Thermo Scientific
SoftMax Pro 7	Measure absorbance in ELISA assays	Molecular Devices
CLC Main Workbench 8.0.1	Analyzing sequences	CLC Bio, Qiagen

3. METHODS

This thesis has been conducted on DNA, RNA, sera and immune cells as biological sources. Several different molecular biology techniques have been applied. An overview indicating the main methods performed, and subjects included, throughout this study is found below.

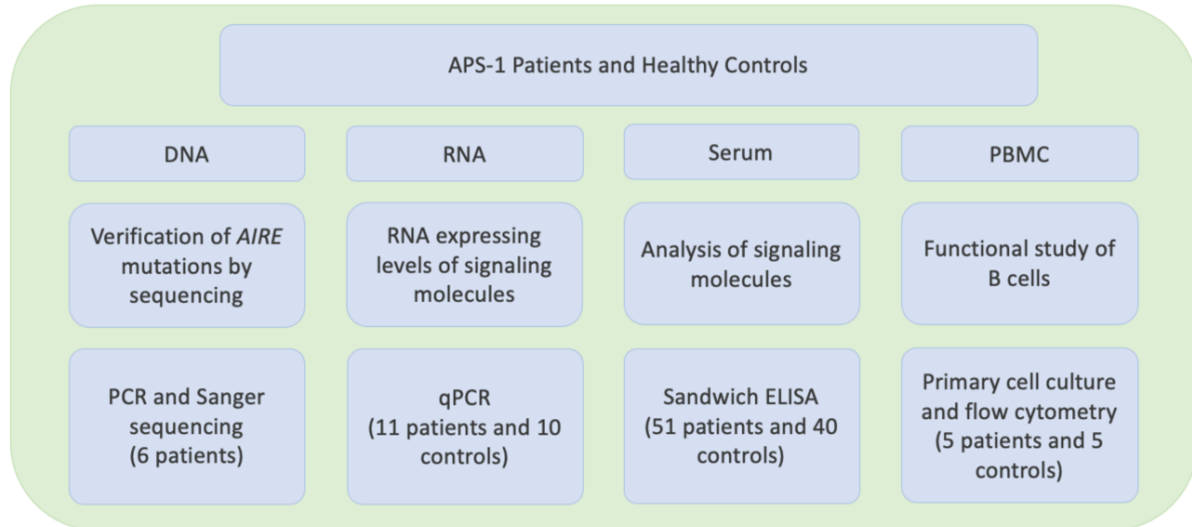


Figure 3.1 – Flowchart for the different methods conducted in this study. The figure shows an overview of the workflow throughout this study, indicating the main methods. For the verification of *AIRE* mutations six APS-1 patients were used (mean age 46.3 years, range 27-77 years). For gene expression of signaling molecules, 11 APS-1 patients (mean age: 49.1, age range: 31-63) and 10 healthy controls (mean age: 39.1, age range: 22-62) were subjected to cDNA synthesis and qPCR. For analysis of the signaling molecules in sera, 51 APS-1 patients (mean age: 44.82, age range: 5-77) and 40 healthy controls (mean age: 44.5, age range: 23-82) were included. For the functional study of B cells, five APS-1 patients (mean age: 43.8, age range: 30-62) and five healthy controls (mean age: 38.8, age range: 24-69) were included.

3.1 ETHICAL CONSIDERATIONS AND THE ROAS REGISTRY

The project has approval from the regional committees for medical and health research ethics (REK), with REK-numbers 2018/1417 and 2009/2555. The patients in this thesis are members of the registry for organ specific autoimmune diseases (ROAS)[98]. The healthy controls were obtained from blood doners at Haukeland University Hospital. Both patients and healthy controls have provided written consent to donate blood to research. ROAS is the world's largest Addison's registry and biobank with information from amongst others 910 live patients with AAD and 46 patients with APS-1 (in 2020), which includes blood samples, sera and peripheral blood mononuclear cell (PBMC) from these patients[99]. The ROAS non-disclosure agreement was signed by the candidate.

3.2 SANGER SEQUENCING

To verify the different mutations in the *AIRE* gene of the included patients, PCR was first performed to amplify the desired DNA sequences. A master mix containing 1x PCR Gold Buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTP, 1.5M Betaine, 8U AmpliTaq Gold DNA polymerase, 0.3 mM forward and reverse primer (Table 2.3), 10 µg DNA and ddH_2O to a total volume of 25 µl was prepared for each reaction. The thermoprofile comprised of pre-incubation, denaturation, annealing and elongation as show in Table 3.1.

Table 3.1: Thermoprofile used to make PCR-product by using the Veriti 96-Well Fast Thermal Cycler.

Thermoprofile	Temperature (°C)	Time	Number of cycles
Pre-incubation	98	10 minutes	-
Denaturation	98	15 seconds	
Annealing	69*	15 seconds	10
Elongation	72	45 seconds	
Denaturation	95	15 seconds	
Annealing	50	15 seconds	30
Elongation	72	45 seconds	
Elongation	72	7 minutes	-
Storage	4	∞	-

* Decreases by 0.5 degrees each cycle.

A 2% agarose gel was made in 1X Tris-Borate-Ethylenediamine tetra acetic acid (EDTA) (TBE) buffer with 1X GelRed to verify the PCR-product. The GeneRuler 50 bp DNA Ladder and the PCR-products with 6X loading dye (50% Glycine, 0.4% bromophenol blue, 0.8% xylene cyanol, 200 mM EDTA), was added to the wells before the gel was run at 100V for 30 minutes (min). The PCR-products were verified by using ImageLab 3.0. Two microliters ExoProStar was added to 5 µl PCR-product before it was run in the Veriti 96-Well Fast Thermal Cycler at 37°C for 15 min, 80°C for 15 min) to clean the PCR-product. Two master mixes containing BigDye 1.1, 1x BigDye Buffer, 2 mM forward or reverse primer and ddH_2O to a total volume of 6.5 µl was prepared. 3.5 µl of the cleaned PCR-product was added to the master mixes and run in the PCR-machine with the thermoprofile shown in Table 3.2 before the samples were sent to sequencing at the sequencing lab at the department of Medical Genetics, University of Bergen. The results were analyzed using CLC Main Workbench 8.

Table 3.2: Thermoprofile for cleaning of the PCR-product by using the Veriti 96-Well Fast Thermal Cycler.

Thermoprofile	Temperature (°C)	Time	Number of cycles
Pre-incubation	96	5 minutes	-
Denaturation	96	15 seconds	
Annealing	50	15 seconds	30
Elongation	60	2 minutes	
Storage	4	∞	-

3.3 ISOLATION OF TOTAL RNA FROM HUMAN WHOLE BLOOD

To isolate RNA, blood from 11 APS-1 patients and 10 healthy controls was drawn directly into PAXgene Blood RNA tubes, before frozen at -80°C after 15-30 min incubation on the bench. RNA was later isolated by using the PAXgene Blood RNA kit from Qiagen by following the manufacturer´s protocol. In short, the PAXgene Blood tubes were centrifuged (3000g, RT, 10 min) and the pellet was washed once with Rnase-free water and centrifuged (3000g, RT, 10 min), before dissolved in resuspension buffer. Binding buffer and proteinase K was added and mixed by vortexing for 5 min, before incubation (500 rpm, 55°C, 10 min) in a shaker-incubator. The lysate was transferred to a Shredder spin column and centrifuged (13000g, RT, 3 min). Ethanol (100%) was added to the supernatant and mixed. The sample was transferred to the PAXgene RNA spin column and centrifuged (13 000g, RT, 1 min), and washed with wash buffer 1. DNase 1 stock solution was mixed with DNA digestion buffer to make a DNase 1 incubation mix, added to the column membrane, and incubated for 15 min on the benchtop. After washing, a dry-spin was conducted to dry the column completely. To elute RNA, elution buffer was placed directly onto the column membrane and centrifuged (13 000g, RT, 1 min). After centrifugation, the eluate was incubated for 5 min at 65°C and chilled immediately on ice afterwards. The RNA was measured using Nanodrop Spectrophotometer ND-1000 and stored at -70°C until further use.

3.4 cDNA SYNTHESIS AND qPCR REACTION

3.4.1 cDNA Synthesis

cDNA synthesis was performed to generate cDNA from the isolated mRNA for the qPCR reaction. For the cDNA synthesis, the mRNA from chapter 3.3 was diluted to 0.3 µg with Diethyl pyrocarbonate (DEPC)-Treated water. The high-capacity RNA-to-cDNA kit from Thermo Fisher Scientific was used to make a master mix containing 2X RT buffer mix and 20X

RT enzyme mix, before it was mixed with the mRNA and run in the PCR-machine (37°C for 60 min, 95°C for 5 min).

3.4.2 qPCR Reaction

To determine if there is any significant difference in RNA expression between APS-1 patients and healthy controls, a qPCR reaction was performed using the Taqman system. For the qPCR reaction, master mixes for each probe (Table 2.2 in Materials) were made containing 20X probe/primer, 2X Taqman gene expression master mix and ddH_2O to a total volume of 26.5 µl. 5 µl cDNA was added to the master mix before distributed in triplicates in a 384-well plate. The plate was vortexed and centrifuged (1 min, 300g) using Centrifuge 5810. Subsequently, the plate was run in the qPCR-machine (50°C for 2 min, 95°C for 10 min, (95°C for 15 seconds, 60°C for 1 min) x40).

3.4.3 Calculating the $2^{-\Delta\Delta Ct}$-Value

The $2^{-\Delta\Delta Ct}$-method was use to analyze the relative changes from the qPCR experiments [100]. The formula is shown below.

$$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ gene)$$
$$\Delta\Delta Ct = \Delta Ct\ (patient\ or\ control) - \Delta Ct\ calibrator$$
$$Relative\ expression: 2^{-\Delta\Delta Ct}$$

This was done by running a qPCR reaction with the samples of interest in triplicates with primers for the genes of interest in addition to two housekeeping genes and subsequently calculate the average of the triplicates for all genes for all samples. The two housekeeping genes used were Human *Beta-2-Microglobulin (B2M)* and Human *Beta-Actin* and the average of them was used as “Ct (housekeeping gene)” in the formula. The average of ΔCt of the controls was used as the calibrator, and all calculations were done in excel.

3.5 SANDWICH ELISA

To determine the concentration of different B cell factors (antigens) in sera and supernatant from APS-1 patients and healthy controls, Enzyme-linked Immunosorbent Assay (ELISA) was used. A sandwich ELISA was performed for all measurements. Briefly, this method utilizes two antibodies, a capture antibody bound to the plate and a detection antibody, where the antigen is bound between the two. Different commercial ELISA kits were available, with slight differences in assay performance as described below. All kits came with a standard curve to

determine the concentration of the antigens, and all incubations were done at room temperature (RT), except for the Human BAFF-R ELISA kit, where all incubations were conducted at 37°C.

3.5.1 DuoSet ELISA, Measuring Levels of EBI3, TACI, APRIL

A 96-well plate was coated with capture antibody solution and incubated at 4°C overnight before the plate was washed with wash buffer. Reagent Diluent was added and incubated for one hour to block the plate, before the plate was washed again. The samples were diluted 1:1 in Assay Diluent and both samples and standards were added in duplicates and incubated for two hours, followed by a new wash step. Detection antibody was added and incubated for two hours, before washing again. Streptavidin-HRP conjugated antibody was then added and incubated for 20 min in the dark.

3.5.2 Quantikine ELISA, Measuring Levels of BAFF, BAFF-R, IL-10 and BCMA

For the Quantikine ELISA´s, the plates were coated with the capture antibody from the supplier. The samples were diluted 1:1, 1:4 or 20-fold with 1x Phosphate Buffered Saline (PBS). The samples and standards were added in duplicates and incubated for 1.5-3 hours, while shaking on a horizontal orbital at 500 rpm. The plate was washed with wash buffer before Human conjugated antibody was added to the wells and incubated for 1-2 hours while shaking. An additional step was included for the BAFF-R and the BCMA kit, where Avidin-Biotin-Peroxidase complex solution and Streptavidin-HRP solution was added, respectively, and incubated for 30-45 min while shaking.

The final following steps were common for all kits. The plate was washed, before Substrate Solution was added and incubated for 20 min-one hour in the dark, depending on the kit. For the IL-10 HS kit, amplifier solution was also added and incubated for 30 min while shaking. Stop solution was added before the absorbance was determined, using the SpectraMax Plus 384 Microplate Reader. The absorbance was measured at 450-490 nm to 570-690 nm, depending on the kit, and the concentrations were acquired by using the SoftMax Pro 7 software.

3.5.3 Making and Interpreting the Standard Curve

The standard curve was made by making a series of 2-fold dilutions. A vial supplied with each kit containing Recombinant Human Standard was reconstituted with 0.5 mL of Reagent Diluent and subsequently diluted 2-fold to a seven-point standard curve in Reagent Diluent. The range of the assays vary from 7.8 – 6000 pg/mL, depending on the kit.

3.6 ISOLATION OF PBMC

To isolate PBMC from human blood from six APS-1 patients and six controls, heparin blood was diluted 1:1 with 1x PBS and mixed by pipetting a few times, before being layered 2:1 on top of Ficoll, and centrifuged (400 g, RT, 30 min, break 1) using Centrifuge 5810. The PBMC layer was transferred to a new tube, washed with PBS, and centrifuged (400g, RT, 10 min). After centrifugation, the supernatant was removed, and PBS was added to resuspend the pellet. The cell suspension was diluted 1:4 in PBS and counted using Millipore cell counter with 40 µM chip. The cell suspension was centrifuged (400g, RT, 5 min) and the supernatant was removed. Human AB serum and Dimethyl Sulfoxide (DMSO) (4:1) was prepared for freezing down PBMC. After centrifugation, the cells were resuspended in AB serum, transferred to cryotubes, and incubated for 10 min at RT before being stored at -80°C for two days in a cell freezing container. This allowed the samples to freeze gradually, before being stored at -150°C until further use. For sample C-PBMC5, Fetal Bovine Serum (FBS) was used, due to lack of AB-serum.

3.7 PRIMARY CELL CULTURE

PBMC isolated from five APS-1 patients and five healthy controls was quickly thawed and added to 10 mL 1xPBS with 5% MACS Bovine serum albumin (BSA) (Flow buffer (FB)) before being centrifuged (300g, RT, 10 min) using Centrifuge 5810. The supernatant was removed, and the pellet was resuspended with FB. The cell suspension was filtered, and FB was added to the filter to make sure there was no cells left in the filter.

3.7.1 Magnetic Labeling and Separation with MS Columns

The cell suspension was centrifuged (300g, RT, 10 min) and the supernatant was removed afterwards. The cell pellet was resuspended in 80 µl of FB, and 20 µl of CD19 Microbeads per 10^7 total cells was added before incubated for 15 min in 2-8°C. The cells were washed with 2 mL FB, centrifuged (350g, RT, 5 min), and resuspended in 500 µl FB. The MS column provided

with the B Cell Expansion Kit was placed in the magnetic field of the suitable MACS separator. The column was rinsed with 500 µl of FB, before adding the cell suspension. The column was washed by adding 500 µl of FB three times onto the column. The column was removed from the magnetic field and placed in a suitable collection tube. 1 mL of 1x PBS was added onto the column and the cells were immediately flushed out by pushing the plunger into the column.

3.7.2 Cell Proliferation Staining

To measure cell division, 5 mM Carboxyfluorescein Succinimidyl ester (CFSE) stock solution and a 10 mM CFSE/PBS solution was prepared. After centrifugation (300g, RT, 10 min), the cells were resuspended in preheated (37°C) PBS, and 1 mL of the 10 mM CFSE/PBS solution was added and incubated at 37°C for 10 min. 2 mL of ice-cold AB-serum was added to the cells to stop the staining and incubated on ice for 5 min, before centrifugation (300g, RT, 10 min).

3.7.3 Activating and Expanding the B Cells

Expansion medium was prepared containing 10 mL Stem MACS hematopoietic stem cell (HSC) Expansion medium XF, 10 µl reconstituted IL4 and 5% AB-serum. CD40-L and crosslink antibody was mixed 1:1 and incubated for 30 min before being added to the medium. The cells were resuspended with 1 mL expansion medium and counted using C-Chip Disposable Hemocytometer by mixing the cells 1:1 with Trypan Blue. For B cell expansion the cells should be resuspended in culture medium at $0.15x10^6$ cells/mL. The cells were divided into wells with the appropriate amount of Expansion medium, according to the table provided by the manufacturer (Table 7.8 in Supplementary data) and incubated at 37°C for seven days. The cells were then harvested, centrifuged (300g, RT, 10 min) and the supernatant was saved for ELISA assays.

3.8 FLOW CYTOMETRY

Flow cytometry was used to phenotypically characterize the B cells after cell culture. B cells isolated from PBMC from five APS-1 patients and five healthy controls were prepared for flow cytometry after being cultured for seven days. The cells were stained with fluorochrome-conjugated antibodies (Table 3.3) and analyzed with an already established inhouse panel by flow cytometry.

Table 3.3: Panel for flow cytometry including targets, fluorochrome, excitations and filter for emittance.

Target	Fluorochrome	Dilution factor	Excitation (nm)	Filter for emittance
Aqua (Live/dead)	BV510	1:1000	407	525/50 band pass
CD20	Pe-Cy5	1:500	561	661/20 band pass
CD24	BV785	1:200	407	780/60 band pass
CD27	BV605	1:500	407	605/12 band pass
CD38	PE	1:200	561	582/15 band pass
CFSE	FITC	As described in section 3.7.2	488	530/30 band pass
IgD	APC-Cy7	1:200	640	780/60 band pass

The harvested cells from the cell culture were resuspended in FB, before adding 0.5 mg Fc block and incubating at RT for 20 min. The cells were washed with FB and centrifuged (450g, 5 min). The supernatant was removed before Fixable live/dead Aqua cell stain mixed 1:1000 with PBS, was added and incubated at RT for 20 min in the dark. The cells were washed again, and the supernatant was removed before a master mix containing all the antibodies (Table 3.3) was added to the cells and incubated for at 4°C for 20 min in the dark. The cells were washed and centrifuged (450g, 5 min) again, before the 1X Fixation/Permeabilization solution was added to the cells and incubated at 4°C for 30 min. The cells were washed with 1X permeabilization wash buffer and centrifuged (450g, 5 min) before the supernatant was removed. The cells were resuspended in remaining buffer and analyzed on the BD LSR Fortessa Flow Cytometer.

Single stain compensation controls were made by adding one drop of UltraComp eBeads to 1 µl of each antibody in the panel, before incubation at 4°C for 20 min and washed. The compensation controls were vortexed and stored at 4°C until use. This panel is an established inhouse protocol, which has been used in the lab before. Further analysis of the cells was done using the FlowJo 10.8.1 software.

3.9 STATISTICS

For the statistical analysis for ELISA in sera, an unpaired Student's t-test was performed by using GraphPad Prism 9.2.0. Pearson correlation analysis using a 95% confidence interval was performed to calculate the correlation between age and B cell factors concentrations in sera using GraphPad Prism 9.2.0. This analysis measures the strength of a linear association between two variables[101]. A two-tailed Wilcoxon test was used for the comparison of healthy controls before and after culturing, while for the other statistical analysis, a two-tailed Mann-Whitney

test was performed by using GraphPad Prism 9.2.0. The latter considers differences in medians, shape and spread between two groups[102]. A non-parametric test was chosen due to the low number of samples where a normal distribution cannot be assumed. The standard deviation (SD) is shown in the graphs. The unpaired t test considers the average of two groups that are independent of each other in order to determine if there is a significant difference between the two groups[103]. Wilcoxon test is a non-parametric test that assumes that the two paired groups come from a dependent population, in this case the same individual[104]. Results were considered as statistically significant when $P < 0.05$. All graphs were created in GraphPad Prism 9.2.0.

4. RESULTS

4.1 VERIFICATION OF *AIRE* MUTATIONS IN APS-1 PATIENTS

In order to confirm mutations in the *AIRE* gene in APS-1 patients, Sanger sequencing was performed using DNA from APS-1 patients and verified on a 2% agarose gel (Figure 7.1 in Supplementary data). The Medical Genetics department at Haukeland University hospital sequence all patients as a part of their routine work, and six APS-1 patients were here chosen to verify the individual mutations (Figure 4.1). See Table 4.1 for an overview of the mutations.

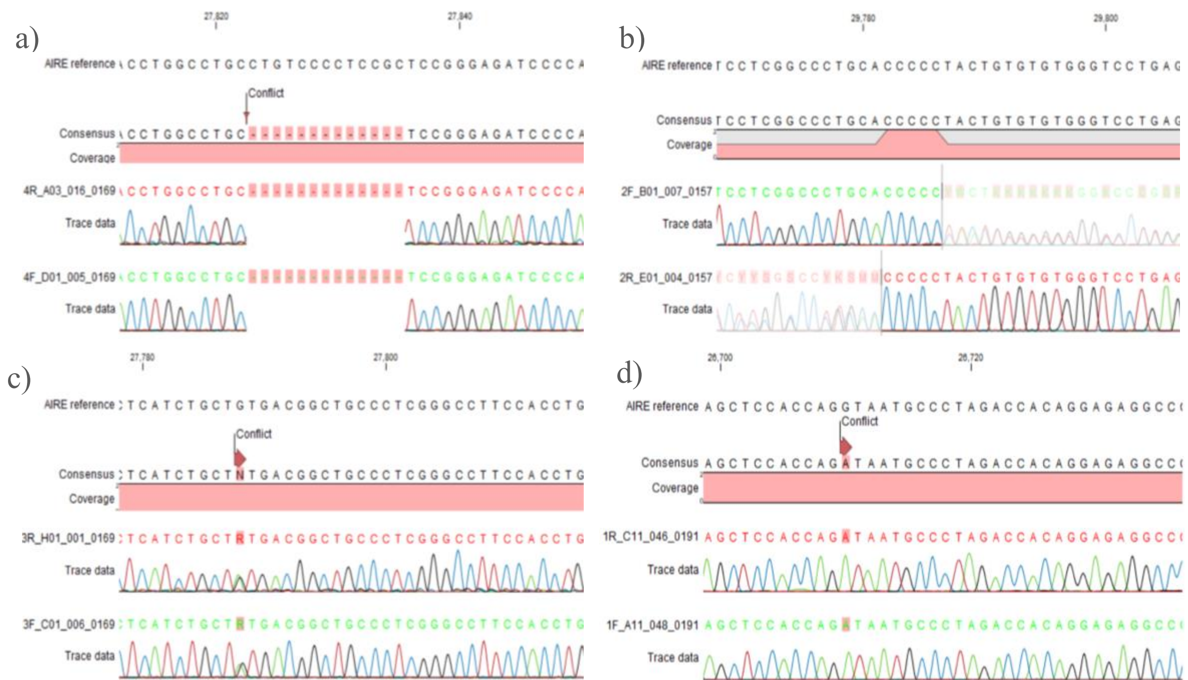


Figure 4.1 – Different mutations in APS-1 patients. These are examples of the various mutations in *AIRE* that patients suffering from APS-1 can harbour. The *AIRE* reference gene is shown at the top of each alignment. a) The mutation shown is a homozygote 13 bp deletion in exon 8 in *AIRE.* Both forward and reverse strand obtain the mutation, making it a homozygote deletion. b) The mutation shown is an insertion of C on one strand, making it a heterozygous mutation in exon 10 in *AIRE*. c) The mutation shown is a heterozygote point mutation in exon 8 in *AIRE*. One of the strands has a G, which is the same as the reference gene, while the other strand has an A, making this a heterozygote mutation. d) The mutation shown is a point mutation in exon 7 in *AIRE*, both strands have substituted a G for an A, making it a homozygote mutation. Figures from CLC Main Workbench 8.0.1.

Table 4.1: The sequencing results indicating type of mutation and exon location in the *AIRE* gene.

Patient no.	Exon	*AIRE* mutation	Zygosity
P-SEQ1	1	c.22C>T	Heterozygote missense point mutation
P-SEQ1	8	c.967-979del13	Heterozygote 13 bp deletion indel
P-SEQ2	7	c.879+1G>A/ c.879+1G>A	Homozygote splice point mutation
P-SEQ3	10	c.1163insA/c.1249dupC	One insertion and one duplication indel
P-SEQ4	8	c.932G>A	Heterozygote missense point mutation
P-SEQ5	8	c.967-979del13/c.967-979del13	Homozygote 13 bp deletion indel
P-SEQ6	6	c.769C>T	Heterozygous nonsense point mutation
P-SEQ6	10	c.1249dup	Duplication of C indel

4.2 GENE EXPRESSION OF B CELL FACTORS

To investigate if the defective AIRE protein in APS-1 patients might affect key B cell factors, the gene expression of *BCMA, TACI, APRIL, BAFF, IL-10, EBI3* and *BAFF-R* was determined using isolated RNA from 11 APS-1 patients and 10 healthy controls. The RNA concentration for the controls was found to vary from 58.3 ng/µl to 126.2 ng/µl with the 260/280 value varying between 2.07 to 2.17 (Table 7.1 in Supplementary data). The RNA concentration for the patients varied from 15.9 ng/µl to 197.1 ng/µl with the 260/280 value between 2.08 to 2.39 (Table 7.2 in Supplementary data).

The isolated RNA was normalized to 0,3 ug and used for cDNA synthesis and subsequently subjected to qPCR. All the investigated B cell factors were found to be expressed in blood, but *BCMA* was the only gene found to have a significant downregulation in patients compared to controls. For *BCMA* the fold-change for the APS-1 patients was found to be 0.564 ± 0.366, and the fold-change for the controls was found to be 1.039 ± 0.298, compared to the mean of the controls, with a P value for the difference between APS-1 patients and controls of 0.008 (Figure 4.2).

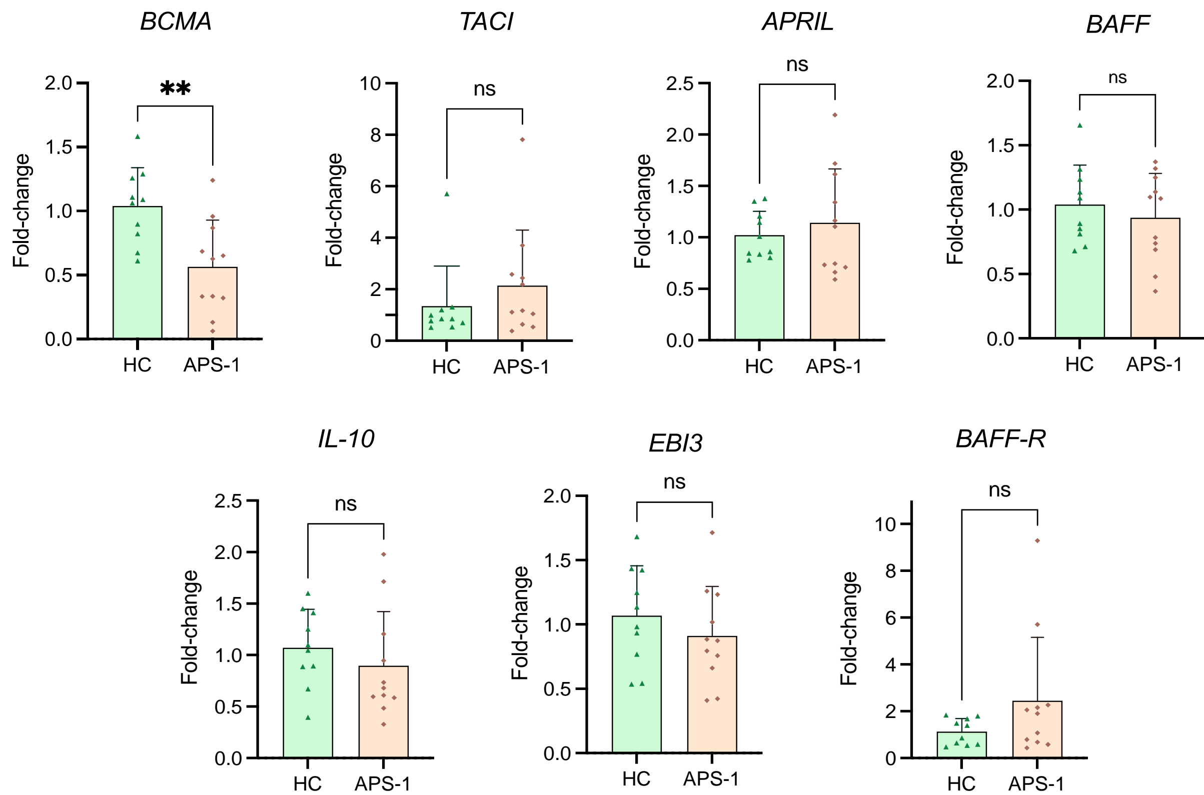


Figure 4.2 – Gene expression of the different B cell factors. Isolated RNA from both APS-1 patients (APS-1) and healthy controls (HC) was subjected to cDNA synthesis for each of the seven genes investigated. The relative changes were analyzed by running a qPCR reaction with the samples of interest in addition to two housekeeping genes (Human *Beta-2-Microglobulin* and Human *Beta-Actin*) and calculated using the $2^{-\Delta\Delta Ct}$-method. Each data point represents one individual APS-1 patient or healthy control. *BCMA* was found to have significant difference (APS-1=0.564 ± 0.366, HC=1.039 ± 0.298, P value: 0.008) of gene expression, while *TACI* (APS-1=2.146 ± 2.145, HC=1.347 ± 1.555, P value: 0.314), *APRIL* (APS-1=1.143 ± 0.524, HC=1.023 ± 0.231, P value: 0.809), *BAFF* (APS-1=0.938 ± 0.343, HC=1.038 ± 0.307, P value: 0.705), *IL-10* (APS-1=0.897 ± 0.526, HC=1.071 ± 0.374, P value: 0.251), *EBI3* (APS-1=0.911 ± 0.383, HC=1.069 ± 0.387, P value: 0.350) and *BAFF-R* (APS-1=2.450 ± 2.707, HC=1.133 ± 0.559, P value: 0.173) was found to be non-significant. Error bars show the standard deviation (SD), and the P value was calculated by using the Mann-Whitney test in GraphPad Prism 9.2.0.

4.4 DETERMINING LEVELS OF B CELL FACTORS IN SERA

In order to determine the protein levels of the B cell factors of interest in circulation, sera from both APS-1 patients and healthy controls were subjected to ELISA analysis. The ELISA assay for BAFF revealed significantly elevated levels in sera in APS-1 patients with a P value of 0.0358. However, the assay for APRIL revealed significantly lower levels in sera in APS-1 patients with a P value of 0.0239 (Figure 4.3, Table 7.3 in Supplementary data (controls) and Table 7.5 in Supplementary data (patients)). TACI and BCMA (Figure 4.3, Table 7.3 (control) and Table 7.5 (patients) in Supplementary data) did not reveal a significant difference, while the ELISA assays for EBI3, IL-10 and BAFF-R (Table 7.4 (controls) and Table 7.6 (patients) in Supplementary data) had too many samples out of range, indicating that the concentrations of the B cell factors were too low in sera to be detected (Figure 7.2 in Supplementary data).

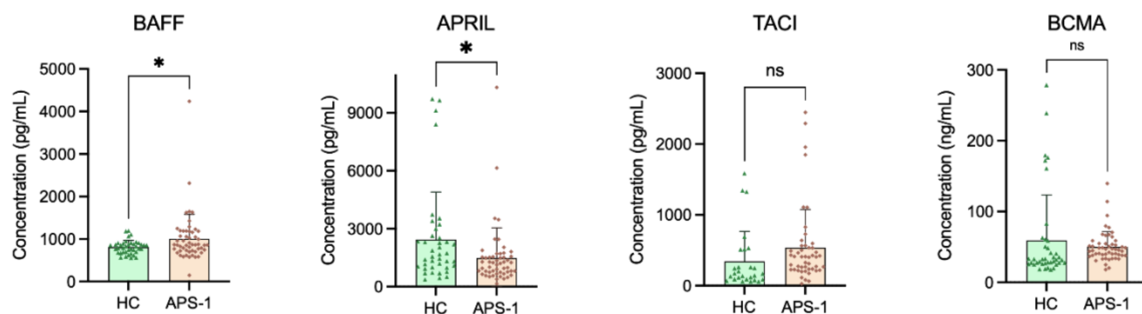


Figure 4.3 – Concentrations of the different B cell factors in sera from APS-1 patients (APS-1) and healthy controls (HC). The 96-well plate was coated with capture antibody before adding the samples, subsequently incubated with detection antibody, and afterwards with substrate solution. The concentrations were acquired using SoftMax Pro 7 software by measuring the absorbance at 450-490 nm to 570-690 nm, depending on the ELISA kit. Each data point represents one individual APS-1 patient or healthy control. BAFF (APS-1=1008 ± 571.7, HC=810 ± 156.7, P value: 0.0358) and APRIL (APS-1=1483 ± 1567, HC=2444 ± 2456, P value: 0.0239) revealed a significant difference, however BCMA (APS-1=50.21 ± 21.52, HC=59.33 ± 63.95, P value: 0.3388) and TACI (APS-1=535.3 ± 543.3, HC=344.5 ± 422.7, P value: 0.1191) was shown to be non-significant. Error bars show the SD, and the P value was calculated by using the unpaired t test in GraphPad Prism 9.2.0.

To investigate if there were any correlations between age and the concentrations of BAFF and APRIL in sera, a Pearson correlation analysis was conducted. Since BAFF and APRIL revealed significantly increased and decreased levels, respectively, only these two were subjected to the analysis. However, there was no clear correlation between age and the concentrations of BAFF and APRIL in sera from either APS-1 patients or healthy controls. (Figure 4.4).

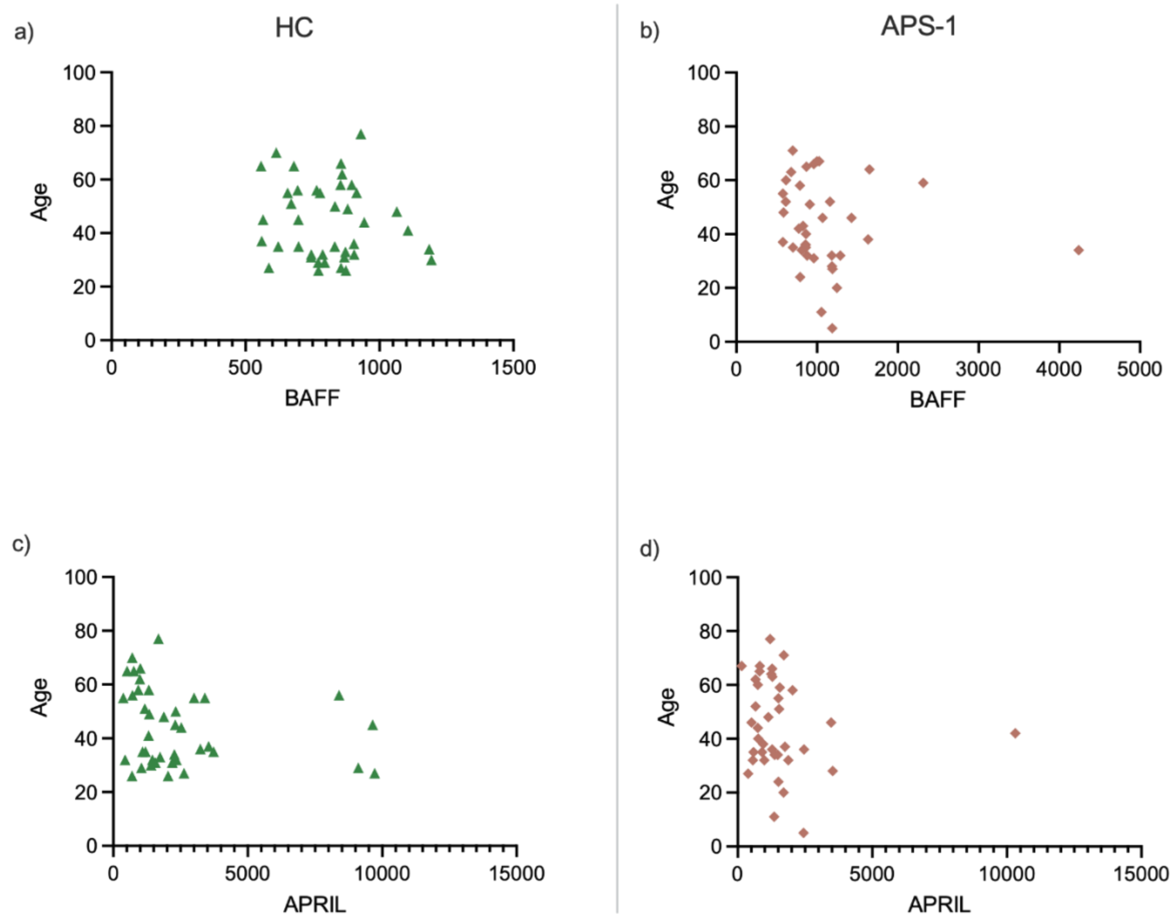


Figure 4.4 – Concentrations of BAFF and APRIL in sera in both APS-1 patients (APS-1) and healthy controls (HC) correlated against age. Concentrations of BAFF and APRIL in sera was correlated against age of the APS-1 patients and healthy controls. a) BAFF concentrations correlated against age of the healthy controls subjected to ELISA. b) BAFF concentrations correlated against age of the APS-1 patients subjected to ELISA. c) APRIL concentrations correlated against age of the healthy controls subjected to ELISA. d) APRIL concentrations correlated against age of the APS-1 patients subjected to ELISA. There was no correlation between age and concentration of BAFF or APRIL in sera from either APS-1 patients or healthy controls. Each data point represents one individual APS-1 patient or healthy control. Pearson correlation coefficient was calculated in GraphPad Prism 9.2.0.

4.5 FUNCTIONAL CHARACTERIZATION OF B CELLS

With the aim to activate and expand the B cells and to analyze them phenotypically, the B cells were isolated from PBMC from five APS-1 patients and five sex- and age-matched controls. The B cells were subsequently cultured and subjected to flow cytometry at the point of isolation and after seven days in culture. As the patient samples were available through ROAS, matching controls were collected from the blood bank at Haukeland University Hospital. The concentration of PBMC from the controls isolated in this study ranged from 2.0-5.8 x 10^6 cells

(Table 7.7 in Supplementary data). The supernatant from the cultures were also analyzed by ELISA to determine the production of B cell factors.

4.5.1 Isolation, Characterization and Expansion of B cells

To expand the B cells after isolating them from PBMC, they were activated in culture for seven days. For the healthy controls, the start number of cells ranged from $10*10^6$-$15*10^6$. While the percentage of B cells isolated from the PBMCs ranged from 1.9-3.8%, and the percentage of live B cells after seven days in culture ranged from 2.2-37.0% (Table 4.3). For the APS-1 patients, the start number of cells ranged from $4*10^6$-$20*10^6$, and the percentage of B cells isolated from these cells ranged from 0.6-1.0%. The percentage of live B cells after seven days in culture ranged from 1.6-12.6% (Table 4.4). This indicates that B cells from the healthy controls manage better in culture, as well as it seems to be more B cells in controls compared to patients (Figure 4.5).

Table 4.3: Isolated B cells from PBMC from blood doners, indicating the sex and age of each healthy control, the amount of all cells, the amount of B cells isolated, and the percentage of live B cells on day 7.

Control no.	Sex	Age	All cells (cells/mL)	B cells day 0 (cells/mL)	Live B cells day 7
C-PBMC1	F	69	$12*10^6$	235.000	37.0%
C-PBMC2	M	39	$10*10^6$	185.000	3.0%
C-PBMC3	M	29	$12*10^6$	275.000	2.2%
C-PBMC4	F	24	$15*10^6$	465.000	3.5%
C-PBMC5	M	33	$12*10^6$	460.000	7.1%

Table 4.4: Isolated B cells from PBMC from APS-1 patients, indicating the sex and age of each APS-1 patient, the amount of all cells, the amount of B cells isolated, and the percentage of live B cells on day 7.

Patient no.	Sex	Age	All cells (cells/mL)	B cells day 0 (cells/mL)	Live B cells day 7
P-PBMC1	F	62	$4*10^6$	40.000	12.6%
P-PBMC2	M	46	$17*10^6$	120.000	3.9%
P-PBMC3	M	30	$9*10^6$	90.000	2.5%
P-PBMC4	F	49	$8*10^6$	50.000	6.3%
P-PBMC5	M	32	$20*10^6$	135.000	1.6%

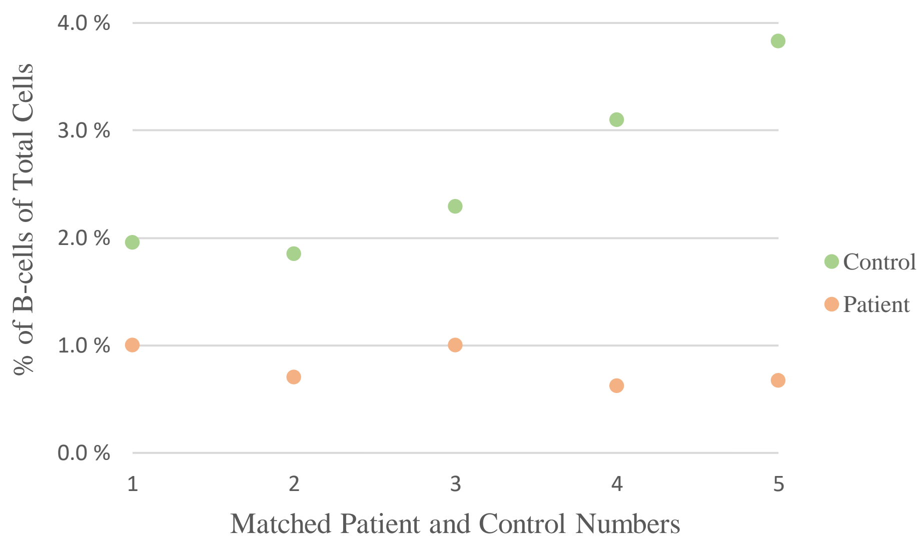


Figure 4.5 – Matched APS-1 patient and healthy control showing the percentage of B cells from total cells on day 0. B cells were isolated from PBMC from APS-1 patients and healthy controls by the means of magnetic labeling and separations using CD19 microbeads. Percentage of B cells isolated from the healthy controls and APS-1 patients range from 1.9-3.8% and 0.6-1.0%, respectively. There is a distinct difference in the amount of B cells between controls and APS-1 patients, where the APS-1 patients clearly have less B cells than controls.

4.5.2 Characterization of B cells by Flow Cytometry

In order to characterize the different types of B cells in the cell culture, the cells from APS-1 patients and healthy controls were subjected to flow cytometry on day 0 (for the samples with enough cells) and after being cultured for seven days. The cells were stained with fluorochrome-conjugated antibodies and analyzed with an already established inhouse panel by flow cytometry (Table 3.3 in Methods). An example of the gating strategi from a control is shown in Figure 4.6, and this gating strategi was used for all APS-1 patients and healthy controls.

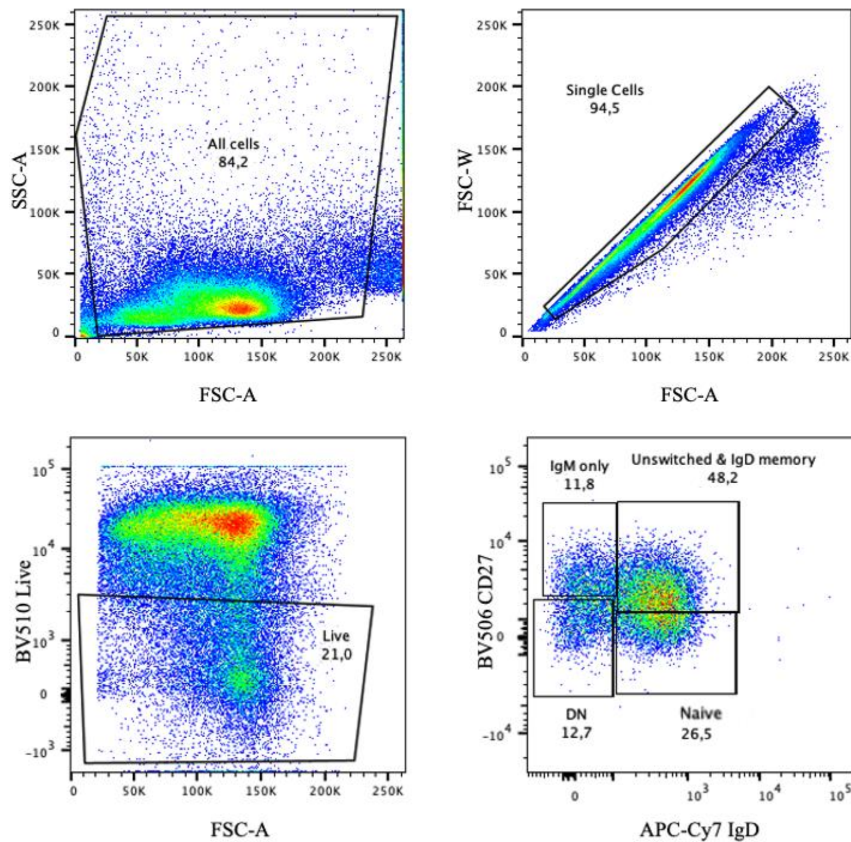


Figure 4.6 – Gating strategy for flow cytometry. All cells, single cells, live cells, B cell subset. The gating was first set to include all cells by plotting forward scatter area (FSC-A) against the side scatter area (SSC-A). Further, the gate was set to single cells from all cells by plotting FSC-A against forward scatter width (FSC-W), and subsequently the gate was set to live cells by plotting FSC-A against BV510 Live, which is the live/dead marker, making sure that the dead cells were not included in the analysis. Here, shown for one control on day 0. From the live population, IgD was plotted against CD27, where naïve cells, cells with only IgM, IgD memory cells and double negative (DN) cells were separated[68]. Figure created in FlowJo 10.8.1.

Analysis by flow cytometry after seven days revealed first and foremost that the majority of B cells were dead in both APS-1 patients and healthy controls (Figure 4.7). On the example showing the gating strategy, there were only 21% live cells (Figure 4.6). CFSE staining revealed that the majority of the cells did not divide, and some cells only divided once (Figure 4.7).

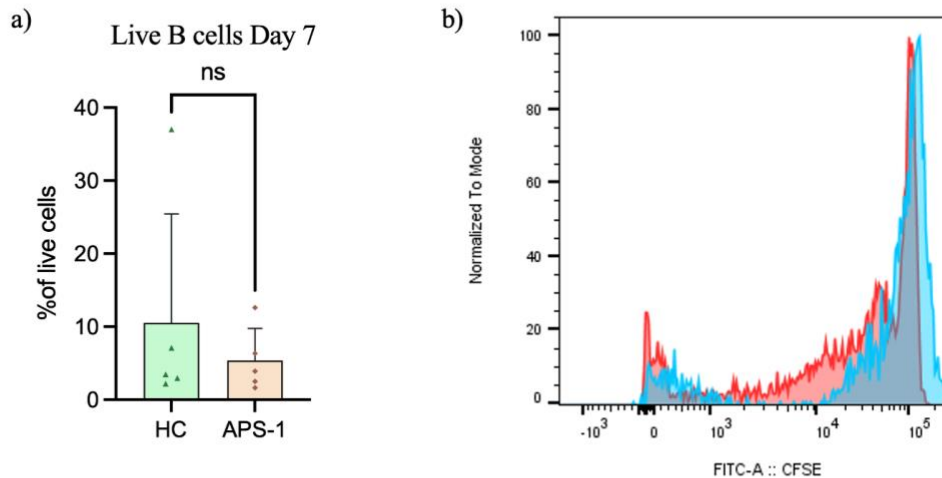
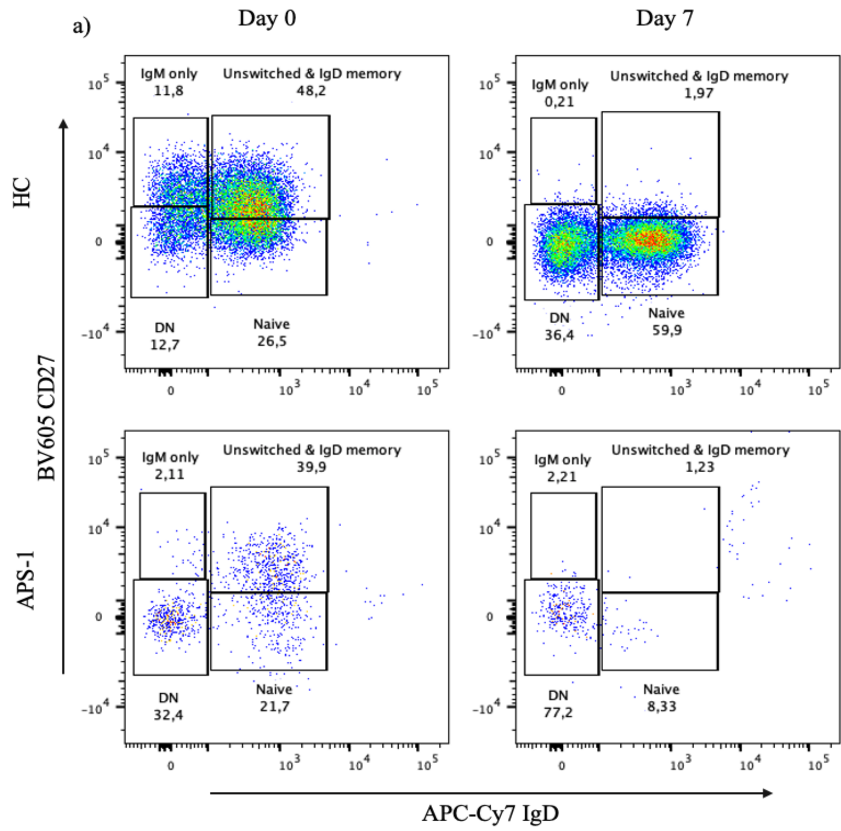
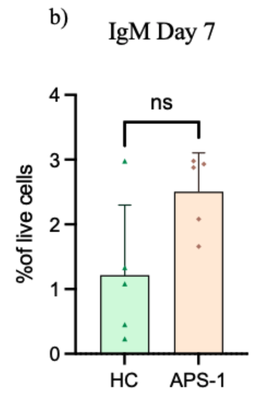
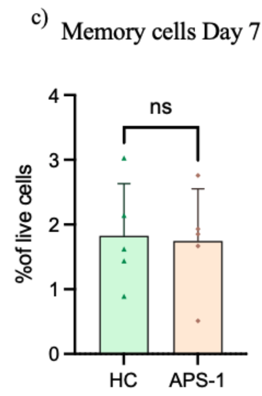


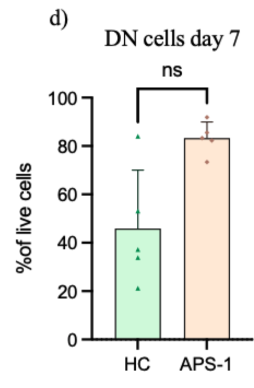
Figure 4.7 – Live B cells and cell proliferation. The B cells isolated from PBMC from APS-1 patients (APS-1) and healthy controls (HC), were analyzed by flow cytometry before being cultured for seven days, and afterwards analyzed again, in order to characterize the different types of B cells. The cells were stained with fluorochrome-conjugated antibodies and analyzed with an already established inhouse panel by flow cytometry (Table 3.3 in Methods). a) Gating strategi was done according to Figure 4.6. Each data point in the graph represents one individual APS-1 patient or healthy control. Error bars show the SD, and the P value was calculated by using the Mann-Whitney test in GraphPad Prism 9.2.0. The percentage of live B cells from APS-1 and HC (APS-1=5.398 ± 4.401, HC=10.56 ± 14.90, P value: 0.841) on day 7 are shown. b) The CFSE staining results for one HC is shown, where the blue graph indicates HC on day 0 and the red graph indicates HC on day 7. The largest peak are the cells before dividing, while the next peak indicates that some of the cells divided once. The peak at approximately 0 are the negative cells that did not obtain the staining. Figure created in FlowJo 10.8.1.

Among the cells that were alive at day 7, there were no significant changes between the APS-1 patients and the healthy controls although there was a trend towards more double negative (DN) cells and less naïve cells in the APS-1 patients (Figure 4.8).


a)
Day 0
Day 7
HC
APS-1
BV605 CD27
APC-Cy7 IgD
IgM only
11,8
Unswitched & IgD memory
48,2
DN
12,7
Naive
26,5
IgM only
0,21
Unswitched & IgD memory
1,97
DN
36,4
Naive
59,9
IgM only
2,11
Unswitched & IgD memory
39,9
DN
32,4
Naive
21,7
IgM only
2,21
Unswitched & IgD memory
1,23
DN
77,2
Naive
8,33


b)
IgM Day 7
ns
%of live cells
HC
APS-1


c)
Memory cells Day 7
ns
%of live cells
HC
APS-1


d)
DN cells day 7
ns
%of live cells
HC
APS-1

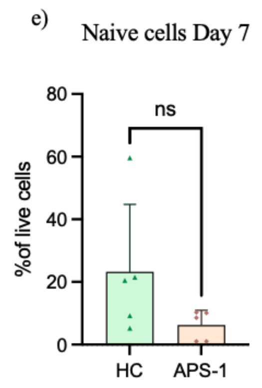

e)
Naive cells Day 7
ns
%of live cells
HC
APS-1

Figure 4.8 – Comparison of APS-1 patients and healthy controls from flow cytometry data. The B cells, isolated from PBMC from APS-1 patients (APS-1) and healthy controls (HC), were analyzed by flow cytometry before being cultured for seven days, and afterwards analyzed again, in order to characterize the different types of B cells. The cells were stained with fluorochrome-conjugated antibodies and analyzed with an already established inhouse panel by flow cytometry (Table 3.3 in Methods). a) Gating strategi was done according to Figure 4.6. Here, one example for both APS-1 and HC is shown for day 0 and day 7. For the HC, two distinct populations (DN cells and naïve cells) are shown on day 7, while for the APS-1 there is one population (DN cells) and a few cells spread in the three other subsets. Figure created in FlowJo 10.8.1. b) The percentage of IgM cells in both APS-1 and HC are shown (APS-1=2.506 ± 0.60, HC=1.214 ± 1.084, P value: 0.056). c) The percentage of memory cells in both APS-1 and HC are shown (APS-1=1.744 ± 0.807, HC=1.824 ± 0.809, P value: >0.999). d) The percentage of DN cells in both APS-1 and HC are shown (APS-1=83.28 ± 6.667, HC=45.88 ± 24.15, P value: 0.103). e) The percentage of naïve cells in both APS-1 and HC are shown (APS-1=6.210 ± 4.771, HC=23.21 ± 21.52, P value: 0.151). Each data point in the graphs represents one individual APS-1 patient or healthy control. Error bars show the SD, and the P value was calculated by using the Mann-Whitney test in GraphPad Prism 9.2.0.

Looking at the differences in the cell populations at day 0 and day 7, only controls could be analyzed as most of the APS-1 patients did not have enough cells to prioritize flow cytometry before culturing. After activation for seven days there was a decrease in both IgM cells and memory cells, while there was an increase in DN cells and naïve cells for most of the controls (Figure 4.9). This indicates that they did not mature and divide into memory cells.

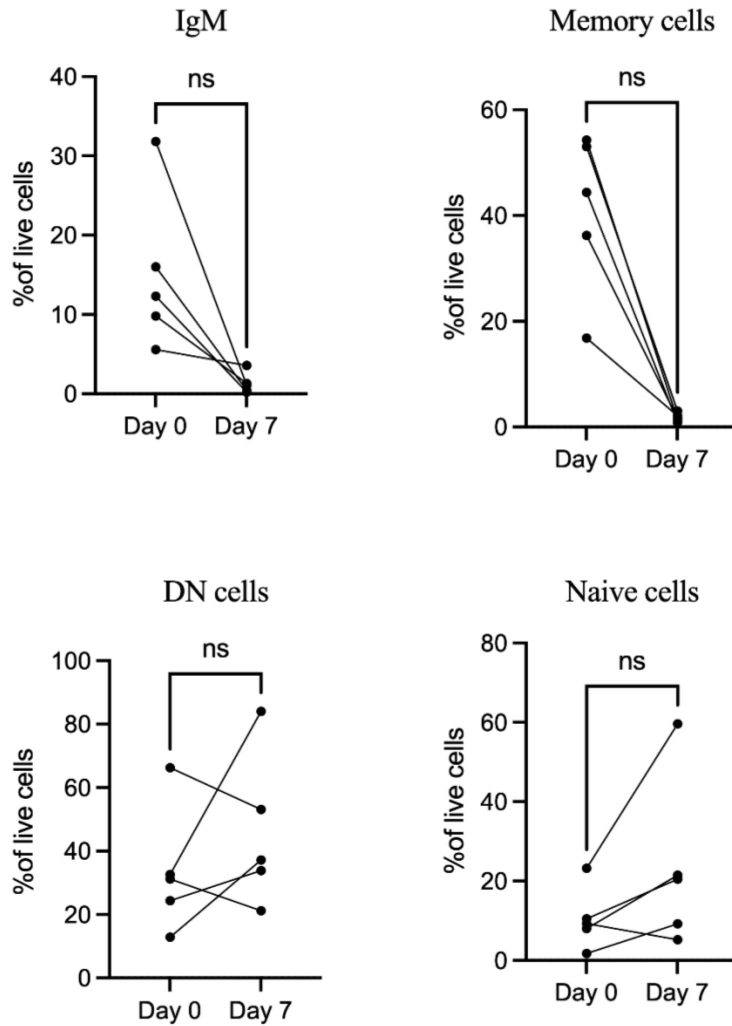


Figure 4.9 – Comparison of day 0 and day 7 for the healthy controls. To obtain the percentages for the graphs, the gating strategi was done according to Figure 4.6 and the flow cytometry data was compared for day 0 and day 7 for the healthy controls. Each data point represents one healthy control. For the IgM cells (Day 0=15.10 ± 10.08, Day 7=1.334 ± 1.333, P value: 0.0625) and memory cells (Day 0=40.94 ± 15.34, Day 7=1.824 ± 0.809, P value: 0.0625) the decrease is quite clear, however it was found to be non-significant. DN cells (Day 0=33.48 ± 19.93, Day 7=45.88 ± 24.15, P value: 0.620) and naïve cells (Day 0=10.56 ± 7.827, Day 7=23.21 ± 21.52, P value: 0.1250) were also non-significant, however the cell number for most of the controls increased. The P value was calculated by using the paired Wilcoxon test in GraphPad Prism 9.2.0.

4.5.3 Levels of B cell Factors in Supernatant After Cell Culture

In order to determine levels of the B cell factors of interest, the supernatant from both APS-1 patients and healthy controls cell cultures were subjected to analysis by ELISA. The ELISA assays for BCMA, APRIL and BAFF (Table 7.14 (controls) and Table 7.15 (patients) in Supplementary data) revealed no major deviations in the concentration in supernatant between APS-1 patients and healthy controls (Figure 4.10). The ELISA assay for TACI (Table 7.14 (controls), Table 7.15 (patients) and Figure 7.3 in Supplementary data) revealed that almost all samples were out of range, indicating that the concentrations were too low to be detected. While in the ELISA assays for EBI3 and IL-10, no samples were detected indicating too low concentrations.

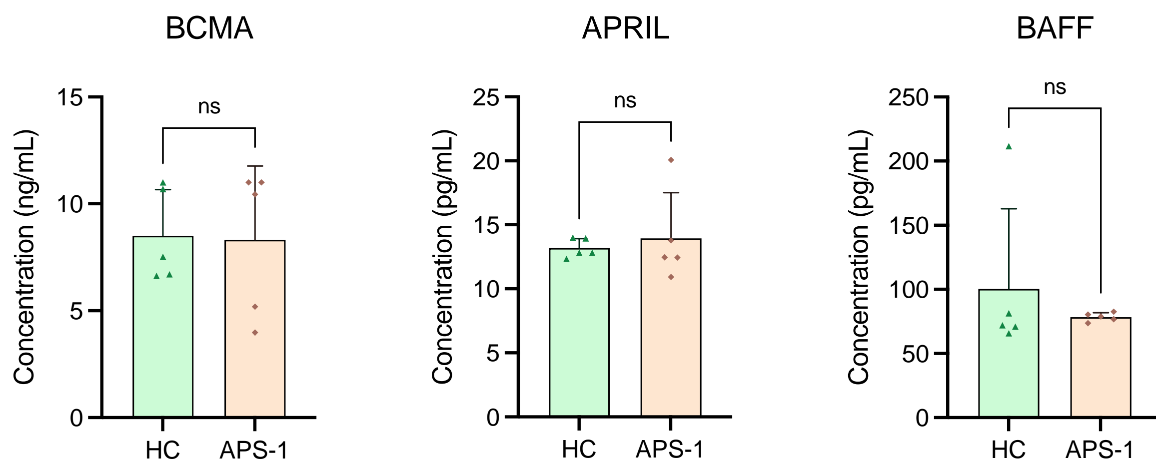


Figure 4.10 – Concentrations of the different B cell factors in supernatant from the five sex- and age-matched APS-1 patients (APS-1) and healthy controls (HC). The 96-well plate was coated with capture antibody before adding the samples, subsequently incubated with detection antibody, and afterwards with substrate solution. The concentrations were acquired using SoftMax Pro 7 software by measuring the absorbance at 450-490 nm to 570-690 nm, depending on the ELISA kit. Each data point represents one individual APS-1 patient or healthy control. BCMA (APS-1=8.324 ± 3.446, HC=8.509 ± 2.162, P value: >0.999), APRIL (APS-1=13.94 ± 3.570, HC=13.19 ± 0.744, P value: 0.651) and BAFF (APS-1=78.41 ± 3.416, HC=100.3 ± 62.43, P value: 0.548) revealed no significant difference. Error bars show the SD, and the P value was calculated by using the Mann-Whitney test in GraphPad Prism 9.2.0.

5. DISCUSSION

APS-1 is a rare, recessively inherited monogenic autoimmune disease that is characterized by mutations in the *AIRE* gene[6,43]. In-depth knowledge about different key B cell factors in APS-1 is needed to further elucidate the role they have in the disease and if they could be used as a way to diagnose or treat APS-1 patients. In the present study, we aimed to investigate how the B cells will respond upon activation, as well as investigate the levels of important B cell factors in both sera and supernatant from APS-1 patients compared to healthy controls. A significant difference of BAFF and APRIL in sera was confirmed, while the gene expression of *BCMA* revealed a decreased expression in APS-1 patients compared to controls. Flow cytometric analysis gave some insight to the phenotype of the live B cell after culturing in both APS-1 patients and healthy controls.

5.1 VERIFICATIONS OF MUTATIONS IN THE *AIRE* GENE

Sanger sequencing of the *AIRE* gene in six APS-1 patients was performed to verify their mutations. Since APS-1 is a recessively inherited disease, a mutation needs to be present in both *AIRE* alleles[21,44,45]. However, dominant mutations have occasionally been identified, although this causes milder phenotypes and symptoms compared to classical APS-1[46]. To this day, there are over 130 mutations identified in *AIRE*[52]. One of the mutations that was verified in this study was a 13 bp deletion in exon 8 in *AIRE,* where both the forward and reverse strand contained the mutation, making it a homozygote deletion. This is the most common mutation in APS-1 patients in Norway[47]. Over the years, the human reference genome has been substantially upgraded. The sequencing methods has furthermore been improved since the early 2000s, which has led to the findings of more variations of the *AIRE* gene as well as other genes. Knowledge of the mutations in the different exons can disclose information in relation to which domain it will affect in the AIRE protein as well as the function of the protein. This again can give us a better understanding of AIRE and for general immunological understanding, since AIRE is essential in immunological tolerance.

5.2 COMPARISON OF B CELL FACTORS

5.2.1 Comparison of B Cell Factor Concentrations in Sera *versus* Supernatant

Sera levels of BAFF was found to be significantly elevated in APS-1 patients compared to healthy controls. This finding is in concordance with previous studies of BAFF in sera in APS-

1 patients[68,105]. BAFF has also shown elevated sera levels in patients suffering from other autoimmune diseases, such as SLE, SS, and rheumatoid arthritis, which indicates that BAFF plays an essential role in the immune system and could be used as a new possible approach for the treatment of patients suffering from APS-1[55-57]. Belimumab, an anti-BAFF monoclonal antibody, neutralize soluble BAFF and has shown to reduce the number of circulating naïve B cells in SLE patients. Treatments with other anti-BAFF antibodies such as blisibimod and tabalumab are also being investigated for treatment of SLE patients. The difference between these two and belimumab is that the latter can only bind to soluble BAFF, while the other two can bind to membrane-bound and soluble BAFF[59]. Since the levels of BAFF in APS-1 patients' sera are elevated such as in SLE, these treatments might be good alternatives to consider for possible treatment of APS-1 patients as well.

In contrast to BAFF, APRIL has not been researched enough in APS-1 patients. However, reports from other autoimmune diseases, such as SLE, have shown significant upregulation of APRIL levels in sera in patients[106]. Studies revealed that soluble APRIL, together with BAFF, can form active BAFF/APRIL heterotrimer complexes that will circulate in sera of patients with rheumatic diseases. It was also shown that the levels of BAFF/APRIL heterotrimer were elevated in patients suffering from SLE and systemic immune-based rheumatic diseases[107,108], which may contribute to the findings of elevated levels of these two ligands in these autoimmune diseases.

One study that measured APRIL in sera in APS-1 patients, revealed no major deviation in the levels of APRIL compared to the controls. The study stated that they used 17 APS-1 patients and matched controls for the ELISA assays. However, by looking at the graph in the study it seemed as there were only 10 patients that were detected with ELISA[105]. In the present study, 51 APS-1 patients and 40 healthy controls were subjected to ELISA, which revealed a significant downregulation of APRIL levels in sera in APS-1 patients, and not an upregulation such as previously showed in other autoimmune diseases. Considering that every autoimmune disease is different, and that the levels of APRIL have found to be elevated in patients suffering from other autoimmune diseases, does not automatically mean that the same is expected in APS-1 patients. This may possibly be a way to separate APS-1 patients from other autoimmune diseases. The significant difference in APS-1 patients compared to healthy controls indicates that APRIL may also be used as a possible approach for treatment of APS-1 such as BAFF. Atacicept, a recombinant fusion protein, has been shown to neutralizes BAFF and APRIL

activity in patients with SLE and rheumatoid arthritis by binding to them, this includes the BAFF/APRIL heterotrimers as well[60]. This treatment, like the other treatments mentioned previously, have not been studied in patients suffering from APS-1. Since a significant difference was found for APRIL in sera, this should also be considered as an alternative for a possible treatment or diagnosis for APS-1 patients.

Correlation analysis between age and the concentrations of BAFF and APRIL in sera revealed no clear deviation from either APS-1 patients or healthy controls. A study done on patients with common variable immunodeficiency (CVID) revealed that there was an inversely correlation between age and BAFF and APRIL in healthy controls, but not in CVID patients[109]. This observation was not confirmed in our healthy control population.

Levels of both BAFF and APRIL in supernatant from *in vitro* stimulated B cells showed no difference between the APS-1 patients and controls. However, it is important to take into consideration that this was done with APS-1 few patients and controls, and the cells did not divide and expand as expected. Another aspect to consider when interpreting the results is that other cell types also produce BAFF and APRIL, such as monocytes, macrophages and dendritic cells[110,111]. In contrast to the assays done with sera, these other cell types were not included in this assay, due to the B cells being isolated before being cultured. This may be an explanation for BAFF and APRIL showing no difference in the levels in supernatant.

In sera, EBI3, BAFF-R and IL-10 were within the assay limits in some samples, but this was still insufficient to see any significant differences and also unreliable. Indication of a downregulation trend of BAFF-R and IL-10 may be argued, but due to the low number of samples detected, this cannot be determined. A high-sensitivity ELISA kit for IL-10 (IL-10 HS) was also performed to increase sensitivity. Since sera from APS-1 patients is very valuable due to APS-1 being a very rare disease, the IL-10 HS ELISA assay was done for the controls to check if the levels would be detectable. This was not successful as only 8 out of 40 controls were detected and therefore IL-10 HS ELISA assay was not performed for the APS-1 patients. The IL10- HS kit was also used for APS-1 patients and controls measuring the IL 10 levels in the supernatant after cell expansion, but again, no samples were detected, indicating too low concentrations.

In supernatant, IL-10, EBI3 and BAFF-R were all found at too low concentrations to be within the assay limits, while TACI was only detected in one APS-1 patient and one matched control. Interestingly, these were the oldest individuals that were analyzed, the APS-1 patient was 62 years old, and the control was 69 years old. There is an age-gap of 13 years to the next oldest APS-1 patient (49 years). This may indicate that age could play a part in the secretion of TACI. However, no previous studies have researched if there is in fact an inversely correlation between age and secretion of TACI in supernatant. Only five APS-1 patients and healthy controls were included for the assays with supernatant, which may be insufficient to notice a significant difference. Although there was a clear difference between the one sex- and age-matched APS-1 patient and healthy control in the TACI assay. One may argue that this difference could have reached significance if there were more samples subjected to the assay.

Levels of TACI and BCMA in sera were equal between patients and controls. No previous studies on TACI and BCMA in APS-1 patients has been done, but one study revealed elevated levels of BCMA in sera in patients with myeloma, which is a blood cancer that develops from plasma cells in the bone marrow[112]. BCMA is expressed in various malignant plasma cells, which would explain the elevated levels of BCMA in myeloma patients investigated in the study[113]. In supernatant, BCMA was equal between APS-1 patients and controls. Further studies of both BCMA and TACI in supernatant and sera in patients suffering from APS-1 needs to be explored, in order to understand the role they play in the disease.

5.2.2 Protein Expression *versus* Gene Expression

As mentioned previously, the BCMA levels in both sera and supernatant were equal between patients and controls. However, the gene expression of *BCMA* was the only factor investigated that revealed a significant decreased expression in APS-1 patients compared to controls. No previous studies on gene expression of *BCMA* in APS-1 patients has been done, but various studies in myeloma have revealed elevated levels of *BCMA* expression[113-115]. The fold-change in APS-1 patients were lower than healthy controls, which may indicate that the differentiation into plasma cells could be affected in APS-1 patients. This would also explain the distinct decrease of memory cells seen in cell culture in APS-1 patients. This is in concordance with another study that have also observed that APS-1 patients had a reduction of total B cells and switched memory B cells[116].

Gene expression of other B cell factors investigated did not show any major deviation between APS-1 patients and healthy controls. BAFF revealed significantly elevated levels in sera, while it was slightly decreased in gene expression. APRIL on the other hand, revealed significantly lower levels in sera and slightly increased in gene expression. RNA levels and protein levels are not always the same, as all RNA does not always translate into a functional protein. Even though the gene is expressed, errors during for instance splicing can occur, which will lead to a non-functional protein, or a protein that has another function than the expected function[117]. This could be the reason for BAFF and APRIL having up- and downregulation in sera, respectively, while the gene expression revealed the opposite.

5.3 PHENOTYPICAL CHARACTERIZATION OF B CELLS

The number of B cells in blood in general is lower than T cells (B cells account for 5-10% of all cells among all peripheral blood cells)[24], which makes it all the more important to culture and expand B cells for downstream experiments. Flow cytometry was done both after B cell extraction and after seven days in culture, in order to establish the total number of B cells upon expansion as well as their phenotypic changes during cell culture. Optimalization with controls were done to establish the method. Here, the cells were cultured for 14 days where the cells were re-stimulated and counted on day 7. This revealed that the number of live B cells increased on day 7, however, on day 14 the number of live B cells started to decrease. Therefore, it was decided to culture the cells for seven days and not 14 days as the protocol recommended. When the experiments with APS-1 patients and controls started, some problems occurred during the isolation and expansion of the B cells. Cell death was observed in the B cells cultures from both APS-1 patients and controls, and they did not seem to expand and divide, especially the B cells from APS-1 patients. This suggests that the B cells from the controls managed better in culture. We do not know if this is due to cell intrinsic factors or factors within the cell culture, but equal conditions were used in the culture of patient and control B cells, as well as equal amount of cells were plated. Crosslinking of the B cell receptor is important for B cell survival, and although the medium used contained IL4, CD40L, and a crosslink antibody, which plays an essential role in activation, differentiation and proliferation B cells[118], the B cells did not divide or expand. One explanation might be that the crosslinking was not fully successful and therefore led to cell death, which has been shown in a study previously[119].

In general, the controls had more B cells than the APS-1 patients considering the starting cell number and how many B cells were successfully isolated from PBMC. This is shown specifically when looking at the sex- and age-matched APS-1 patient and control 5 (C-PBMC5 and P-PBMC5). C-PBMC5 started with $12*10^6$ PBMCs and 460.000 B cells were successfully isolated, whereas P-PBMC5 started with almost the double the amount of cells ($20*10^6$ cells) but only 135.000 B cells were successfully isolated, which is less than half of the amount that were successfully isolated from C-PBMC5.

When comparing the APS-1 patients and controls on day 7 the data must be interpreted with care as there were few live B cells. There was no major deviation between the two conditions, but perhaps if the culturing of the B cells were more successful, it would have been a significant difference between APS-1 patient and control. However, there is a clear trend towards more IgM cells and DN cells in APS-1 patients compared to controls, while there were less naïve cells in APS-1 patients compared to controls. The percentage of memory cells in both APS-1 patients and controls were close to equal. The flow cytometric analysis showed two clear populations of DN and naïve cells for controls on day 7, while when comparing the controls to the APS-1 patients, there was only one clear population of DN cells for the APS-1 patients. A previous study revealed that DN cells is associated with renal impairment in SLE, where the patients had significantly elevated levels of DN B cells[120].

The differences in the cell population at day 0 and day 7 was only analyzed for the controls, as most of the APS-1 patients did not have enough live B cells at day 0 for both cell culture and flow analysis. The comparison of day 0 and day 7 for the controls revealed a distinct trend toward less IgM cells and memory cells, however it did not reach significance. This is most likely due to the low number of samples included in this experiment, and one may argue that if there were more samples included, that there probably would have been a significant difference.

For unknown reasons a majority of B cells died in culture. One might assume that this is due to the CFSE staining used to determine the cell proliferation of the B cells, as this is slightly toxic to the cells[121]. The flow cytometric analysis also showed that a few of the B cells divided once, whereas the majority the B cells did not divide at all. When new medium, interleukins, and CD19 beads were tested, it still resulted in low number of B cell and increase in cell death compared to the first optimalization. In the lab where this study was conducted, CFSE has been used for several different subsets, and some have also experienced the same issues with their

cells. Another explanation is that the B cells were marked with CD19 beads before being cultured. These beads are still bound to the B cells in the culture and might cause an interference so that for instance CD40L cannot bind. However, there are methods to avoid this, such as either removing the CD19 beads after B cell isolation or untouched B cell kits, where all the other cells get marked instead of the B cells, which leaves them untouched.

5.4 CONCLUSIONS

In-depth knowledge about different key B cell factors in APS-1 is needed to further elucidate the role they have in APS-1 and if they could be used as a way to diagnose or treat APS-1 patients. This study focused on investigating how the B cell responded upon activation, as well as examining the levels of important B cell factors in both sera and cell culture supernatant from APS-1 patients compared to healthy controls.

To summarize, a significant difference of BAFF and APRIL in sera was found, where APS-1 patients had elevated levels of BAFF and lowered levels of APRIL compared to healthy controls. These findings indicate that these two could be good candidates for a possible new way of targeted treatment for APS-1 patients. The gene expression of *BCMA* revealed a decreased expression in APS-1 patients compared to controls, which may affect the differentiation into memory and plasma cells in APS-1 patients.

Activation and expansion of B cell *in vitro* had some challenges due to undisclosed reasons, however the CFSE staining of the cells remains as the main hypothesis. Further optimization is needed to make this a viable approach for future studies of B cells. Flow cytometric analysis gave insight to the phenotype of the live B cell after culturing. However, this must be interpreted with caution due to the low number of B cells and the low number of APS-1 patients included in the analysis. As already emphasized, APS-1 is a very rare disease, which means that obtaining blood samples, PBMC and sera samples from APS-1 patients is challenging in itself.

5.5 FUTURE PERSPECTIVES

Although a rare disease, APS-1 is a good model disease to study immunological tolerance and might hold information that will be transferable to other endocrine autoimmune diseases. AIRE is central in the development of T cells and to gain better knowledge about AIRE and its functions, it would be beneficial to obtain a crystal structure of the protein. There is however a

crystal structure of plant homeodomain 1 (PHD1) in *AIRE*[122], but not the whole gene. This would be an important aspect to work towards in the future in order to gain a better understanding of how AIRE works and to gain insight into how the different mutations affect the function of the protein.

Further optimization is needed for activation and expansion of B cells *in vitro* in order to get a more sufficient result. As crosstalk between B and T cells is important for B cell survival[118], a co-culture system with other immune cells could aid in the proper activation and expansion of the B cells. To establish why there was so much cell death in the cultures, levels of apoptosis markers could have been measured using qPCR or by flow cytometry. To avoid expanding the number of B cells, approaches like single cell analysis have the benefit of reduced sample volumes, which can be useful and well suited for this type of study, where there are few B cells and few patients.

Gaining more knowledge about the key B cell factors and the role that they play in APS-1 is critical for the development of future treatments and overall understanding of autoimmune diseases. The current study was performed using whole blood, serum or blood cells, and access to secondary lymphoid tissue or tissue where the autoimmune process is ongoing would likely give a more correct picture of the autoimmune reaction. This is however difficult to organize as there are few patients and many of these tissues are not easily available. Prioritizing further studies on B cells is imperative, as is characterizing of the cells in the different stages of activation as well as what happens to the B cells when administering drugs to knockdown or increase different factors.

To this day, diagnosis of APS-1 patients is determined by the presence of two out of the three hallmark manifestations. The treatment of the patients is challenging as they have many different manifestations and symptoms, and the treatments strategies have not evolved much since the 1950s. Research on APS-1 is imperative to obtain better treatment for the APS-1 patients as well as to implement these findings into other more common autoimmune diseases.

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7. SUPPLEMENTARY DATA

7.1 VERIFICATION OF *AIRE* MUTATIONS IN APS-1 PATIENTS

The PCR product was verified on a 2% agarose gel before subjected to Sanger sequencing.

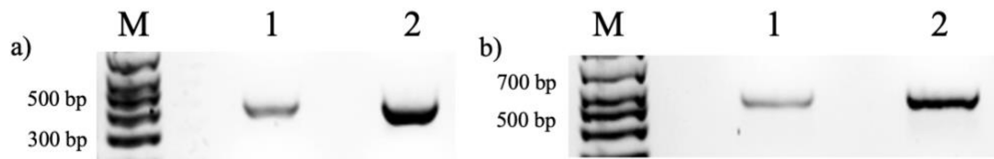

Figure 7.1 – Verification of the PCR product using agarose gel electrophoresis. The PCR product was analyzed by electrophoresis in a 2% agarose gel to verify that there in fact was a PCR product before being subjected to Sanger sequencing. The DNA ladder (M) is indicated by the base pair (bp) size of selected fragments. a) Lane 1 shows an APS-1 patient with mutation in exon 7, while Lane 2 shows exon 7 of a healthy control. b) Lane 1 shows an APS-1 patient with mutation in exon 6, while Lane 2 shows exon 7 of a healthy control. Image taken with Gel Doc EZ Imager and analyzed using ImageLab 3.0.

7.2 RNA ISOLATION

RNA isolation was performed using the PAXgene Blood RNA kit from Qiagen by following the manufacturer´s protocol.

Table 7.1: Isolated RNA from 10 healthy controls, indicating the concentration, 260/280 value, 260/230 value, sex and age for each control.

Control no.	**Sex**	**Age**	**Concentration (ng/µl)**	**260/280**	**260/230**
C-RNA1	F	29	92.2	2.14	1.83
C-RNA2	M	59	74.7	2.07	1.13
C-RNA3	F	22	118.5	2.10	1.57
C-RNA4	F	46	110	2.14	2.04
C-RNA5	F	25	112.9	2.15	0.76
C-RNA6	F	31	126.2	2.11	1.77
C-RNA7	F	28	110.9	2.17	1.96
C-RNA8	M	46	58.3	2.14	0.71
C-RNA9	M	62	63.5	2.11	1.25
C-RNA10	M	43	69.0	2.07	1.79

Table 7.2: Isolated RNA from 11 APS-1 patients. indicating the concentration, 260/280 value, 260/230 value, sex and age for each APS-1 patient.

Patient no.	Sex	Age	Concentration (ng/µl)	260/280	260/230
P-RNA1	F	52	197.1	2.13	2.05
P-RNA2	F	47	114.1	2.16	2.19
P-RNA3	M	56	114.7	2.14	1.15
P-RNA4	M	46	44.3	2.16	1.76
P-RNA5	F	31	75.8	2.20	2.04
P-RNA6	M	35	120.1	2.12	1.60
P-RNA7	F	63	130.3	2.13	1.31
P-RNA8	M	60	15.9	2.39	0.23
P-RNA9	M	76	60.1	2.08	1.96
P-RNA10	F	50	242.8	2.14	1.75
P-RNA11	M	36	105.3	2.22	2.14

7.3 CONCENTRATIONS FROM ELISA ANALYSIS WITH SERA

ELISA analysis was performed in order to determine the concentrations in sera in both APS-1 patients and healthy controls.

Table 7.3: Raw data from ELISA shown for BAFF, APRIL, TACI and BCMA using sera from healthy controls, which was used to create Figure 4.3 in Results.

Control no.	Concentration (pg/mL) BAFF	Concentration (pg/mL) APRIL	Concentration (pg/mL) TACI	Concentration (pg/mL) BCMA
C-ELISA1	942.54	2529.244	261.639	27.935
C-ELISA2	896.15	1323.731	58.841	20.556
C-ELISA3	914.995	3006.033	170.160	18.516
C-ELISA4	1105.857	1309.744	263.980	33.492
C-ELISA5	1185.246	2274.671	513.891	35.621
C-ELISA6	1064.666	1879.143	115.117	28.434
C-ELISA7	765.612	8399.810	-	160.957
C-ELISA8	744.932	1565.551	-	176.038
C-ELISA9	855.957	1003.236	511.030	32.221
C-ELISA10	930.719	1676.550	-	19.151
C-ELISA11	778.061	3404.097	244.768	27.689
C-ELISA12	881.256	1340.010	1325.757	35.521

C-ELISA13	868.403	2196.466	58.889	31.633
C-ELISA14	1194.037	1402.969	143.857	17.824
C-ELISA15	860.824	988.855	86.441	27.777
C-ELISA16	795.097	1044.851	132.255	25.394
C-ELISA17	787.661	440.550	67.500	26.053
C-ELISA18	872.733	1737.642	91.506	29.629
C-ELISA19	833.655	2324.926	531.337	50.561
C-ELISA20	904.296	3234.409	1585.587	172.117
C-ELISA21	614.287	704.080	136.557	33.371
C-ELISA22	697.209	2303.893	247.584	25.755
C-ELISA23	772.349	2037.967	79.533	28.392
C-ELISA24	680.621	511.690	-	29.384
C-ELISA25	560.498	3543.618	1343.563	60.539
C-ELISA26	905.652	2344.954	58.388	25.031
C-ELISA27	856.291	9718.402	-	179.289
C-ELISA28	565.490	9649.469	-	238.870
C-ELISA29	697.651	3733.001	203.665	32.923
C-ELISA30	587.267	2633.245	92.124	27.913
C-ELISA31	832.733	1084.174	-	19.934
C-ELISA32	771.646	9114.815	-	278.607
C-ELISA33	670.604	1168.679	-	27.218
C-ELISA34	745.113	1451.215	168.074	40.635
C-ELISA35	558.034	761.801	118.469	41.619
C-ELISA36	621.993	1199.207	-	48.450
C-ELISA37	854.513	930.803	691.458	58.932
C-ELISA38	694.316	715.251	-	32.941
C-ELISA39	657.416	370.189	225.267	63.174
C-ELISA40	874.787	699.251	1628.327	83.236

Table 7.4: Raw data from ELISA shown for BAFF-R, IL-10 HS, EBI3 and IL-10 using sera from healthy controls, which was used to create Figure 7.2. Only 20 healthy controls were subjected to the EBI3 and IL-10 analysis.

Control no.	**Concentration (pg/mL) BAFF-R**	**Concentration (pg/mL) IL-10 HS**	**Concentration (pg/mL) EBI3**	**Concentration (pg/mL) IL-10**
C-ELISA2	-	-	-	18.490
C-ELISA3	-	-	-	6.339
C-ELISA4	-	-	-	11.322
C-ELISA7	10854.408	-	-	44.621
C-ELISA8	1611.715	1.213	-	12.775
C-ELISA9	-	-	-	0.987
C-ELISA11	-	7.204	-	0.239
C-ELISA12	-	1.862	-	-
C-ELISA13	-	2.821	-	-
C-ELISA15	-	-	-	49.643
C-ELISA16	-	2.372	-	-
C-ELISA18	-	-	495.907	-
C-ELISA19	-	-	-	-
C-ELISA20	-	-	-	7.535
C-ELISA21	-	82.162	*	*
C-ELISA27	-	0.894	*	*
C-ELISA28	204.215	-	*	*
C-ELISA31	-	1.777	*	*

* Not run due to many samples out of range

Table 7.5: Raw data from ELISA shown for BAFF, APRIL, TACI and BCMA using sera from APS-1 patients, which was used to create Figure 4.3 in Results.

Patient no.	**Concentration (pg/mL) BAFF**	**Concentration (pg/mL) APRIL**	**Concentration (pg/mL) TACI**	**Concentration (pg/mL) BCMA**
P-ELISA1	1288.763	563.071	409.637	**
P-ELISA2	575.984	1513.040	1849.866	67.989
P-ELISA3	825.385	666.117	829.773	54.542
P-ELISA4	959.845	1203.503	26.254	24.876
P-ELISA5	1158.645	1710.888	639.891	40.701
P-ELISA6	863.328	761.197	266.925	54.982
P-ELISA7	774.137	10315.370	2445.348	79.635
P-ELISA8	698.262	-	**	**
P-ELISA9	1425.135	515.519	464.976	37.891
P-ELISA10	860.455	900.493	**	44.102
P-ELISA11	1247.192	1707.534	533.225	33.047
P-ELISA12	1026.138	148.131	410.466	50.174
P-ELISA13	1191.529	388.944	**	44.768
P-ELISA14	790.365	1512.604	199.576	33.983
P-ELISA15	4240.626	1367.731	-	18.053
P-ELISA16	1632.330	945.997	311.049	33.536
P-ELISA17	1066.761	3469.641	425.561	50.954
P-ELISA18	1185.933	3531.743	11044.979	114.210
P-ELISA19	1188.451	2448.661	2291.546	66.477
P-ELISA20	2315.661	1559.421	502.292	39.164
P-ELISA21	612.710	663.377	262.008	36.611
P-ELISA22	959.548	1275.673	225.730	48.908
P-ELISA23	810.361	1487.696	214.904	32.056
P-ELISA24	999.837	819.622	564.249	64.352
P-ELISA25	876.965	1880.228	210.085	33.195
P-ELISA26	908.442	1540.397	432.020	40.754
P-ELISA27	583.929	1135.349	410.079	40.125
P-ELISA28	678.987	1288.712	236.080	48.604
P-ELISA29	867.518	808.852	234.803	45.699
P-ELISA30	**	748.923	1106.162	58.863
P-ELISA31	1054.938	1356.475	273.442	33.625

P-ELISA32	1648.607	1247.892	114.945	51.452
P-ELISA33	858.659	1281.185	225.471	58.805
P-ELISA34	615.963	749.386	271.846	48.762
P-ELISA35	788.032	2039.722	63.467	38.249
P-ELISA36	1621.124	812.464	202.865	53.547
P-ELISA37	1046.562	644.234	406.629	47.029
P-ELISA38	766.828	6144.603	14936.614	94.434
P-ELISA39	1231.339	1489.875	729.761	50.666
P-ELISA40	854.637	521.847	172.593	38.062
P-ELISA41	1180.867	987.649	490.161	47.419
P-ELISA42	678.726	479.505	294.595	70.657
P-ELISA43	701.795	590.595	567.430	39.765
P-ELISA44	735.922	409.613	325.118	77.424
P-ELISA45	747.428	823.998	596.308	67.269
P-ELISA46	728.842	1811.136	1108.033	40.378
P-ELISA47	853.919	2458.781	265.499	43.760
P-ELISA48	575.450	1750.026	402.016	39.218
P-ELISA49	723.769	1242.026	80.813	20.365
P-ELISA50	866.917	1527.330	535.393	42.449
P-ELISA51	584.420	801.173	1956.138	139.634
P-ELISA52	149.899	948.479	471.497	46.733
P-ELISA53	1198.817	569.307	420.021	30.502
P-ELISA54	613.159	1013.503	217.723	52.534

** Not run due to lack of sample

Table 7.6: Raw data from ELISA shown for BAFF-R, EBI3 and IL-10 using sera from APS-1 patients, which was used to create Figure 7.2. Only 20 APS-1 patients were subjected to the EBI3 and IL-10 analysis.

Patient no.	**Concentration (pg/mL) BAFF-R**	**Concentration (pg/mL) EBI3**	**Concentration (pg/mL) IL-10**
P-ELISA1	-	-	15.252
P-ELISA2	-	-	8.423
P-ELISA3	-	112.391	-
P-ELISA4	-	-	6.916
P-ELISA5	-	310.391	-
P-ELISA6	-	-	10.394
P-ELISA7	-	44.412	-
P-ELISA8	-	90.806	-
P-ELISA9	-	-	1.776
P-ELISA11	-	-	15.362
P-ELISA12	-	1263.980	-
P-ELISA13	-	-	9.147
P-ELISA14	-	-	15.406
P-ELISA15	-	-	3.993
P-ELISA16	-	100.591	-
P-ELISA17	-	-	2.186
P-ELISA18	278.582	-	13.433
P-ELISA19	-	-	8.134
P-ELISA20	46.328	-	6.426
P-ELISA34	46.328	-	-
P-ELISA39	42.645	-	-

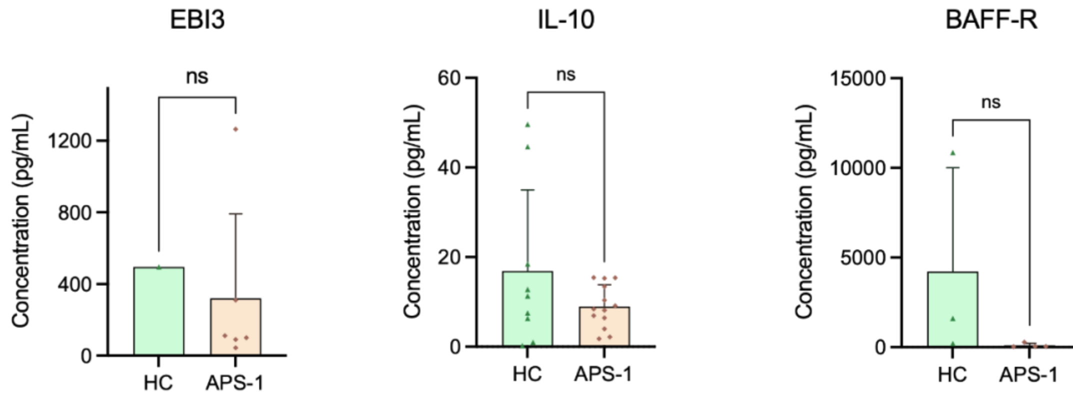

Figure 7.2 – Concentrations of the different B cell factors in sera from APS-1 patients and healthy controls. The 96-well plate was coated with capture antibody before adding the samples, subsequently incubated with detection antibody, and afterwards with substrate solution. The concentrations were acquired using SoftMax Pro 7 software, by measuring the absorbance at 450-490 nm to 570-690 nm, depending on the ELISA kit. Each data point represents one individual APS-1 patient or healthy control. EBI3 (APS-1=320.4 ± 471.4, HC=495.9 ± 0, P value: not determined due to only one HC sample detected), IL-10 (APS-1=8.988 ± 4.813, HC=16.88 ± 18.10, P value: 0.146) and BAFF-R (APS-1=100.4 ± 118.9, HC=4223 ± 5786, P value: 0.200) was shown to be non-significant, but there was many samples with too low concentrations that were not detected, making this result insufficient. Error bars show the SD, and the P value was calculated by using the unpaired t test in GraphPad Prism 9.2.0.

7.4 FUNCTIONAL CHARACTERIZATION OF B CELLS

7.4.1 Isolation of PBMC from APS-1 patients and controls

PBMC was isolated from blood that were obtained from healthy controls from the blood bank at Haukeland University Hospital.

Table 7.7: Isolation of PBMC from nine healthy controls, indicating the sex, age and number of cells isolated. Only five controls (C-PMBC1 - C-PMBC5) that were sex- and age-matched with APS-1 patients were used for cell culture.

Control no.	Sex	Age	Number of cells
C-PBMC1	F	69	$4.7*10^6$
C-PBMC2	M	39	$2.0*10^6$
C-PBMC3	M	29	$4.9*10^6$
C-PBMC4	F	24	$5.8*10^6$
C-PBMC5	M	33	$2.5*10^6$
C-PBMC6	M	71	$5.7*10^6$
C-PBMC7	-	-	$4.0*10^6$
C-PBMC8	F	18	$4.3*10^6$
C-PBMC9	F	22	$4.5*10^6$

7.4.2 Cell Culture Plating

The manufactures protocol showed how many cells should be added to the appropriate culture plate with the appropriate amount of medium.

Table 7.8: Culture plate sizes suitable for different cell numbers and appropriate amounts of medium to be added is indicated as well.

Total cell number (high density)	Total cell number (low density)	Final medium volume	Culture plate
$0.5x10^6$	$0.075x10^6$	0.5 mL	48 well
$1.0x10^6$	$0.15x10^6$	1 mL	24 well
$2.0x10^6$	$0.3x10^6$	2 mL	12 well
$4.0x10^6$	$0.6x10^6$	4 mL	6 well

7.4.3 Raw Data from Flow Cytometry

Raw data from flow cytometry for healthy controls (unstained and stained) and APS-1 patients (stained) from before after culturing.

Table 7.9: Raw data from flow cytometry shown for the five healthy controls on day 0 (stained), indicating the percentage of single B cells, live B cells and B cell subsets.

Stained day 0						
Control number	**All single cells**	**Live**	**DN**	**IgM only**	**Naive**	**Unswitched**
C-PBMC1	94.5 %	25.3 %	12.9 %	12.3 %	23.2 %	53.0 %
C-PBMC2	90.6 %	15.0 %	32.6 %	9.8 %	1.8 %	54.3 %
C-PBMC3	92.8 %	4.8 %	66.3 %	5.6 %	10.5 %	16.8 %
C-PBMC4	95.4 %	3.3 %	31.2 %	16.0 %	9.3 %	44.4 %
C-PBMC5	94.9 %	3.9 %	24.4 %	31.8 %	8.0 %	36.2 %

Table 7.10: Raw data from flow cytometry shown for the one healthy control on day 0 (unstained), indicating the percentage of single B cells, live B cells and B cell subsets.

Unstained day 0						
Control number	**All single cells**	**Live**	**DN**	**IgM only**	**Naive**	**Unswitched**
C-PBMC5	93.60 %	4.17 %	75.60 %	19.80 %	0 %	0 %

Table 7.11: Raw data from flow cytometry shown for the five healthy controls on day 7 (stained), indicating the percentage of single B cells, live B cells and B cell subsets.

Stained day 7						
Control number	**All single cells**	**Live**	**DN**	**IgM only**	**Naive**	**Unswitched**
C-PBMC1	85.2 %	37.0 %	37.2 %	0.2 %	59.6 %	3.0 %
C-PBMC2	91.0 %	3.0 %	84.0 %	1.3 %	9.2 %	1.4 %
C-PBMC3	93.5 %	2.2 %	53.1 %	3.6 %	20.6 %	2.1 %
C-PBMC4	95.7 %	3.5 %	21.2 %	0.5 %	5.5 %	0.9 %
C-PBMC5	92.0 %	7.1 %	33.9 %	1.1 %	21.5 %	1.6 %

Table 7.12: Raw data from flow cytometry shown for three of the APS-1 patients on day 0 (stained), indicating the percentage of single B cells, live B cells and B cell subsets.

Stained day 0						
Patient number	**All single cells**	**Live**	**DN**	**IgM only**	**Naive**	**Unswitched**
P-PBMC1	-	-	-	-	-	-
P-PBMC2	79.5 %	19.3 %	85.6 %	2.8 %	3.7 %	7.7 %
P-PBMC3	-	-	-	-	-	-
P-PBMC4	74.7 %	25.2 %	87.6 %	6.0 %	2.0 %	4.8 %
P-PBMC5	91.1 %	8.8 %	26.2 %	2.1 %	21.6 %	48.6 %

Table 7.13: Raw data from flow cytometry shown for the five APS-1 patients on day 7 (stained), indicating the percentage of single B cells, live B cells and B cell subsets.

Stained day 7						
Patient number	**All single cells**	**Live**	**DN**	**IgM only**	**Naive**	**Unswitched**
P-PBMC1	83.6 %	12.6 %	85.5 %	2.1 %	10.3 %	1.9 %
P-PBMC2	88.5 %	3.9 %	91.9 %	3.0 %	1.1 %	1.9 %
P-PBMC3	92.3 %	2.5 %	83.3 %	2.9 %	10.1 %	2.8 %
P-PBMC4	91.2 %	6.3 %	82.3 %	1.7 %	1.0 %	0.5 %
P-PBMC5	91.5 %	1.6 %	73.4 %	2.9 %	8.6 %	1.7 %

7.4.4 ELISA Analysis after Cell Culture

ELISA analysis was performed in order to determine the concentrations in supernatant in both APS-1 patients and healthy controls.

Table 7.14: Raw data from ELISA shown for BAFF, APRIL, TACI and BCMA using supernatant from healthy controls, which was used to create Figure 4.10 in Results. The IL-10 HS and EBI3 analysis was also performed, however every sample had too low concentration, resulting in them not being detected.

Control no.	Concentration (pg/mL) BAFF	Concentration (pg/mL) APRIL	Concentration (pg/mL) TACI	Concentration (pg/mL) BCMA
C-PBMC1	70.991	12.827	290.888	6.709
C-PBMC2	72.036	12.827	-	6.634
C-PBMC3	81.397	12.352	-	7.513
C-PBMC4	211.533	14.008	-	10.689
C-PBMC5	65.662	13.949	-	11.000

Table 7.15: Raw data from ELISA shown for BAFF, APRIL, TACI and BCMA using supernatant from APS-1 patients, which was used to create Figure 4.10 in Results. The IL-10 HS and EBI3 analysis was also performed, however every sample had too low concentration, resulting in them not being detected.

Patient no.	Concentration (pg/mL) BAFF	Concentration (pg/mL) APRIL	Concentration (pg/mL) TACI	Concentration (pg/mL) BCMA
P-PBMC1	80.319	12.479	33.718	11.000
P-PBMC2	73.625	20.067	-	11.000
P-PBMC3	82.551	13.772	-	10.448
P-PBMC4	82.551	10.935	-	5.188
P-PBMC5	78.831	12.438	-	3.985

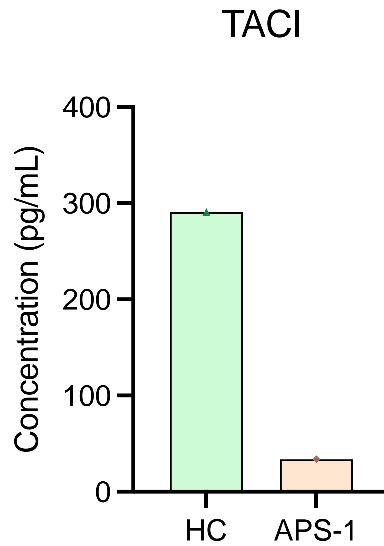

Figure 7.3 – Concentrations of TACI in supernatant from the one sex- and age-matched APS-1 patient and control. The 96-well plate was coated with capture antibody before adding the samples, subsequently incubated with detection antibody, and afterwards with substrate solution. The concentrations were acquired using SoftMax Pro 7 software by measuring the absorbance at 450-490 nm to 570-690 nm, depending on the ELISA kit. Statistical analysis for TACI (APS-1=33.72, HC=290.9) was not done due to only one APS-1 patient and one control being detected. Figure created in GraphPad Prism 9.2.0.