The *in vitro* effect of cytokine autoantibodies on Th17-differentiation and function

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Selective abbreviations

AB	Antibody
AIRE	Autoimmune regulator
APECED	Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy
APS-1	Autoimmune polyendocrine syndrome type 1
CD	Cluster of differentiation
CTLA-4	Cytotoxic T lymphocyte antigen-4
cTEC	Cortical thymic epithelial cell
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
FoxP3	Forkhead box P3
GATA3	Gata binding protein 3
IFN	Interferon
IL	Interleukin
mTEC	Medullary thymic epithelial cell
PBMC	Peripheral blood mononucleated cells
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PRR	Pattern recognition receptor
ROAS	Registry and biobank for organ specific autoimmune disorders
RORγt	Retinoic acid-related orphan receptor gamma t
STAT3	Signal transducer and activator of transcription3
T-bet	T-box protein expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor β
TRA	Tissue restricted antigen
Treg	Regulatory T cell
Th	Helper T cell

Summary

Autoimmune polyendocrine syndrome type 1 (APS-1) is a rare genetic disorder characterized by multi-organ autoimmunity and is caused by mutations in the autoimmune regulator (*AIRE*) gene. The impact and symptoms of APS-1 can differ among individuals, with varying degrees of severity leading to a decreased quality of life and a higher risk of premature death. In this autoimmune disorder, in addition to autoantibodies targeting specific tissues and organs, there is growing recognition of the presence of neutralizing autoantibodies against T helper 17 (Th17)-related cytokines including anti-IL-17, anti-IL-22, as well as type I IFNs antibodies that can possibly modulate Th17 cell differentiation and function. Dysregulation of these cytokines may contribute to the aberrant Th17 cell responses observed in APS-1 patients. Therefore, further research is needed to uncover the precise mechanisms involved and to explore potential therapeutic interventions aimed at restoring immune homeostasis in APS-1 disorder.

The development of Th17 cells, which play a crucial role in mucosal immunity against fungal infections like chronic mucocutaneous candidiasis (CMC), is initiated by the activation of innate immune responses triggered by *Candida albicans* through pattern recognition. The role of Th17 cells and their cytokines in human host defence against this fungus has been confirmed by in-depth studies of groups of patients with heightened vulnerability to CMC due to Th17 cell deficiency. This altered plasticity of Th17 cells can contribute to the susceptibility to chronic candidiasis in these individuals. These investigations have also provided a valuable understanding of the intricate mechanisms involved in Th17 cell development.

Although studies conducted in mice have greatly advanced our understanding of Th17 biology, elucidating the molecular mechanisms and signalling pathways utilizing simplified and controlled *in vitro* experimental settings could be less challenging and more informative for human biology. In this project, we aimed to study the effects of APS-1 cytokine autoantibodies on *in vitro* cultures of PBMCs and expanded Th17 cells from APS-1 patients and healthy controls. Functional and phenotypical analyses of cultured T cell subtypes were conducted using techniques such as flow cytometry, quantitative polymerase chain reaction (qPCR,) and enzyme-linked immunosorbent assay (ELISA). Although commercial kits were used for qPCR and ELISA, the cell culture conditions and parts of the flow cytometry panel needed to be optimized in the start of this project.

Overall, flow cytometry data indicated very limited changes in Th17 cell behavior in isolated CD4+ T cell cultures in response to commercial antibodies, which were further confirmed

through qPCR and ELISA analyses. From flow results of Th17 cultures after addition of healthy control (HC) HC and APS-1 sera and IgG trends towards reduced Th17 cell development was observed when exposed to sera, either from patients or controls. However, no differences were seen between the patients and controls.

When further examining the effects of heat killed *Candida albicans* (HKCA) in the presence or absence of HC and APS-1 sera/IgG on PBMCs derived from healthy individuals, a drop in the proliferation of Th17 cells within the CD4+ population due to HKCA addition was revealed, but with no large effects with the addition of sera/IgG. Similarly, Th17 cells within the CD4+ population of APS-1 patient PBMCs showed a non-significant effect of sera/IgG when exposed to HKCA. To ensure the validity and generalizability of these results, future studies should prioritize the inclusion of a larger cohorts of APS-1 patients, enabling more robust and reliable conclusions regarding the effects of APS-1 cytokine autoantibodies on Th17 cells in an *in vitro* setting.

1. Introduction

1.1. The immune system

The immune system is made up of an immense network of cells, tissues, and organs that cooperate to rid their host organism of potential threats from a variety of infections, tissue damage, and self-cancerous cells, and its coordinated reaction to infectious agents is known as an immunological response (Abbas and Lichtman 2009). As the initial line of defence in this process, the body's epithelial surfaces function as a physical and chemical barrier to avoid bacteria, viruses, parasites, and other foreign particles from easily entering the body. If microbes manage to breach the first-line physical defence, they come into contact with the cells and proteins of the unspecific innate immune branch that responds quickly to a wide variety of pathogens. The second line of immune defence, referred to as the adaptive immune system, being slower but capable of targeting specific pathogens, then comes into play (Parkin and Cohen 2001; Rogatsky and Adelman 2014; Marshall et al. 2018).

1.1.1 The innate immune system

This defence mechanism consists of physical barriers that serve as the interface between an organism and its environment and in addition, cellular and chemical elements such as white blood cells, complement proteins, and cytokines, all operate in concert to locate, neutralize, or kill pathogens. It is not necessary to be exposed to a specific pathogen in order for the innate system to function; it is capable of quickly and efficiently detecting foreign substances in an unspecific manner from birth. Defence cells of this rapid response system include macrophages, dendritic cells (DCs), granulocytes, natural killer (NK) cells, and innate lymphoid immune cells (ILCs); these are all classified as leukocytes. These immune cells are produced in the bone marrow and then spread throughout the body via blood and lymph flow. Macrophages and other innate immune system cells can immediately recognize pathogens through their pattern recognition receptors (PRRs) on the exterior of the cells. PRRs generally recognize pathogen-associated molecular patterns (PAMPs) that are specific conserved molecular motifs only present on microbes. Consequently, PPR engagement with PAMPs prompts a downstream signalling cascade that results in leukocyte recruitment, further driving inflammation (Newton and Dixit 2012; Bianchi 2007; Mogensen 2009).

1.1.2 The adaptive immune system

Unlike the innate immune system, the adaptive immune system requires extensive knowledge of the pathogenic agents to effectively eradicate a variety of pathogens. This immune branch is made up of white blood cells known as B and T cells that are originated from the hematopoietic stem cells in the bone marrow and circulate through the blood and lymphatic vessels. After an individual's immune system encounters foreign invaders and recognizes them as non-self (antigens), these adaptive immune cells elicit highly specific and targeted immune responses against that antigen. This type of response is acquired through antigen binding by the highly diverse antigen receptors on the respective cells, the B cell receptor (BCR) and the T cell receptor (TCR), which are specifically constructed and selected to act on their specific target. Both B and T-lymphocyte commitment originates from multipotent progenitors. B cells are in charge of making antibodies, which are proteins that can recognize and kill certain diseases brought on by a variety of pathogens. Additionally, when B cells come into contact with an unfamiliar antigen, they can get activated and differentiate into memory B cells, which then remain in circulation after an infection has been cleared, with the tendency to react quickly and strongly in the case of re-exposure (Hagman, Ramírez, and Lukin 2012; Bonilla and Oettgen 2010). T cells, the focus of our study, contrarily, have a more intricate role in the immune response (detailed in 1.2).

1.2 T cells

One of the key characteristics that set T cells apart from other lymphocytes is their expression of the TCR. T cells mature in the thymus, a specialized primary lymphoid organ located above the heart. Based on the expression of distinct effector molecules and signalling co-receptors, T cells can be categorized as helper/effector, cytotoxic, memory, regulatory, or gamma delta (γδ) T cells (Dong 2021). The cellular response is triggered by the exposure of naïve T cells to antigen-specific ligands and costimulatory molecules presented by antigen-presenting cells (APCs) like DCs. As a result, T cells proliferate and differentiate into effector cells prepared to assist in the eradication of pathogens. T lymphocytes are mostly located in lymphoid tissues, such as the spleen, bone marrow, tonsils, and lymph nodes, and in substantial levels in the mucosal sites, but can also be found in other organs and tissues throughout the body (Kumar, Connors, and Farber 2018).

1.2.1 T cell development and Maturation

During the individual's lifetime, common lymphoid progenitors with T lineage potential, migrate from the bone marrow to the thymus gland. Two lobes that make up the thymus, have distinct functions throughout the early stages of thymopoiesis. Each of these lobes separated into a cortex and a medulla, with the outer layer cortex plays a role in the differentiation of T cell lineages and the establishment of self- major histocompatibility complex (MHC)-restriction. MHC molecules, divided into two classes: MHC class I and MHC class II, play a crucial role in the immune system by presenting antigens to T cells and triggering the immune response. The inner layer medulla, on the other hand, is responsible for central tolerance induction and subsequent thymocyte maturation (overview in Fig 1.1). Thymus development and function are controlled by a variety of phenotypically and functionally different thymic epithelial cells (TECs) including cortical TECs (cTECs) and medullary TECs (mTECs), with the cTECs located in the outer cortex, while mTECs are located in the inner medulla (Boyd et al. 1993; Klein et al. 2014; Nitta et al. 2008). A population of double-negative (CD4-CD8-) (DN) cells are early thymocyte progenitors that migrate and expand towards certain thymus niches and do not express CD4 or CD8 proteins. These immature thymocytes go through several rounds of transitions of DN stages after interacting with cTECs that provide fundamental signals for their survival and development which results in the T-lineage commitment of immature thymocytes. Cytokines, chemokines, and adhesion molecules are expressed by TECs and interact with thymocyte progenitors. These signals aid in guiding early thymocyte progenitors' migration into the mid cortex where the β-chain locus is rearranged and paired with the α-chain locus to form and express the pre-T cell surface receptors (pre-TCRs) and maintain T-lineage specification and proliferation. Subsequently, the rapid rise in cell proliferation leads the cells to become double-positive (DP)(CD4+CD8+) thymocytes, soon ready to recognize their antigen and initiate an immunological response (Fig 1.1)(Klein et al. 2014; Koch and Radtke 2011).

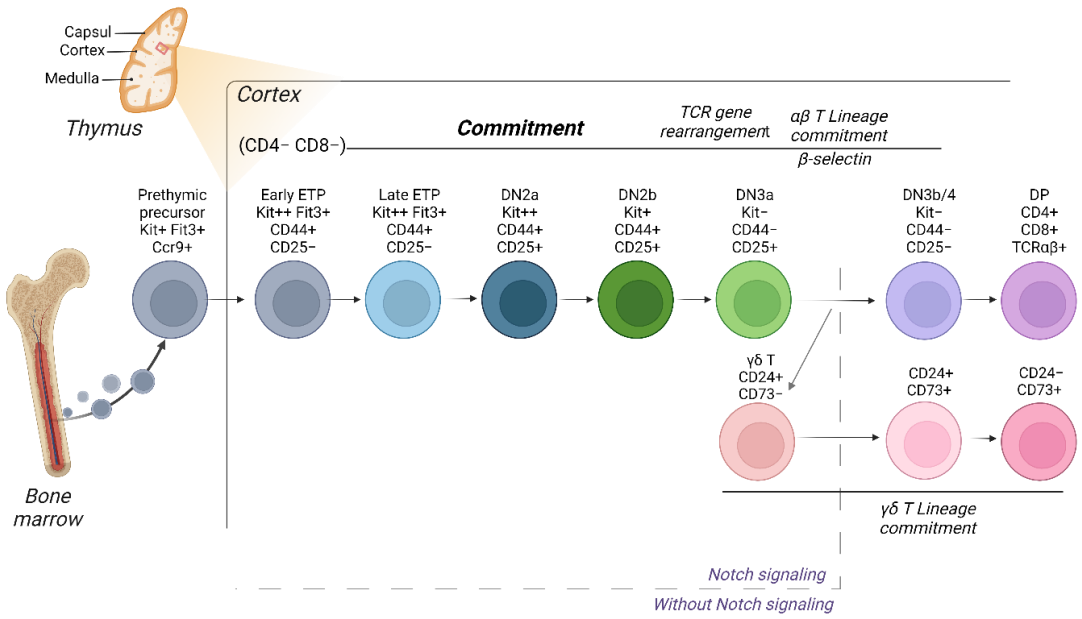

Figure 1.1 T cell development and maturation Primary thymocytes are classified as a double-negative (CD4-CD8-) (DN) cell. In early thymopoiesis, being guided by Notch1 signalling, thymocytes go through several rounds of transitions of DN stages. In this process, the β-chain locus is rearranged and paired with the α-chain locus to form and express the pre-T cell surface receptors (pre-TCRs) and trigger T cell proliferation. The rapid rise in cell proliferation leads the cells to acquire both CD4 and CD8 expression to become double-positive (DP)(CD4+CD8+) thymocytes. Notch signalling commits precursors to two distinct T-cell lineages (CD4 or CD8) and elicits the expression of genes required for the synthesis of the αβ or γδ chains of the T-cell receptor (TCR). Depending on the pre-TCR rearrangement, αβ, and γδ T cells are hypothesized to develop from a similar T cell precursor of late Double negative (DN)2 or DN3 thymocytes. The αβ versus γδ lineage diversion is determined by the TCR signal intensity rather than TCR isotypes. While a weak signal via the pre-TCR leads cells toward the αβ T cell lineage, a strong signal directs cells to enter the γδ T cell lineage. The figure was modified from (D'Acquisto and Crompton 2011) and created in Biorender.com.

1.2.2 Positive selection

Following T cell maturation and expression of a TCRαβ heterodimer, DP thymocytes that survive within the thymus migrate deep into the thymic cortex, where they encounter self-antigens that are complexed with MHC class I and class II proteins displayed on the surface of cTECs. Accordingly, double-positive T cells that fail the expression of TCRαβ or express receptors with a negligible affinity for self-antigens do not bind to self-MHC proteins and become eliminated (death by neglect). T cells that are bound with moderate avidity to the self-MHC proteins, transmit a survival signal and undergo positive selection that allows them to survive and mature (Klein et al. 2014). During this process, DP thymocytes that bind to MHC class II proteins lose the expression of CD8 cell surface receptors and become CD4+ helper single-positive cells while double-positive cells selected on MHC class I molecules mature into CD8+ cytotoxic single-positive cells by downregulating the expression of CD4 cell surface receptors (Fig 1.2). Thus, the cell-surface phenotype and functional potential of mature

thymocytes are determined in this step (Duong et al. 2019; Mayerova and Hogquist 2004; McCaughtry et al. 2012).

1.2.3 Thymic central tolerance (negative selection)

After being positively selected, T cells that are rescued from apoptosis move into the thymic medulla (Ross et al. 2014; Ehrlich et al. 2009; Witt et al. 2005). In the medulla, the medullary (m)TECs and thymic DCs together regulate the final stages of T cell maturation including central tolerance and induction of T regulatory cells (Tregs) (Fig 1.2). T cell tolerance is achieved to prevent autoimmunity by preserving immunological tolerance to self-antigens and efficiently removing foreign antigens. This occurs through the presentation of a wide variety of tissue-restricted self-antigens (TRAs), including those that are frequently expressed in distant organs, to developing T cells (Klein and Kyewski 2000). The thymic expression of TRA genes is facilitated by a transcriptional activator known as Autoimmune Regulator (*AIRE*), enabling the immune system to recognize and tolerate these antigens as self and lowering the risk of autoimmune reactions (Mathis and Benoist 2009). The negative selection of self-reactive T cells is triggered by the interaction of TCRs with self-peptides supplied by MHC molecules. During this process, high avidity binding of the self-reactive TCR of either CD4 or CD8 cells to self-antigens presented directly by MHC^{high} mTECs and by cross-presentation of DCs leads to the transmission of a strong cell death signal. This robust signal transduction leads to deletion of autoreactive T cells from the repertoire (Fig 1.2)(Aschenbrenner et al. 2007; Birnberg et al. 2008; Gallegos and Bevan 2004). However, it has been demonstrated that negative selection may also eradicate T cells with moderately avidity TCRs for self-antigens, albeit less successfully than T cells with highly avid TCRs. This is due to the induction of weaker signals generated by moderate interaction of these T cells with TRAs which is inadequate to trigger apoptosis. Weaker signals also lead thymocytes to either become mature or differentiate into Treg, a T-cell subset responsible for regulating or suppressing other autoreactive immune cells due to their moderate affinity towards self-antigens (Goverman 1999; Klein and Kyewski 2000; Mayerova and Hogquist 2004). In consequence, CD4+ and CD8+ single-positive cells reach their last stage of maturation in the thymic medulla after surviving both positive and negative selection, ready to leave the thymus and circulate as antigen- naïve mature T cells into the periphery (Xing and Hogquist 2012).

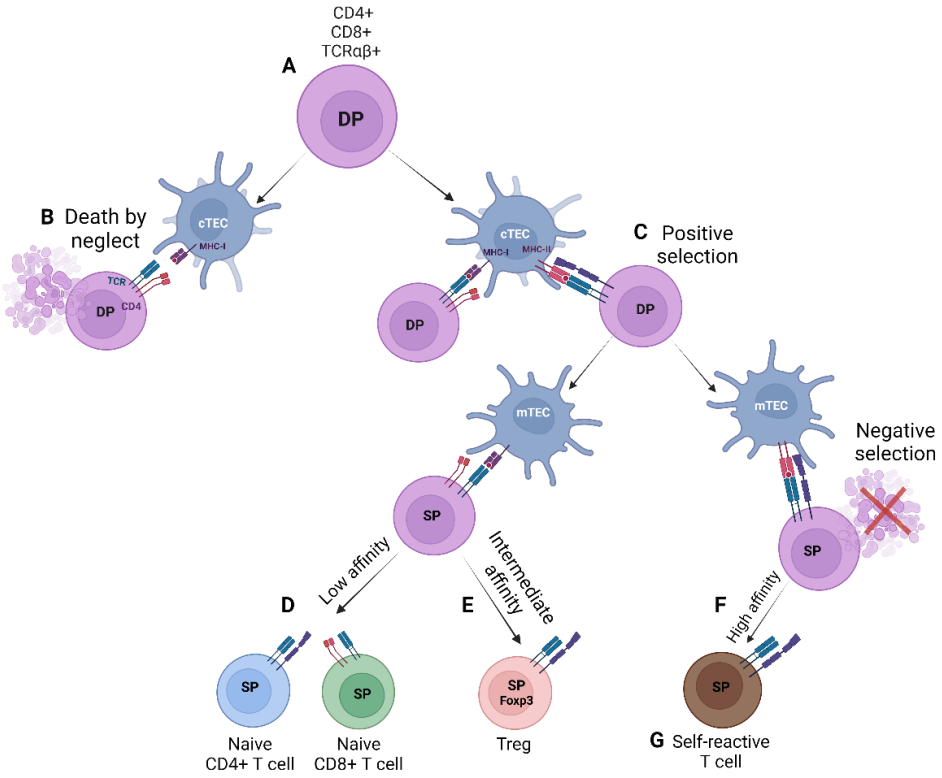

Figure 1.2. T cell development and central tolerance A) Double positive (DP) thymocytes that have developed into CD4+CD8+ precursor cells expressing a TCR and the CD4/CD8 co-receptors. **B**) DP thymocytes in the thymic cortex, with no affinity for self-MHC die by neglect, while **C**) the ones that exhibit TCR affinity towards self-MHC undergo a process where they downregulate either CD4 or CD8, becoming single positive (SP) cells, migrating to the thymic medulla. Medullary thymic epithelial cells (mTECs) expressing autoimmune regulator (*AIRE*) that enables them to express a broad range of self-antigens that are typically restricted to specific tissues, helps in educating and selecting T cells with self-tolerance. **D**) low affinity TCR interactions with self-antigens differentiate into naïve CD4+ or CD8+ T cells. **E**) Intermediate affinity interactions promote the generation of regulatory T cells (Tregs), while **F**) high affinity interactions with self-antigens indicate a higher likelihood of being self-reactive, and they are eliminated through apoptosis to prevent autoimmune responses. **G**) Some thymocytes may escape negative selection and enter the peripheral circulation. These cells, if they encounter cognate antigens in peripheral tissues, may contribute to autoimmune responses. The figure was created in Biorender.com.

1.2.4 Peripheral tolerance

The tolerance mechanisms of the thymus are not perfect, and some of the self-reactive T cells, will escape negative selection and emerge into the periphery as naïve functional T cells. Some of these are low-avidity autoreactive T cells and others have high avidity TCRs towards self-antigens that are not sufficiently expressed in mTECs. Peripheral mechanisms serve as a safety net to obtain and keep immune tolerance. The regulation of T cell clonal activation and establishment of peripheral tolerance depend on both negative and positive costimulatory signalling as well as TCR signalling. The TCR-DC engagement in the absence of co-stimulatory signalling induces an unresponsive state in T cells termed anergy (Campbell et al. 2009; Telander and Mueller 1997). This achieves through negative regulation of T cells by several TCR targeted E3 ubiquitin ligases which lead to suppression of TCR signalling and

Interleukin-2 (IL-2) synthesis resulting in long-term loss of T cell effector functions (Mueller 2004; Bandyopadhyay et al. 2007). Additionally, anergy can be induced through negative costimulatory factors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and the programmed death-1 (PD-1) protein. CTLA-4 and PD-1 are both inhibitory receptors, which block further TCR engagement and maintain T cells in an unresponsive state (Nurieva et al. 2006; Sharpe 2005; Tivol et al. 1995). Along with these mechanisms, Tregs also contribute to peripheral tolerance by controlling immune responses to preserve immune homeostasis and avert autoimmune disorders. These cells produce immunosuppressive cytokines including IL-10 and TGF-β in the periphery, which prevents effector T cells and APCs from acquiring proliferation and activation. Additionally, they engage in direct interactions with other immune cells through cell surface molecules including CTLA-4 and PD-1 to limit their activation and expansion (Hutloff et al. 1999; Massey et al. 2002).

1.2.5 T cell activation

In the periphery, when TCRs are engaged with proper costimulation a cascade of signalling events trigger T cell activation is triggered. This process is required for development of effective immune responses as it plays a crucial role in stimulating the production of high-affinity antibodies by B cells and the establishment of cytotoxic T-cell responses. The initial step of T cell clonal activation begins when a T lymphocyte recognizes its antigen in the form of short peptides presented by MHC class I or class II molecules found on the surface of APCs (Bianchi 2007; Kawai and Akira 2010). The expression of MHC molecules and antigen presentation is stimulated by inflammatory cytokines produced by activated innate immune cells. IL-1, tumor necrosis factor-alpha (TNF-α), and interferon-γ (IFN-γ) are examples of cytokines responsible for these upregulations and required for full T cell activation. T lymphocytes that detect MHC class I are CD8 coreceptor expressors and serve as killer cells, whereas those that recognize MHC class II express the CD4 coreceptor not CD8 and function as helper cells. Besides binding to the MHC antigen, a variety of negative and positive costimulatory signals need to be activated in order for both cytotoxic and helper T cells to respond efficiently to the threat and for the responses to be properly regulated. The positive signal is provided by CD28-mediated costimulation. Transmembrane protein CD28, a member of the immunoglobulin gene superfamily, is expressed constitutively on T cells. This molecule on the T lymphocytes binds to one of its two ligands CD80 (B7-1) and CD86 (B7-2) on the APCs such as DCs, monocytes, and activated B cells, which subsequently initiates T-cell proliferation. This process leads to production of millions of T cells, all capable of the same

antigen recognition (Sansom 2000; Mosmann et al. 1986; Romagnani 1991). To regulate the response, T cells begin to express the coinhibitory surface molecule, CTLA-4, a competitor of CD28. High-affinity CTLA-4/B7 engagement inhibits the interaction between these costimulatory molecules on antigen-presenting cells and CD28 on T cells. Thus, it reduces the signals sent to the T cell by the other molecules in the system (Wing et al. 2008).

1.3 CD4+ T helper cells

CD4+ T helper cells are diverse and can carry out various functions. After activation and differentiation into distinct effector subtypes, the members of this group influence the functioning of other crucial immune cells such as B cells, macrophages, and CD8+ cytotoxic T cells through the release of their specific cytokines. They are also in charge of chemoattraction signals that draw innate or antigen-specific cells to the sites of infection and inflammation and boost macrophages' ability to kill bacteria. Following antigen recognition, several variables, also known as non-cytokine factors, affect the lineage-specific fate decision of a naïve CD4 T cell that desires to differentiate into a distinct effector subtype. Some examples of these include the presence of a certain types of PAMPs, nature of APCs, strength of TCR stimulation and costimulation, change in cytokine magnitude, and the induction of certain types of transcription factors. In addition, the nature of signal transducer and activator of transcription factor (STAT) signalling is taken into account to determine T helper fate. Each effector subset is also regulated by a distinct lineage specific transcription factor to be identified as T-bet+ T helper 1 (Th1) cells, GATA+ T helper 2 (Th2) cells, Th17 cells, RORγt+ AhR+ T helper 22 (Th22) cells, and BCL6+ follicular helper T cells (Tfh) (Fig 1.3 and 1.4) (Bhaumik and Basu 2017; Zhu and Paul 2010; Basu, Hatton, and Weaver 2013). As this project is mainly concerned with Th17 cells and the Th17-Th1-Tregs balances, these are the cell types that will be covered in detail in this thesis.

1.4 Th17 cells

Naïve CD4 T cells are transformed into Th17 cells after they encounter APCs that are pulsed with fungal or extracellular bacterial antigens (Fig 1.3). The potent pro-inflammatory cytokines IL-1β, IL-6, IL-21, IL-23, and transforming growth factor β (TGF-β), as well as IL-12, are essential for Th17 differentiation (Basu, Hatton, and Weaver 2013; Miossec, Korn, and Kuchroo 2009; Bettelli and Kuchroo 2005). The activation of the Th17 differentiation program relies entirely on the presence and function of STAT3, the transcription factor retinoic acid receptor-related orphan receptor gamma-T (RORγt) and the initial stage of differentiation

triggered by combination of TGF-β and IL-6 (Veldhoen et al. 2006; Bettelli and Kuchroo 2005; Mangan et al. 2006). IL-21 produced by Th17 cells themselves are responsible for the self-amplification step, and IL-23 mediates the stabilization of Th17 cells (Bettelli and Kuchroo 2005). In addition, IL-1β can promote clonal expansion of Th17 cells (Sutton et al. 2006). Human Th-17 cells are identified based on the expression of surface markers including CCR6, CCR4, and IL-23R, as well as the production of the pro-inflammatory cytokines IL-17A, IL-17F, IL-21, and IL-22 (Rouvier et al. 1993; Durant et al. 2010) (Bettelli and Kuchroo 2005; Chen et al. 2006; Chang and Dong 2007). Cytokine secretion by activated Th17 cells induce inflammation by mobilizing, recruiting, and activating neutrophils in a process pivotal for the host's defence against external pathogens (Zenobia and Hajishengallis 2015).

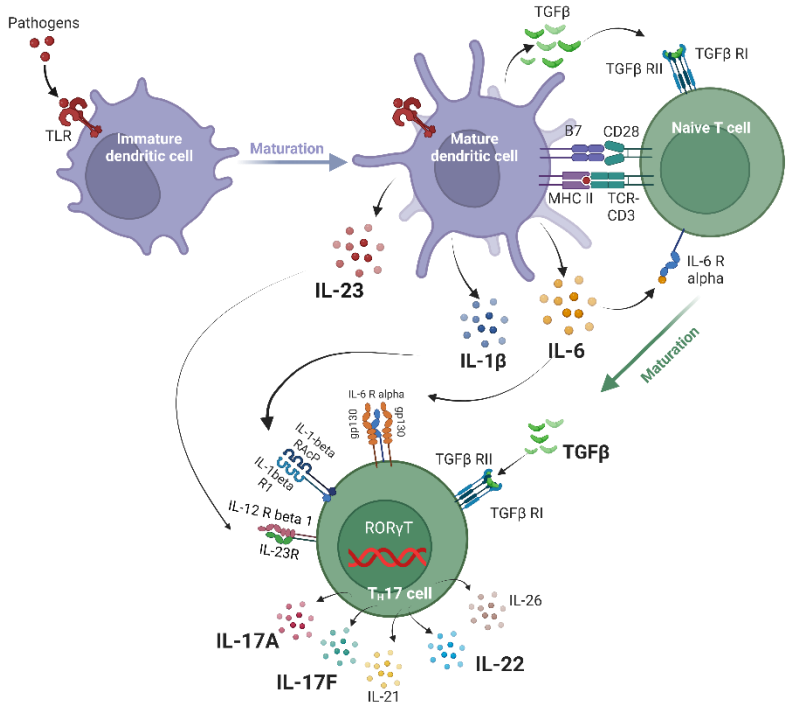

Figure 1.3. Differentiation of Th17 cells. Th17 cells are generated from naïve CD4+ T cells that are activated in the presence of TGF-β/IL-6. Downstream of TCR-signalling following the cytokine/receptor interaction, STAT3 becomes activated which then leads to the expression of the Th17 master transcription factor RORγt. Activated Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22, and IL-26. Th17 cells are important for maintaining homeostasis and integrity of mucosal tissues and contribute to the immune defence against fungal and bacterial infections. Nevertheless, they are also responsible for inducing significant tissue inflammation in numerous autoimmune disorders. The figure was created in Biorender.com.

1.4.1 IL-17 family of cytokines

The IL-17 family of cytokines is made up of six related proteins (IL-17A-F) (Yao et al. 1995; Li et al. 2000; Starnes et al. 2002; Lee et al. 2001) and five members of IL-17 receptor (IL-17R) family (IL-17RA–IL-17RE) (Yao et al. 1995; Shi et al. 2000; Lee et al. 2001; Haudenschild et al. 2002; Moseley et al. 2003) generated by a number of immune cells, including Th17 cells, γδ T cells, and ILCs (Jin and Dong 2013) and have a variety of roles in both immunity and disease processes. These small proteins contribute significantly to development of inflammatory diseases, autoimmune disorders, and cancer through activation of IL-17R signalling (Ferretti et al. 2003; Fossiez et al. 1996; Yang et al. 2014; Zhang et al. 2006; Tartour et al. 1999). With the highest sequence homology between IL-17A and IL-17F, the biological role and regulation of these two cytokines are the best understood of all the members (Wang et al. 2012). These two cytokines are mostly produced by T cells that belong to the Th17-effector class (Park et al. 2005; Harrington et al. 2005). The activation of IL-17R-mediated signal transduction induce several downstream signalling pathways involved in inflammation, immune cell recruitment, and tissue remodeling. On mucosal surfaces, IL-17 stimulates chemokines to encourage neutrophil recruitment to sites of inflammation or upregulates antimicrobial proteins to suppress microbial proliferation (Kao et al. 2004; Huang et al. 2007; Miossec and Kolls 2012). In individuals with APS-1 disorder, the process of clearing systemic and oropharyngeal candidiasis, which is crucially dependent on IL-17-mediated neutrophil recruitment, is disturbed (Huang et al. 2004; Conti et al. 2009). Thus, novel therapeutic approaches that have been created with the objective of raising or neutralizing the levels of IL-17 have displayed promising therapeutic benefits in clinical settings, specifically in the management of several inflammatory disorders (Patel et al. 2013).

1.4.2 IL-22 cytokine

The presence of IL-22 coexpression with IL-17 in mice has led to its widespread recognition as a Th17 cytokine (Liang et al. 2006). Among the cytokines from the IL-20 subfamily (IL-19, IL-20, IL-22, IL-24, and IL-26), IL-22 has received the greatest scientific attention due to its act on a variety of cells, including epithelial cells, fibroblasts, and immune cells, to stimulate the production of antimicrobial peptides and other immune mediators (Zheng et al. 2007; Ouyang et al. 2011; Lindahl and Olsson 2021). Other innate and adaptive immune system cells can also produce this cytokine. For instance, IL-22 is generated independently of IL17 coexpression, defining a distinct subset of Th22 cells (Eyerich et al. 2009). These small proteins

get released by Th17 cells in response to cytokines, mostly IL-23 but also IL-6 and IL-1β. Accordingly, mice lacking IL-23 had drastically reduced levels of IL-22 production. IL-22 secretion is also regulated by the aryl hydrocarbon receptor (AhR) and Notch pathways (Rutz, Eidenschenk, and Ouyang 2013).

1.4.3 Th17 differentiation pathway

The STAT3 signalling pathway, activated by IL-6, IL-21, and IL-23 signals, plays a crucial role in Th17 differentiation (Fig 1.3) (Rouvier et al. 1993; Durant et al. 2010). Following the cytokine/receptor engagement, STAT3 become activated through Janus kinase-mediated (JAK) phosphorylation (Lee et al. 2009; Parham et al. 2002; Pflanz et al. 2002). Once phosphorylated, the STAT3 dimer has the ability to translocate into the nucleus to initiate transcription of many genes crucial to Th17 differentiation, including IL-17A, IL-17F, IL-21, IL-23R, and RORc, the gene encoding the master transcription factor RORγt. STAT3 cooperates with RORγt to induce optimal Th17 differentiation. This developmental pathway can be inhibited by cytokines that activate Th1 cell subset such as type I IFNs, IFN-γ and IL-27 and by the Th2 cytokine IL-4 (Guo, Chang, and Cheng 2008; Pflanz et al. 2002; Deimel et al. 2021).

1.5 Th17 plasticity

CD4 T cell subsets are developmentally related and have plasticity towards one another dependent on the cytokine milieu they are in. Compared to Th1 and Th2 cell lines, which are considered fairly stable, Th17 cells exhibit a high degree of plasticity. As an example, Treg cells have overlapping signalling pathway with Th17 as a result of their common requirement for TGF-β and IL-6 throughout development. Moreover, studies suggest that IL-12 and IL-23 induce a conversion from Th17 cells to the Th1 phenotype in the absence or with low amounts of TGF-β, and to maintain a Th17 phenotype when combined with adequate TGF-β quantities (Fig. 1.4) (Guery and Hugues 2015; Trinchieri, Pflanz, and Kastelein 2003). Currently, the trans differentiation ability of Th17 cells remains unsolved and appears to occur most frequent during persistent autoimmune inflammation, and not in acute infection. This points to a possible therapeutic potential of skewing of these pathways (Hirota et al. 2011).

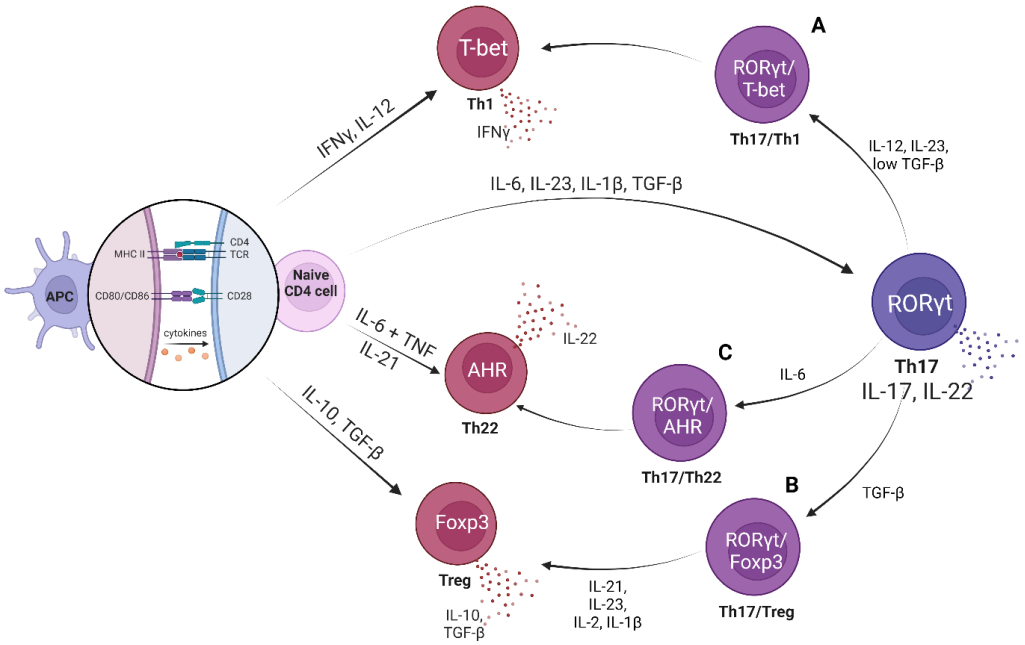

Figure 1.4. Th17 cell plasticity. Th17 cells possess the ability to undergo conversion into different lineage subsets depending on the specific microenvironment they encounter. **A**) Th17/Th1 plasticity: IL-23 and IL-12 cytokines can trigger the differentiation of both Th17 and Th1 cell subsets upon naïve CD4+ T cell activation. **B**) Th17/Treg plasticity: activated naïve CD4+ T cells exposed to TGF-β can lead to either Th17 or Treg cell commitment. **C**) Th17/Th22 plasticity: activated naïve CD4+ T cells can differentiate towards either Th17 or Th22 cells in the presence of IL-6. The figure was modified from (Guery and Hugues 2015) and created in Biorender.com.

1.5.1 The Th17-Treg axis

The immune system relies on TGF-β, expressed by Treg amongst other cell types for both survival of naïve T cells and maintenance of peripheral tolerance. This cytokine inhibits the proliferation of self-reactive T cells and induces DCs to retain tolerance. (Korn et al. 2009; Taylor et al. 2006) According to this TGF-β-dependent reciprocal regulation, there is a partial overlap in differentiation programs between Th17 and Treg CD4+ T cells. By itself, TGF-β triggers the anti-inflammatory Tregs, while combined with other cytokines such as IL-6 or IL-21 in an inflammatory microenvironment, inhibits Treg cell differentiation and induces the Th17 cell differentiation (Korn et al. 2009). Th17-Treg plasticity was initially observed in mice, when, in the presence of TGF-β, IL-6 together with IL-1β induced FoxP3+ Treg conversion to Th17 cells (Xu et al. 2007). It has also been demonstrated that, in the presence of low concentrations of IL-6, increasing TGF-β concentrations can promote Foxp3 levels while lowering IL-23R expression, causing the differentiation of Th cells from Th17 to Tregs. Accordingly, FoxP3 can suppress RORγt and RORα through direct physical binding to these Th17 lineage-specific transcription factors. Expression of IL-6, IL-21 and IL-23, on the other hand, relieve Foxp3-mediated inhibition on RORγt, thereby promoting Th17 cell differentiation (Zhou et al. 2008). Overexpression of Foxp3 showed no effect on RORγt and

RORα, while inhibiting Th17 secreted cytokine expression including IL-17, IL-21 and IL-22, in a process that is facilitated by an IL-2-mediated mechanism (Yang, Nurieva, et al. 2008; Moisan et al. 2007). There are indications that, despite the paucity of evidence, this plasticity may be crucial for immune system regulation, allowing a swift transition from suppression to active immunity that might be a critical factor for the development of autoimmune diseases.

1.5.2 The Th17-Th1 axis

The immune system's response to infection relies heavily on a T-cell subtype referred to as a Th1 cell line. In cellular immunity, Th1 cells are in charge of activating macrophages, which are crucial for eradicating intracellular pathogens. In addition, they promote antibody synthesis by B-cells. The generation of Th1 cells from naive CD4+ T cells requires a complex interaction of signalling pathways and transcription factors, primarily including IFN-γ and IL-12 signals as well as the transcription factor T-bet. The signature cytokine of this cell line, IFN-γ, is produced in abundance by fully differentiated Th1 cells in addition to tumor necrosis factor (TNF), lymphotoxin, and IL-2 that are all aid in antimicrobial defence. IFN-γ secretion activates macrophages and DCs, protects body against intracellular pathogens, such as Mycobacterium tuberculosis. The IL-12 heterodimer is made up of two subunits, p35 and p40, that is generated upon Toll-like receptor (TLR) activation of APCs by fungal antigens, but not bacterial antigens (Hsieh et al. 1993). This cytokine is further involved in the trans differentiation of Th17 into Th1 cell line (Trinchieri, Pflanz, and Kastelein 2003). In mouse studies, the Th1-associated cytokines IL-12 and IFN-γ, suppress differentiation of Th17 cells suggesting that there is a functional dichotomy existings between Th1 and Th17. (Acosta-Rodriguez et al. 2007) Accordingly, IL-12 combined with another cytokine, IL-27, promote the differentiation of Th1 committed cells, leading to IFN-γ production by inducing the expression of the Th1-related transcription factors STAT4 and T-bet, while inhibiting the Th17 lineage-specific transcription factor RORγt. (Manetti et al. 1994) IL-12 share proinflammatory properties with another cytokine ,IL-23, which is produced by DCs and macrophages. The IL-23 signature cytokine appears to play an important role in enhancing and/or maintaining the Th17 phenotype. IL-12 and IL-23 are identical in that they both have the p40 subunit, but differ in the second subunit, as IL-12 possesses the p35 subunit, but IL-23 is composed of a different subunit, p19 (Acosta-Rodriguez et al. 2007). The simultaneous induction of Il-12/IL-23 production in DCs by their stimuli will favor Th1 responses despite IL-12 promoting IL-23p19 synthesis by APCs promotes Th17 differentiation (Vignali and Kuchroo 2012). TGF-β is another essential cytokine for Th17 differentiation, and it works best when combined with IL-

6 to promote the development of the Th17 cell line. The conversion of Th17 cells to Th1 cells can be accelerated by the absolute absence or low concentrations of TGF-β, as in the case of overexpression of Smad7 (a TGF-β inhibitor). In the absence of TGF-β, IL-23 can promote the development of IFN-γ-producing cells and lead to cease the IL-17F and IL-17A expression (Rizzo et al. 2014; Annunziato, Cosmi, Santarlasci, Maggi, Liotta, Mazzinghi, Parente, Fili, et al. 2007). This provides evidence that Th17 plasticity can be inhibited by the presence of TGF-β. In sum, T cell mechanisms which are properly regulated are essential for a functional immune response towards microbes, but if homeostasis and tolerance are broken, T cells can also turn against essential self-molecules ending up in autoimmune disorders (Guéry and Hugues 2015).

1.6 Autoimmunity

Autoimmune diseases are brought on by the immune system's inability to differentiate between non-self and self-antigens and hence, start an immunological reaction towards the hosts' own tissue. During thymic T cell development in a healthy host, the thymic medulla negatively selects and eliminates lymphocytes that may be reactive to self-peptides. Significantly, mature T cells undergo secondary selection (peripheral tolerance) after leaving the thymus, during which the majority of leaking self-reactive T cells are suppressed or become anergic (Wang, Wang, and Gershwin 2015). Therefore, a key issue for the underlying abnormalities of autoimmune disorders is a perception of the loss of self-tolerance in both central and peripheral mechanisms. It is likely that declines in the proportion of active Tregs or resistance of autoreactive effector T cells to regulation have an impact in the onset of T cell-dependent inflammatory autoimmune diseases (Rosenblum, Remedios, and Abbas 2015). An intricate interplay of genetic, environmental, and immunological variables may be the origin of autoimmunity (Ermann and Fathman 2001). Evidence for the genetic impact relies on an increased tendency to develop autoimmune diseases associated to genetic polymorphisms that mostly lie in the regulatory regions of genes whose products are believed to influence immune function. For instance, among all the genes linked to autoimmune disorders, those with specific *HLA* alleles, encoding the MHC-molecules which present peptides to T cells, had the greatest and longest-standing correlations (Fernando et al. 2008). Genetic variations in cytokines and cytokine receptors are also associated with the development and severity of many autoimmune disorders e.g., IL-23R, which is critical for Th17 differentiation through binding to the cytokine IL-23. Variants in the gene encoding IL-23R may lead to an increased activation of immune cells and a pro-inflammatory response, contributing to the development of several autoimmune

diseases, including psoriasis, inflammatory bowel diseases, systemic lupus erythematosus, Sjögren syndrome, and type 1 diabetes (Rosenblum, Remedios, and Abbas 2015; Bunte and Beikler 2019). The finest example of environmental factors as a contributor to autoimmunity is the possibility for certain antigenic determinants of microorganisms to mimic host epitopes and hence be able to induce an autoimmune response. Additional to viral or bacterial infections, there are other environmental agents such as psychological stress, hormonal imbalance, certain dietary factors, exposure to ultraviolet radiation from the sun, and smoking that are associated with the development of autoimmune diseases (Wang, Wang, and Gershwin 2015). Besides this, disrupting certain immunoregulatory pathways in the context of immunological regulation can result in the emergence of disorders with autoimmunity characteristics (Ermann and Fathman 2001). Recently, the well-known adverse effect of immune checkpoint inhibitor therapy in cancer, causing autoimmune disorders, is an example of this (Paschou et al. 2021).

Autoimmune disorders are categorized into organ-specific and systemic categories depending on whether the autoantigen is primarily associated with a particular tissue or is present in multiple organs throughout the body (Wang, Wang, and Gershwin 2015). Rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis are examples of frequent systemic autoimmunity illnesses (Shi et al. 2013). Type I diabetes, autoimmune thyroid disease, autoimmune adrenocortical failure (Addison's disease) and autoimmune polyendocrine syndromes are examples of organ specific disorders (Lesage and Goodnow 2001).

1.7 Autoimmune Polyendocrine Syndrome type 1 (APS-1)

The APS-1 syndrome is a monogenic organ specific autoimmune disorder which can be used as model for failure of central immunological tolerance and what happens on the subcellular level when tolerance becomes unregulated.

1.7.1 Clinical signs

In the wake of the discovery of the link between chronic candidiasis and hypoparathyroidism, which was then confirmed in a child with chronic mucocutaneous candidiasis (CMC), hypoparathyroidism, and adrenal insufficiency in 1929 (THORPE and HANDLEY 1929), three forms of autoimmune polyendocrine syndromes were described, each with a unique relationship between the clinical manifestations in 1980 (Neufeld, Maclaren, and Blizzard 1980). APS-1 or Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy

(APECED) is a rare monogenic inherited disease (OMIM#, 240300) that cause autoimmune destruction of certain organs (Lesage and Goodnow 2001). One in every two to three million infants are born with this multi-organ autoimmune disorder, however it is more prevalent in Iranian Jews (1/9,000), Sardinians (1/14,000), and Finns (1/25,000), and less so in Norwegians (1/80,000) and Poles (1/129,000). Furthermore, spanning from 0.2 to 18 years, the age of the initial manifestation varies greatly (Bello and Garla 2023; Zhan and Cao 2021). Despite the fact that the clinical features of APS-1 are quite diverse (Fig 1.5), the clinical diagnosis of APS-1 has relied on the emergence of at least two of the classical triad components, including CMC, hypoparathyroidism, and primary adrenal insufficiency (Addison's disease), or the appearance of only one component if a sibling has already been diagnosed (Husebye, Anderson, and Kämpe 2018). These clinical characteristics mainly belong to one of two categories: "classical" or "non-classical," as determined by the range of phenotypes linked to *AIRE* mutations. APS-1 is often autosomal recessive (classical APS-1) with high titer IFN antibodies although heterozygous dominant-negative variations with less IFN antibodies and relatively late-onset age (non-classical APS-1) have also been noted (Guo et al. 2018; Oftedal et al. 2015; Abbott et al. 2018).

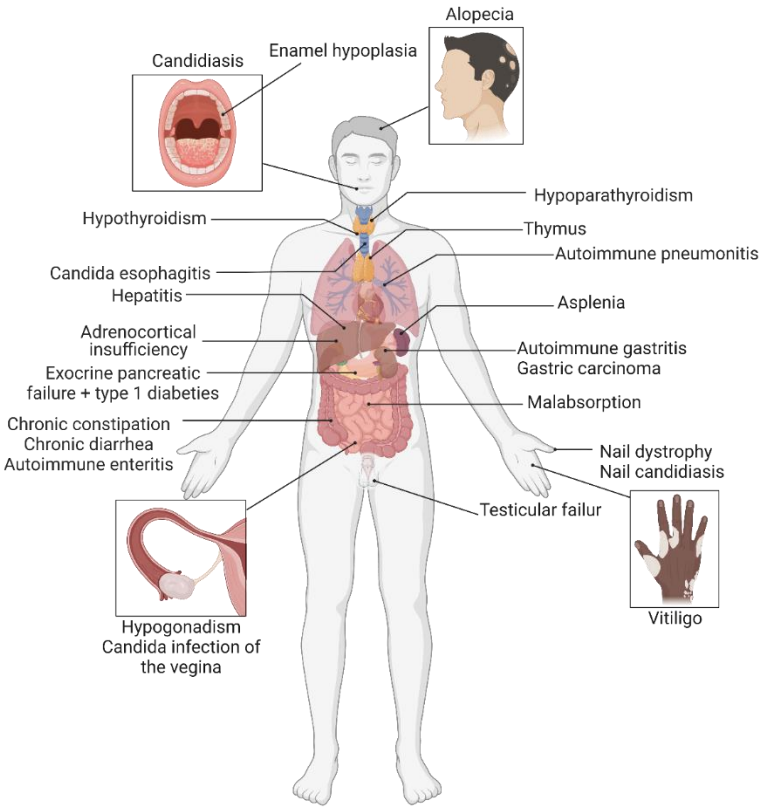

Figure 1.5. Illustration of the typical clinical symptoms of individuals with APS-1. Patients with APS-1 exhibit a diverse range of clinical manifestations characterized by autoimmune dysfunction in multiple organs. These organ-specific autoimmune symptoms vary in frequency and with differences in the age of symptom onset The figure was modified form (Capalbo et al. 2012) and created in Biorender.com.

1.7.2 AIRE

AIRE was determined to be the responsible gene when a genetic connection between APS-1 and variants located on chromosome 21q22.3 was discovered in 1994 (Aaltonen et al. 1994). Wild-type (WT) Autoimmune regulator (*AIRE*) protein with a full-length of 545 amino acids, is expressed mostly in mTECs by the unmutated 14 exons *AIRE* gene (Björses et al. 1999). The WT *AIRE* contains the homogenously staining region (HSR) domain also known as caspase recruitment domain (CARD) present at the N terminus, which are thought to be important in dimerization and/or caspase recruitment. Other motifs and domains include a nuclear localization signal (NLS) essential for the nuclear localization, a central SAND (Sp100, *AIRE*-1, NucP41/75, DEAF-1) domain that is considered to be involved in DNA binding, two plant homeodomains (PHDs) mediate histone binding and transcriptional activation, a proline rich region (PRR) interact with a number of proteins involved in transcriptional regulation, and four interspersed LXXLL motifs that are present in nuclear receptor co-activators and are crucial for controlling the expression of other genes outside of the thymus (Fig 1.6) (Ilmarinen et al. 2006; Sparks et al. 2016). Despite the 14 exon *AIRE* gene's ability to encode the WT *AIRE*

protein, it has been discovered that both in humans and other species, alternative splicing of the *AIRE* pre-mRNA results in transcripts with various exon configurations, each with different functional domains and varied lengths. (Ruan et al. 1999). The Human Gene Mutation Database (HGMD) lists more than 180 distinct rare disease-causing *AIRE* mutations, including missense, nonsense, frameshift, splice-site, and gross deletions (Stenson et al. 2017). The first dominant mutation linked to the dominant form of APECED in both humans and mice was the SAND domain mutation c.682T>G (p.G228W) which then was shown to be associated with the high risk for hypothyroid autoimmune thyroiditis in the patients (Cetani et al. 2001; Su et al. 2008). Some of other mutations have been identified as commonly affecting APS-1 patients, including the nonsense mutation c.769C>T (p.Arg257Ter), which causes an early stop codon and is found in the SAND domain in Finnish, Central European, and Eastern European populations (Nagamine et al. 1997; Cihakova et al. 2001). The c.967_979del (p.Leu323fs) mutation is the most prevalent frameshift mutation in British, North American, Irish, and Norwegian patients (Pearce et al. 1998; Heino et al. 1999; Dominguez et al. 2006; Wolff et al. 2007). In addition, the missense mutation c.254A>G (p.Tyr85Cys) is common among Persian Jews (Björses et al. 2000), and the c.415C>T (p.Arg139Ter) mutation predominates in Sardinian families (Rosatelli et al. 1998).

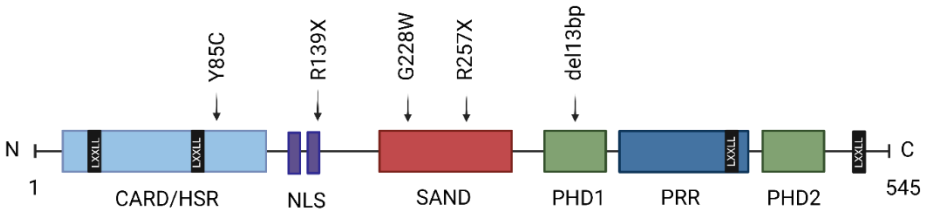

Figure 1.6. Functional domains and most common mutations of the *AIRE* gene. The *AIRE* protein structure is represented by boxes of different colors, each corresponding to a specific domain, and the names of these domains are indicated below the protein structure. The most frequently observed mutations in APS-1 patients are indicated by arrows positioned above the protein structure. The domains of the *AIRE* protein include: HSR (homogenous staining region), CARD (caspase-recruitment domain), NLS (nuclear localization signal), SAND (Sp100, *AIRE*-1, NucP41/75, and DEAF-1), PHD (plant homeodomain), and PRR (proline-rich region). The numbers represent the amino acid positions of the protein. The figure was adopted from (Bruserud, Oftedal, Wolff, et al. 2016) and created in Biorender.com.

1.7.3 Pathogenesis of APS-1

As stated in 1.3.3, *AIRE* has been found to facilitate the ectopic gene expression of a variety of TRAs, while also regulating the expression of non-TRA genes, inside mTECs and lead to negative selection of self-reactive T cells (Su et al. 2008; Kyewski and Klein 2006; Radhakrishnan et al. 2016). Mutations in *AIRE* gene cause a defect in the thymus's ability to properly eliminate autoreactive T cells, which results in the onset of APS-1 illness. Following

this, self-reactive CD4+ T cells egress from the thymus into the periphery (Fig 1.7), suffices to trigger autoimmune tissue infiltration, destruction, and production of autoantibodies (Niki et al. 2006; Anderson et al. 2002) (Ramsey et al. 2002). In the final stage of development, mTECs become mature into Hassall's corpuscles, which are crucial for maintaining immunological homeostasis and preventing autoimmunity by aiding in differentiation and the positive selection of Tregs with a high affinity for self-antigens (Wada et al. 2011). In the absence of *AIRE*, the morphology and distribution of mTECs are aberrant, and the medulla lacks Hassall's corpuscles (Metzger et al. 2013), implying *AIRE*'s significance in mTEC maturation. Likewise, *AIRE*-dependent self-antigens trigger antigen-specific Tregs by polyclonally forming their TCR repertoire in order to positively select Tregs with high affinity TCRs for self-antigens (Perry et al. 2014). Notably, *AIRE*-deficient mouse models exhibit autoimmune reactions to self-antigens that are not controlled by *AIRE*, indicating the existence of additional *AIRE*-independent factors mediating tolerance (Anderson et al. 2002; Niki et al. 2006).

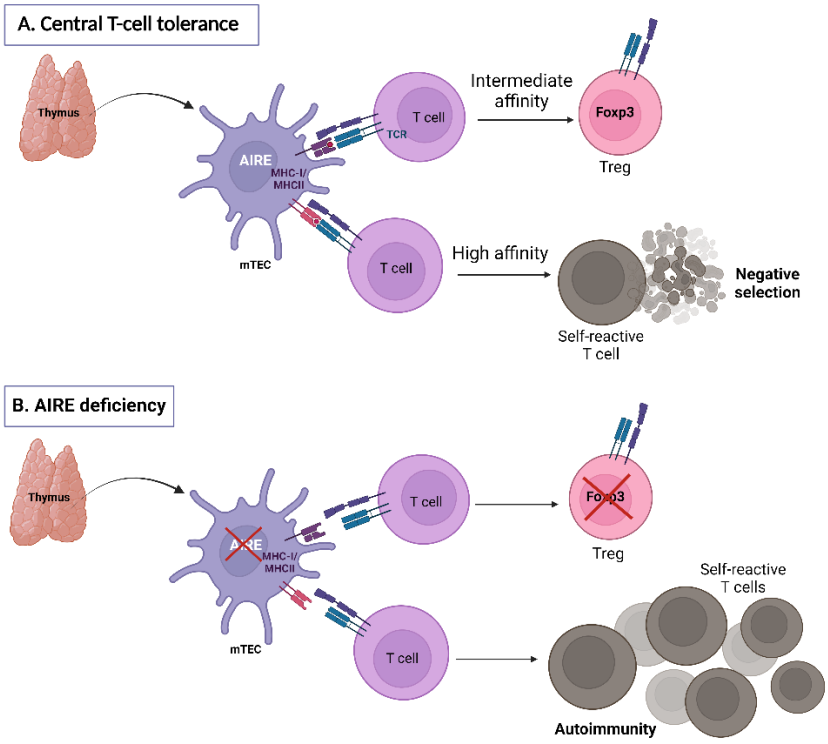

Figure 1.7. *AIRE* deficiency mechanism. *AIRE*, a protein expressed in a specific subset of medullary thymic epithelial cells (mTECs), plays a crucial role in the expression and presentation of self-antigens derived from peripheral tissues (TSAs). **A**) This process is important for the deletion of self-reactive T cells with high affinity for TSAs through apoptosis or their differentiation into regulatory T cells (Tregs). **B**) In individuals with *AIRE* deficiency, the expression and presentation of TSAs on mTECs are impaired. As a result, self-reactive T cells escape negative selection in the thymus and enter the periphery. These T cells can then infiltrate various tissues, leading to the autoimmune manifestations observed in APS-1 patients. The figure was created in Biorender.com.

1.7.4 Immunological defects (Autoantibodies (autoAB))

Autoimmunity generated by antibodies (ABs) against self-antigens in APS-1, along with other manifestations, can be utilized as a diagnostic tool to track illness development and response to therapy. These autoantibodies (autoABs) can be categorized into organ-specific, non-organ-specific, and cytokine ABs (Perniola et al. 2000; Constantine and Lionakis 2019; Wolff et al. 2013). The emphasis of this study is on neutralizing cytokine ABs in APS-1 patients including high-titre autoAbs to type 1 IFNs, and Th17 cell-associated cytokines such as IL-22, and IL-17 A/F. Type I IFNs are essential components of the innate immune response to viral infections. AutoABs that neutralize these cytokines can suppress the antiviral response and put individuals at risk for recurrent infectious diseases. More than 95% of APECED patients have neutralizing autoABs against type-I IFNs, the majority of which target IFN-ω and IFN-α subtypes. Conversely, IFN-β-specific autoABs are only found in ~20% of patients, and autoABs against IFN-ε are rarely observed (Meager et al. 2006; Meloni et al. 2008; Bastard, Orlova, et al. 2021). Early recognition of IFN-ω autoABs in children with suspected APECED has great diagnostic value since these cytokine-directed autoABs can be detected with high sensitivity and are highly specific for the condition before the onset of clinical signs (Meager et al. 2006; Meloni et al. 2008). Regardless of having autoABs against type-I IFNs, individuals with APECED do not show severe viral infections that is reported in patients with congenital complete deficiencies of two subunits of the type I IFN receptor, IFNAR1 and IFNAR2 (Bastard, Manry, et al. 2021; Hernandez et al. 2019). Clinical evidence points to the possibility that residual type-I IFNs still act as compensating mediators, or other type-I IFN-independent immune defences may be effective in preventing infection-causing viruses in these patients (Wu et al. 2020; Yum et al. 2021). Moreover, neutralizing autoABs against IL-22 are present in ~70–90% of APECED patients, while these ABs are less frequently seen for IL-17F and IL-17A and absent for IL-17B and IL-17C (Ferre et al. 2016; Orlova et al. 2017; Kisand et al. 2010; Puel et al. 2010; Ahlgren et al. 2011; Bruserud, Oftedal, Landegren, et al. 2016). The cytokines IL-17 and IL-22 function on epithelial cells by promoting the synthesis of chemokines and antimicrobial peptides, which assist in the developing resistance to fungi, outlined in 1.5.1 and 1.5.2 (Littman and Rudensky 2010). This thus supports the possibility that IL-22 and anti-IL-17 A/F neutralizing autoABs could facilitate the emergence of chronic candidiasis in APS-1 patients (Kisand et al. 2010; Puel et al. 2010).

1.7.5 Chronic Mucocutaneous Candidiasis (CMC)

Chronic Mucocutaneous Candidiasis (CMC) is a rare, inheritable disease that impairs the immune system's ability to resist fungal infections driven by the *Candida* species. Despite the fact that approximately 20 distinct *Candida* species are responsible for causing candidiasis, but *C. albicans* still accounts for the bulk of cases (Puel 2020). CMC is one of the initial and most prevalent clinical signs found in APS-1 individuals. Within the first year of life, CMC affects 24 to 52% of APS-1 patients, and as people age, it becomes more frequent, impacting more than 80% of these patients (Puel et al. 2010; Ferre et al. 2016; Bruserud, Oftedal, Landegren, et al. 2016; Constantine and Lionakis 2019). CMC often manifests as oral, esophageal, genital mucosae, skin, nails, and/or scalp infections and it can vary as a mild, recurrent infection or cause persistent hypertrophic or atrophic lesions (Constantine and Lionakis 2019; Bruserud, Oftedal, Landegren, et al. 2016). As recently revealed by the genetic analysis of CMC, IL-17-mediated immunity has a crucial role in the prevention of mucocutaneous candidiasis. A potential involvement for IL-17A/F in mucocutaneous defence against *Candida* has been proposed by studies of the molecular and cellular basis of syndromic CMC in individuals with various types of deficiencies who all had low levels of circulating IL-17A/F-producing T cells (Ferre et al. 2016; Orlova et al. 2017; Puel et al. 2010; Kisand et al. 2010; Bruserud, Oftedal, Landegren, et al. 2016). These patients subsequently had a higher propensity for developing recurrent *Candida* infections of their mucous membranes, skin, and nails. Additionally, a limited percentage of APS-1 patients have been confirmed to inherit the genetic defect STAT1 gain-of-function (GOF), which enhances the cellular response to IFN-γ and is currently the most common genetic cause of syndromic CMC. The formation and maintenance of Th17 cells are dependent on STAT3-dependent cytokines such IL-6, IL-21, and IL-23. Th17 cells lack in individuals with STAT1 GOF mutations, which may be due to elevated STAT1 signalling downstream from these cytokines, enhanced STAT1 signalling downstream from type I and II IFNs, which is known to hinder the development of Th17 cells via STAT1, or a combination of these two mechanisms. Therefore, the increased STAT1 signalling in these patients may further restrict the growth of Th17 cells, adding to the observed decline in Th17 cell proportion (Liu et al. 2011; van de Veerdonk et al. 2011; Toubiana et al. 2016; Puel 2020).

The extensive research conducted on the functionality and pathogenesis of Th17 cells in APS-1 patients has played a crucial role in advancing our knowledge of this autoimmune disease. The valuable insights gained from these studies have sparked a profound curiosity, driving further research efforts to uncover additional findings related to the pathogenesis of the Th17 cell subset in APS-1. By delving deeper into the characteristics and functionality of Th17 cells, our aim is to unravel the intricate mechanisms underlying this autoimmune disorder, which may hold the potential to improve the diagnosis, management, and treatment strategies for individuals affected by this condition.

2. Aims

We hypothesized that cytokine autoABs (anti-IL-22, anti-IFNα, and anti-IFNω) present in high levels in APS-1 patients' sera have the potential to affect Th17 cell differentiation and function. We therefor aimed to investigate the effect of addition of cytokine ABs to *in vitro* T cell culture Th17-polarized T cell culture conditions from APS-I patients compared to healthy controls.

Specific aims:

1. Test the ability of naïve CD4+ T cells from healthy controls to differentiate to Th17 cells in conditions with:

   a. Commercial type 1 anti-IFN and anti-IL-22 ABs
   b. Sera or purified IgG from APS-1 patients with high levels of these APS-1 hallmark cytokine autoABs.

2. Evaluate how polarised Th17 cells from APS-I PBMCs respond to addition of heat killed C. albicans (HKCA) with or without added serum or IgG from APS-1 patients or healthy controls.

3. Materials

3.1 Reagents, antibodies, and primers

Reagent name	Producer	Cat. Number
2x PowerTrack SYBR Green Master Mix	Thermo Fischer	A46109
Commercial antibodies		
Anti-Hu/Mo IL-22, clone: IL22JOP	Invitrogen	16-7222-82
Anti-Human IFN-omega	Invitrogen	BMS1021
Anti-IFN-alpha	Invitrogen	M710
Cytokines (Th17-polirizing cytokines)		
Human IL-1β	Miltenyi Biotec	130-093-895
Human IL-6	Miltenyi Biotec	130-095-352
Human IL-23	Miltenyi Biotec	130-095-757
Human TGF-β1	Miltenyi Biotec	130-095-067
Cytokine antibodies		
For PBMC and T cell cultures		
IFNγ antibody, clone: 45-15	Miltenyi Biotec	130-095-743
IL-4 antibody, clone: 7A3-3	Miltenyi Biotec	130-095-743
DEPC Treated Water	Ambio (Invitrogen)	AM9915G
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich	D2650
Dulbecco's phosphate buffered saline (PBS)	Sigma Aldrich	D8537
Ficoll-Paque PLUS	Cytiva	17-1440-03
Flow antibodies		
For PBMC and T cell panel		
Anti-CD3, Brilliant Violet510, clone UCHT1	Biolegend	300448
Anti-CD4, Alexa Fluor700, clone: RPA-T4	BD Biosciences	557922
Anti-ROR gamma(t), APC, clone: AFKJS-9	Invitrogen	17-6988-82
Anti-FoxP3, PE-CF594, clone: 236A/E7	BD Biosciences	563955
Anti-Gata3, eFluor450, clone: TWAJ	Invitrogen	48-9966-42
Anti-T-bet, Alexa Fluor488, clone: eBio4B10 (4B10)	Invitrogen	53-5825-82
Anti-CD183 (CXCR3), PE, clone: G025H7	Biolegend	353706
Anti-CD194 (CCR4), PerCP-efluor 710, clone: D8SEE	Invitrogen	46-1949-42
Anti-CD196 (CCR6), PE-Cyanine7, clone: R6H1	Invitrogen	25-1969-42
Anti-Eomes, APC eFlluor780, clone: WD1928	Invitrogen	47-4877-42
HKCA (Heat killed *Candida albicans*)	Invivogen	Tlrl-hkca

Isotype controls Mouse IgG1 kappa Isotype control (CCR4), PerCP-efluor 710, clone: P3.6.2.8.1 Mouse IgG1 kappa Isotype control (CCR6), PE-Cyanine7, clone: P3.6.2.8.1 Mouse IgG1 kappa Isotype control (CXCR3), PE, clone: MOPC-21 Rat IgG2a kappa Isotype control (RORγt), APC, clone: eBR2a Mouse IgG1 kappa Isotype control (FoxP3), PE-CF594, clone: X40 Rat IgG2b kappa Isotype control (GATA3), eFluor450, clone: eB149/10H5 Mouse IgG1 kappa Isotype control (T-bet), Alexa Fluor488, clone: P3.6.2.8.1	 Invitrogen Invitrogen Biolegend Invitrogen Biolegend Invitrogen Invitrogen	 46-4714-80 25-4714-80 981804 17-4321-81 562292 48-4031-82 53-4714-80
Lipopolysaccharide (LPS)	Thermo Fischer	00-4976-93
PBS Tablets	Merck	524650-1EA
Primers for T cell panel (primer sequences in appendix IIII) **Gene target** β-actin CCR4 CXCR3 EF-1 Eomes FoxP3 GATA3 RORγt T-bet	 Merck Eurogentec Eurogentec Merck Merck Eurogentec Eurogentec Merck Merck	
Rinsing buffer: MACS BSA Stock Solution	Miltenyi Biotec	130-091-376
Superscript™ IV VILO™ Master mix with ezDNase	Invitrogen (Thermo Fischer Scientific)	11766050
TexMACS medium	Miltenyi Biotec	130-097-196
Trypan blue solution 0,4%	CORNING	25-900-CI
UltraComp eBeads Compensation beads	Invitrogen	01-2222-42

3.2 Consumables, equipment, and kits

Product name	Producer	Cat. Number
384 PCR-plate vollrand	starstedt	72-1984-202
C-Chip disposable hemocytometer Burker B	NanoEntek	2B18250
Coolcell freezing container	Corning	432001
Cryotubes 1,2 mL	VWR	479-1254
Disposable Glass Pasteur pipettes	150mm	VWR 612-1701
eBioscience FOXP transcription factor Fixation/Permeabilization Kit	Invitrogen	00-5521-00
Eppendorf tubes Safe-lock Tube 1,5 mL	 Eppendorf	 0030-120-086
Falcon serological pipettes - 10 mL - 25 mL	Corning	 357551 P8250
Falcon tube - 15 mL - 50 mL	 VWR Sarstedt	 525-1068 62-547-254
Human IL-17 ELISA Kit	R&D Systems	P304050
LIVE/DEAD Fixable Yellow Dead Cell Stain Kit	Invitrogen	L34959
LS column	Miltenyi Biotec	130-042-401
MiniMACS separator	Miltenyi Biotec	130-042-102
MicroAmp Optical 96-Well reaction plate	Applied Biosystems (by Thermo Fischer Scientific)	N8010560
Microtube 2 mL	Sarstedt	72.694.006
Naïve CD4+ T cell isolation kit II	Miltenyi Biotec	130-094-131
Pipetboy acu 2 controller Integra	Integra Biosciences	
QIAshredder (250)	Qiagen	79656

QuadroMACS separator	Miltenyi Biotec	130-098-308
Rneasy mini kit (250)	Qiagen	74106
Scepter Sensors 40 uM	Millipore	PHCC40050
Twin.tec PCR plate 384	Eppendorf	F166876J
Vacuette Lithium Heparin tubes 9 mL	Greiner bio-one	455084

3.3 Instruments

Instrument name	**Producer**
BD LSR Fortessa	BD Biosciences
Centrifuge 5810	Eppendorf AG
CO2 incubator	Sanyo
GeneAmp PCR system 9700	Thermo Fisher
Multifuge 3SR+ Centrifuge	Thermo Scientific
Nanodrop ND-1000 Spectrophotometer	BD
Olympus CKX53 microscope	Olympus
Sceptre handheld automated cell counter	Merck (Millipore)
Vacusafe inspiration system	Integra Biosciences
Vortex 1 S000	Ika
Quant Studio 5 Real-Time PCR Intrument (384- Well Block)	Thermo Fischer Scientific

3.4 Software

Software name	Developer
BD FACS Diva	BD Biosciences
Microsoft Excel v.2208	Microsoft
Flow Jo 10.8.1	FlowJo LLC
GraphPad Prism 9.5.1	GraphPad
QuantStudio Design & Analysis Software1.6.0	Thermo Fischer
Softmax Pro Software	Molecular Devices
Thermo Fisher connect	Thermo Fisher Scientific

3. Methods

3.1. Experimental pipeline

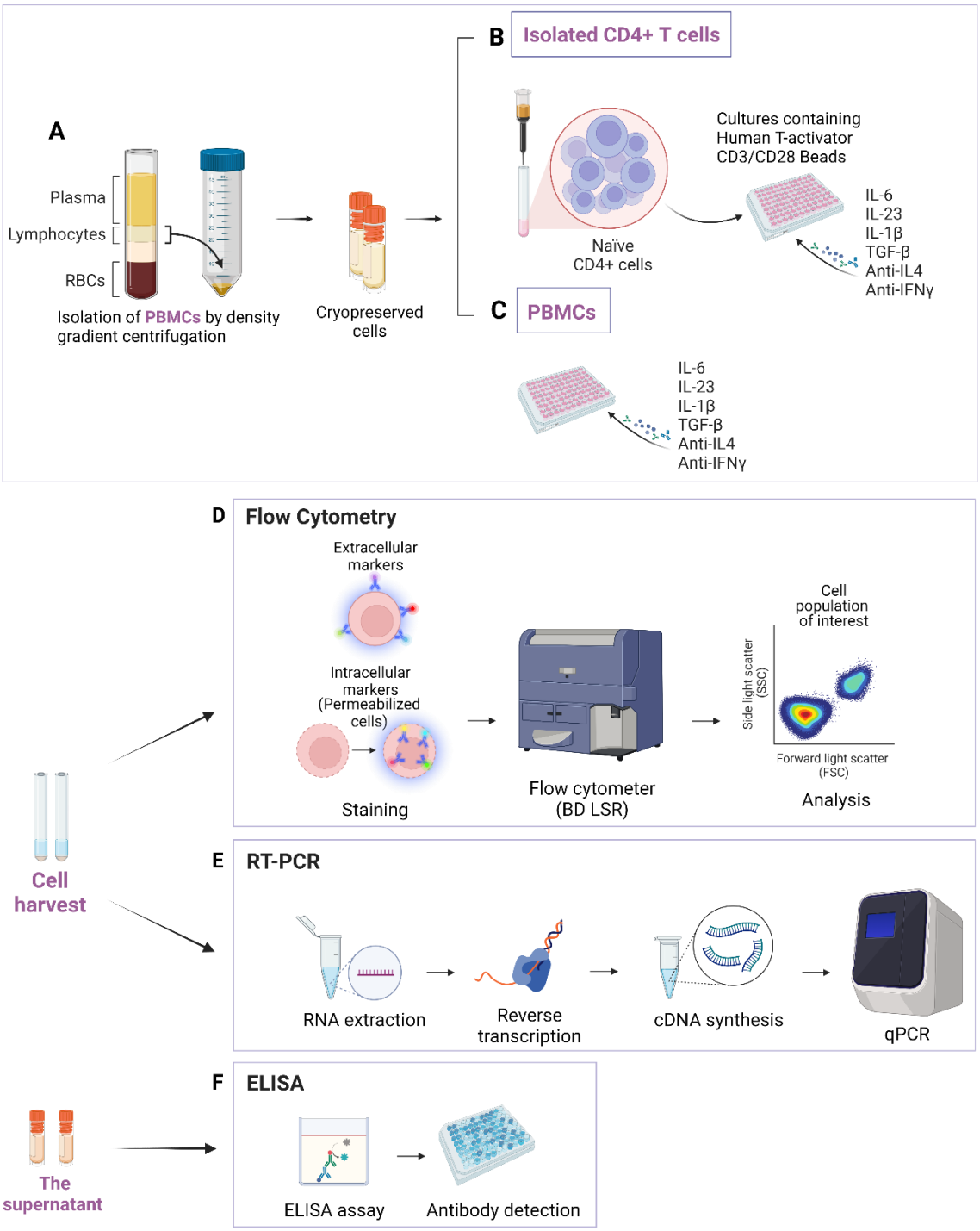

Figure 3.1. The experimental pipeline consists of a number of key methods (highlighted in black) that make use of particular biological materials (highlighted in purple). A) Isolation of PBMCs, **B**) Isolation and cell culture of CD4+T cells with Th17-polarizing cytokines and human T-activator CD3/CD28 beads, **C**) Culturing PBMCs with Th17-polarizing cytokines with heat killed *Candida albicans*. Following with endpoint assays

examining the survival, phenotype, and functionality of T cell subsets after culture using **D**) Flow cytometry, **E**) qPCR **F**) ELISA. The figure is created in Biorender.com.

In this project, we opted for two approaches to evaluate how Th17-cell differentiation and function may be impacted by cytokine autoABs in APS-1 patients. To start, we utilized *in vitro* cultures of CD4+ T cells isolated from whole blood of healthy control subjects (Fig 3.1A/B/C), including ten controls (without any criteria), added Th17-differentiating cytokines and then applied:

1. Serum from APS-I patients (N=6) with type-I IFN and IL-22 autoABs

2. Serum from APS-I patients (N=2) without IL-22 autoAB

3. Purified IgG from APS-I patients (N=3) with type-I IFN and IL-22 autoABs

4. Commercial type1 anti-IFN ABs

5. Commercial anti-IL-22 AB

Further, we estimated how these cytokine autoABs affected the response to C. albicans in *in vitro* cultures of PBMCs (Fig 3.1C), which are composed of different lymphocytes and monocytes and were isolated from both APS-1 patients and healthy controls. To monitor possible changes in the different T cell subtypes and function after seven days of Th17-inducing culture (cultures with isolated CD4+ T cells), we incorporated various well-established endpoint assays including flow cytometry, Real time polymerase chain reaction (qPCR), and enzyme-linked immunosorbent assay (ELISA) (Fig 3.1D/E/F).

3.2 Theory behind the methods

3.2.1 Flow cytometry

Flow cytometry is a powerful tool effective for studying cell populations at the single level based on their physical and chemical properties, that has revolutionized our understanding of intricate biological processes. This potent technology was employed in our project to swiftly evaluate and gauge the characteristics of different CD4 T cell subsets. In this procedure, a cell sample is first treated with fluorescent dyes that bind to specific cellular markers on the exterior or interior of cells. Cells in fluid samples are then arranged into a stream of individual particles

that guide them one by one through single or multiple laser beams and each fluorophore-labelled individual particle then can be probed separately for visible light scatter and one or more fluorescence parameters that is detected by instrument sensors. Subsequently, signals from each fluorochrome are captured and the scattered light and fluorescence generated by each cell is measured (Fig 3.2). Lastly, the data acquired by the cytometer can be used to study information on particle properties that include size, shape, granularity, and fluorescence intensity. One consideration about utilizing several fluorophores in this method is bleeding of signals into other channels due to overlapping emitting spectral properties. To account for these overlaps, a mathematical process called compensation is needed, which subtracts overlapping signals using complex matrices. A gating strategy is then used to evaluate the data, allowing one to distinguish between cell populations that are positive for each marker with sequential methodology, specific for the information that is wanted. This information is important to distinguish specific cell subsets.

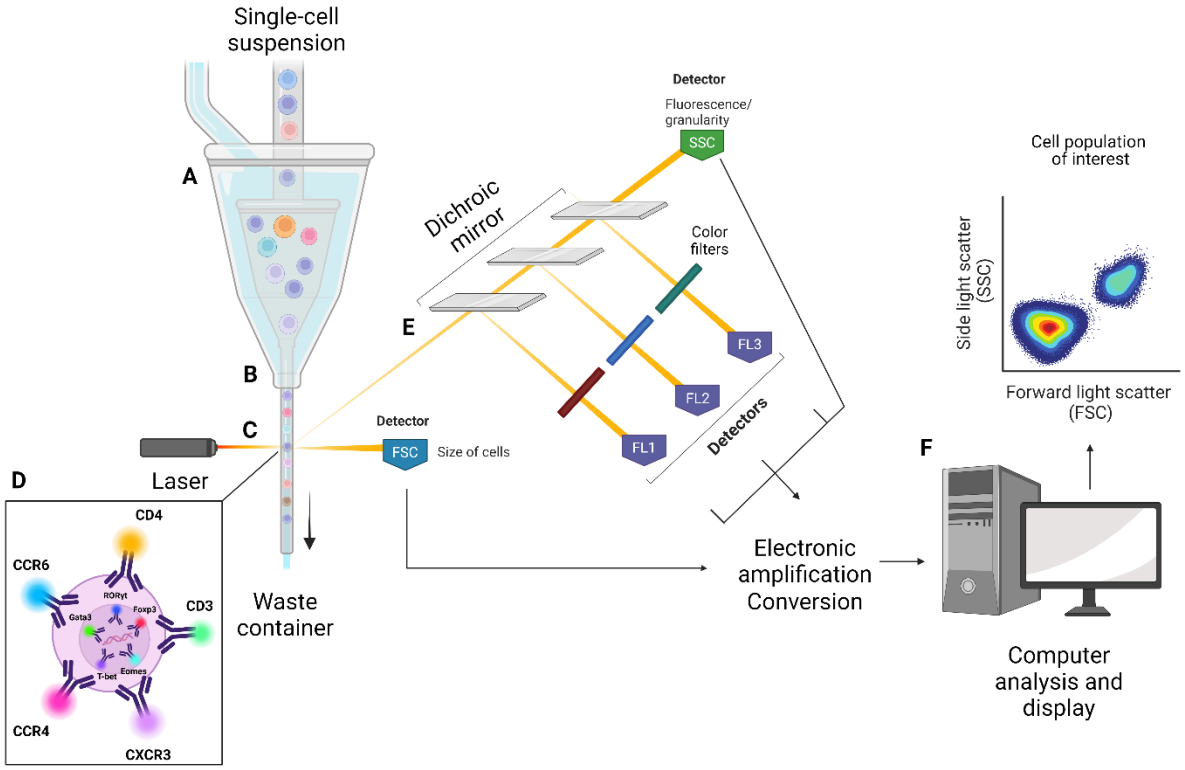

Figure 3.2. The fundamental principles of flow cytometry. A) Cells are introduced into the cytometer and focused into **B**) a single-cell stream using a fluidics system that makes the cells pass through the cytometer one at a time. **C**) cells are exposed to lasers that excite fluorophores attached to ABs targeting specific phenotypical or functional markers. **D**) here exemplified by a CD4+ T cell, extra- and intracellular markers of different T cell subtypes that we utilized in experiments in this study. **E**) Upon excitation, each fluorophore emits light with a longer wavelength, which is then directed through a series of mirrors and bandpass filters. These filters select a narrow range of wavelengths that correspond to specific fluorophores and direct them towards corresponding detectors (e.g., FSC: forward scatter channel, FL: Fluorescence channels). **F**) The detectors convert the received

light energy into voltage pulses, which are then captured and analyzed by the software, providing data for further analysis on plots. The figure was modified from (O'Neill et al. 2013) and created in Biorender.com.

3.2.2 Real time quantitative polymerase chain reaction

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) is a laboratory technique that combines reverse transcription and PCR to detect and quantify RNA molecules in a sample. RT-PCR was carried out in this instance as an additional technique to measure the expression of genes involved in CD4 T cell differentiation, activation, and function. Measuring the gene expression in CD4 T cells from different individuals and under various experimental circumstances, enables insight into molecular mechanisms underlying CD4 T cell biology and immune responses. Using this method in some of our experiments, we quantified the expression of lineage-specific genes such as RORγt, FoxP3, T-bet, and GATA3, along with the expression of extracellular receptor genes including CCR6, CCR4, and CXCR3 (as listed as primers in Table 3.1).

The first step of RT-PCR is reverse transcription, where RNA is converted into complementary DNA (cDNA) using a reverse transcriptase enzyme. The target sequence of interest can then be amplified using cDNA as the starting material in a dye-based (SYBR green) or probe-based (TaqMan assay) PCR reaction. A SYBR Green PCR approach is utilized in this project, in which the number of products made is quantified based on the fluorescent dye SYBR Green which binds to generated double-stranded cDNA. To add specificity to the reaction, a pair of PCR primers are designed that specifically amplify the wanted-cDNA sequence. As the DNA is amplified during the qPCR procedure, the quantity of double-stranded cDNA rises and hence the intercalated SYBR Green dye between the cDNA strands also rises, causing an increase in fluorescence intensity. The cycle at which the fluorescence signal reaches a specific threshold, which is usually defined as when the increase rate of the fluorescence signal is most linear, is called the cycle threshold (Ct). The Ct value is used to calculate the amount of cDNA in the sample, which can then be related to the initial RNA level of this particular gene. A high Ct indicates a low initial concentration of RNA while a low Ct-value (below 35) indicates a high expression of that particular gene in the indicated sample. The delta-delta Ct method was employed to determine the fold changes in gene expression for each sample. In this method, he delta Ct value is a measure of the variation between two or more samples that is determined by comparing the threshold cycle (Ct) values of the target gene and a house keeping reference

gene. Thus, the delta Ct value is the relative abundance of each sample's target gene, corrected to that of the reference gene. Further, the delta-delta Ct technique compares the delta Ct value of the target gene in one sample to the delta Ct value of the target gene in another sample, which makes it possible to calculate the fold changes in gene expression for each sample.

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

The functioning of the Th17 cell subsets was evaluated using sandwich ELISA assays to determine the secretion of its hallmark cytokine, IL-17, following the cell expansion under various circumstances with or without addition of cytokine ABs in medium. The ELISA assay requires microplates that are precoated on a surface, with an immobilized capturing ABs that will specifically bind their target antigen of interest once the samples are added onto the plate. After any unbound materials have been removed by washing, the detection AB, which is specific for a distinct epitope on the same target antigen and is conjugated to an enzyme, is then added, and given time to attach to the antigen, creating a sandwich complex. When a substrate is introduced, this enzyme-linked second AB, such as Avidin-Horseradish-Peroxidase (HRP), reacts with the chromogenic substrate and provides a quantifiable signal, which is commonly a color change or fluorescence. Chromogenic substrate that is typically used to react with HRP is tetramethylbenzidine (TMB), a colorless compound that is oxidized by hydrogen peroxide (H_2O_2) in the presence of HRP to yield a blue-colored end product. The addition of a strong acid called stop solution, which transform the product's color from blue to yellow, can finally terminate the reaction. Thus, the intensity of the resultant color, which directly correlates to the level of enzyme activity and, consequently, the presence or quantity of cytokines in the samples, is measured using a spectrophotometer.

3.3 APS-1 patients and healthy controls

Blood was collected in serum separator tubes for serum and in heparin-tubes for subsequent isolation of PBMCs and frozen at -80 ºC for one to three days, before being placed for long time storage at -150 ºC. Sera, purified IgG, and cells from APS-1 patients were available from the Norwegian national registry of organ specific autoimmune disorders (ROAS), and they had all signed written consents for participation in this project (N=8, mean age: 39 years, age range of 17-67, 2 females, 6 males) (Appendix I). This study was approved by the Regional Committee for Medical and Health Research Ethics (REK), Western Norway (biobank approval number 2013/1504 and project approval numbers 2018/1417 and 2009/2555).

Blood from 70 healthy donors were available from the blood bank of Haukeland University hospital, Bergen, Norway. All donors had agreed to participate in research projects, and they were anonymised in this study. From these donors, three sex-matched controls with APS-1 patients (mean age: 55 years, age range of 41-63, 3 males) were used for culturing the last experiment on PBMCs.

3.4 Isolation and freezing of human peripheral blood mononuclear cells

PBMCs were isolated by density centrifugation using Ficoll-Paque™ Plus. Fresh whole blood was collected in two 9 mL heparin tubes, and the blood was then diluted with PBS at a 1:1 ratio. The diluted cellular whole blood fraction was overlaid onto 12 mL of the Ficoll-Paque™ Plus and then subjected to 400xg for 30 min at room temperature (RT) with low break acceleration ("1"). The PBMC layer was extracted, topped with PBS up to 50 ml and washed at 400xg, RT for 10 min. The supernatant was aspirated, and the pellet resuspended in 10 mL PBS. The Scepter™ 2.0 Handheld Automated Cell Counter with 40uM sensor was used to count the cells. The samples were then washed at 400xg in RT for 5 min, and the cell pellet resuspended in human AB serum containing 10% (v/v) DMSO. The cells were left at RT for 10 min in a CoolCell cryogenic container and the containers were then frozen at -80 ºC for one to three days, before being placed for long time storage at -150 ºC.

3.5 Magnetic CD4+ T cell isolation and expansion

Frozen PBMC samples were rapidly thawn in hand and transferred to 15ml falcon tubes for wash to remove residual DMSO. The cells were rinsed 2X with up to 10 ml (PBS w/0.5% BSA) by centrifugation at 350xg for 10 min at RT and then counted manually by Trypan Blue and light microscope for total count. Naïve CD4+ T cells were isolated from PBMCs using the Human Naïve CD4+ T cell Isolation kit II from Miltenyi according to the protocol of the manufacturer. In brief, per ten million isolated CD4+ T cell, 40 µl of (PBS w/0.5% BSA) buffer and 10 µl of Biotin AB cocktail II were added and incubated at 2-8 ºC for 5 minutes. Addition of 30 µl of (PBS w/0.5% BSA) buffer and 20 µL of microbead cocktail, per ten million cells, was the next step followed by incubation at 4 ºC for 10 minutes.

An LS column was washed with 3 ml (PBS w/0.5% BSA), before application of the cells. Cells were transferred to the column with 3 mL (PBS w/0.5% BSA) and collected from columns in 15ml tubes. The newly isolated CD4+ T cells were counted manually by Trypan Blue for total

count and washed by centrifugation at 350xg for 10 min at RT. The pellet was resuspended in culture medium (TexMACS medium).

3.6 Cell culture and Th17-differentation conditions

Purified CD4+ T cells were resuspended to $2*10^5$ cells/mL in TexMACs medium and cultured in 96-well plates in presence of 1.5 µL pre-washed Dynabeads Human T-Activator CD3/CD28 (1:2 bead-to-cell ratio). Th17-polarizing cytokines were added to the culture, including IL-1β (0.002ng/µL), IL-6 (0.003ng/µL), IL-23 (0.003ng/µL), and TGF-β (0.25ng/µL), as well as anti-IFNγ (100ng/µL) anti-IL-4 (250 ng/2.5 µL) in 100µL TexMACs medium. Cells were incubated for seven days at 37 °C, in an atmosphere of 5% CO2, without any media exchange.

The day after seeding (day 1), an additional 100 µL fresh culture medium, and same concentrations of Th17-polarizing cytokines, anti-IL-4 and anti-IFNγ were added. Cell growth was visually monitored during the course of the seven days of expansion.

Sera, IgG or commercial cytokines were then added to the cultures in different amounts. Due to the cumbersome assays and availability of precious sera and IgG from patients, these concentrations were pre-decided based on other studies conducted in the group in the past. IgG had been prepared from a few APS-I patients and healthy controls prior to this master project and was available to the Candidate in this project. Commercial cytokines were added in different amounts (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL). Additionally, two different concentrations (2 µL and 5 µL) of sera from healthy controls, 2 µL of APS-1 sera and 1 µL of purified IgG from both healthy controls and APS-1 patients were included in the experimental cultures.

3.7 PBMC expansion and cell culture conditions

Frozen PBMC samples were rapidly thawed by hand and transferred to 15ml falcon tubes to remove residual DMSO through a wash step. The cells were washed twice with up to 10 ml of PBS containing 0.5% BSA by centrifugation at 350xg for 10 min at RT. After washing, the cells were manually counted using Trypan Blue and a light microscope to determine the total cell count. The cells were then resuspended to a concentration of $2*10^5$ cells/mL in TexMACs medium and cultured in 96-well plates. Th17-polarizing cytokines, including IL-1β (0.004ng/µL), IL-6 (0.006ng/µL), IL-23 (0.006ng/µL), and TGF-β (0.5ng/µL), as well as anti-IFNγ (200ng/µL) anti-IL-4 (500 ng/5 µL) ABs in 200µL TexMACs medium, were added to

the culture on day 0. The cells were then incubated for seven days at 37 °C, in an atmosphere of 5% CO2, without any media exchange.

3.8 Flow cytometry

3.8.1 Staining of harvested PBMCs and T cells for flow cytometry

Both PBMC and T cell samples from Th17 cultures were stained in the same manner. After being harvested, the cells were washed at 350xg, 20 ºC for 10 min with up to 3 mL PBS. Cells were resuspended in the remaining buffer (approx. 100 µL), and the supernatant was collected and kept at -80 ºC for later ELISA investigation. The Anti-CCR6 AB was added to the cell samples and incubated for 45 minutes in the dark at an optimal staining temperature of 37 °C (described in 4.3.3). Subsequently, cells were rinsed in up to 3 mL PBS at 350xg, 20 ºC for 10 min and resuspended in 1 mL PBS. In keeping with the instructions provided by the manufacturer, dead cells were stained using LIVE/DEAD Fixable Yellow Dead Cell Stain Kit. After another incubation for 30 min at RT in the absence of light, the cells were washed with 1 mL flow buffer (PBS w/0.5% BSA) at 350xg, 20ºC for 7 min. Cells were resuspended in the leftover buffer (about 100 µL) after the supernatant was removed. A master mix of ABs specific for extracellular markers as instructed in Appendix III, was made and applied to samples. Each cell sample was then incubated in the dark at 4 ºC for 30 min before washing at 350xg, 20ºC for 7 min with flow buffer. After aspiration, the cells were fixed and permeabilized using the eBioscience™ FOXP3/Transcription Factor Fixation/Permeabilization Kit after the supernatant was taken out pertaining to the directions issued by the manufacturer. Accordingly, 1 mL of Fix/perm buffer working solution was added to each sample and stored for 1 hour in the dark at 4 ºC. Subsequent to the incubation, the samples were washed with 2 mL 1X permeabilization buffer from the same Kit, and centrifuged at 400xg, 20ºC for 10 min. Supernatant was taken out and cells were resuspended in about 100 µL of the remaining buffer. Then a master mix of ABs targeting intracellular markers was prepared (as per Appendix III preparation instructions) and added to each sample. The samples were incubated for overnight at 4 ºC in the dark. The following day, the samples were rinsed with 2 mL 1X permeabilization buffer from the eBioScience FOXP3 Fixation/Permeabilization Kit and drained off and the cells resuspended in 250 µL permeabilization buffer. Eventually, after performing the analysis with a BD LSR Fortessa flow cytometer, the data was analyzed using FlowJo version 10.8.1.

Most of the flow protocol including voltage settings for Fortessa had been optimised previously in the laboratory. Therefore, this master thesis did not titrate ABs.

3.8.2 Optimizing flow cytometry: The importance of controls for accurate analysis

eCompensation beads were tested to assess single stained (compensation) controls to solve fluorescence spillover. Considering this, 1 µL of each AB except for anti-CD3 stain (2 µL) were added to 100 µL of flow buffer and compensation beads in separate tubes (with one tube left unstained) and kept for 20 min in the dark. To prepare for flow analysis, beads were then washed and re-suspended in about 300 µL of flow buffer. These were then used for compensation in FlowJo.

Moreover, using Th17 cell harvest after 7 days of culture, fluorescence minus one (FMO) controls of all ABs were generated, where one fluorochrome-conjugated AB is excluded from the multicolor staining panel for each stain. During flow data analysis, this was done to accurately differentiate the background fluorescence resulting from non-specific binding of the other ABs in the panel to make sure that the specific AB, which was omitted from the control sample, was gated precisely. Since we observed large autofluorescence signals for several of the fluorophores, we also used isotype controls to ascertain that any binding results from specific interactions between the experimental AB and the target antigen. Isotype controls are identical to the target experimental AB, but typically specific to a different protein or antigen that is absent in the sample being tested. In this case, any signal observed from the isotype control can be taken to be false positive or background noise. Accordingly, the level of specific binding can then be assessed with greater precision by excluding this background signal from the intensity of fluorescence captured in the experimental channel.

3.9 Relative quantification analysis of gene expression levels in harvested T cells

3.9.1 RNA isolation

RNA isolation was performed on harvested culture cells with the same conditions as cells collected for flow staining. Cells, for this manner, were collected and washed at 350xg, 20 ºC for 10 min with up to 3 mL PBS, and then the pellets were resuspended and frozen at -80 ºC in 350 µL of RLT lysis buffer from the RNeasy Mini Kit (Qiagen). On the day of RNA analysis, cells were thawn and proceeded with the isolation. The RNeasy Mini Kit (Qiagen) was followed according to the manufacturer's protocol. Briefly, the cell suspension was transferred

to columns from QiaShredder kit and centrifuged at 13.300 rpm for 2 min to homogenize the lysate. Cell lysates were then added to a gDNA eliminator spin column. The flow through was blended with 350 µL of 70 % ethanol and transferred to a RNeasy MiniElute column, followed by washing once with 700 µL RW1 buffer and twice with 500 µL RPE buffer. The extracted RNA was collected in a new tube following double elution in 35 µL DEPC-treated water. Using a Nano-drop ND-1000 spectrophotometer, the RNA concentration in each sample was calculated, before being frozen at -80 ºC or directly continuing towards cDNA synthesis.

3.9.2 cDNA synthesis

cDNA synthesis was carried out using Superscript IV VILO Kit in accordance with the instructions provided by the kit with input RNA concentrations of 200 ng/mL of each sample that were diluted, if necessary, in DEPC-treated water. For the synthesis, the GeneAmp PCR System 9700 was used, and the following temperature settings were applied: 25 ºC for 10 min, 50 ºC for 10 min, and 85 ºC for 5 min. The cDNA samples were kept at -80 ºC in case they weren't immediately utilized for qPCR.

3.9.3 Optimization of the qPCR SYBR green assay

Preliminary qPCR tests verified the optimal function of the following primers, at a concentration of 10 µM, for evaluating the expression of genes unique to T-cell lineages. Sequences for these primers are included in Appendix IIII: RORγt, FoxP3, Eomes, GATA3, T-bet, EF-1, β-actin, CCR4, and CXCR3. Accordingly, cDNA was added to the reaction mixtures in 3 dilutions: 1000 ng/µL, 200 ng/µL, 20 ng/µL, and one blank (0 ng/µL), followed by a melting curve to check the specificity of the primers for each target gene. This was done to evaluate the effectiveness of the assay with reference to various cDNA inputs. Since all conditions gave adequate response curves, we chose the middle concentration, i.e., 200 ng/µL, to be used as input for subsequent investigations.

3.9.4 RT-PCR SYBR green assay of T cell gene panel

The T cell gene panel was subjected to qPCR using a SYBR Green-based assay. The 2x PowerTrack SYBR Green Master Mix was used, diluted from stock to a concentration of 10 mM, and then combined with forward and reverse primers (5% v/v from 1:10 dilution each), and DEPC treated water (34% v/v) to create a master mix. In the following step, the cDNA (10% v/v, diluted 1:10 from 3.9.2) and yellow sample buffer 40X (2,5% v/v) were merged, and

this mixture was then added to the aforementioned master mix in a 96well-plate to generate a reaction mix with a total volume of 33 µL, of which 10 µL triplicates were taken and transferred to a 384-well plate. Subsequently, the QuantStudio 5 Real-time PCR system was used to execute a run consisting of a preparative phase at 95 ºC for 2 min, 40 cycles of denaturation at 95 ºC for 15 s, and annealing/elongation step amplifying for 60 s at 60 ºC. In addition to housekeeping genes, each reaction mix contained a non-template control made up of a mixture of DEPC-treated water with a master mix of primers described above. All samples were tested in triplicates.

The assay design including plate and run preparation were provided by Quant Studio Design and Analysis Software, capable of normalizing the data using normalization approaches, such as the delta-delta Ct method. This technique allows one to calculate the ΔCt value for each sample by subtracting the housekeeping gene(s) Ct value(s) from the Ct values (mean of Ct values from three replicates) of the genes of interest. After that, the ΔCt value of the calibrator was deducted from the ΔCt value of the sample, to determine the ΔΔCt value for each sample, which followed by utilizing the equation 2^(-ΔΔCt) to measure the fold change in the expression of the target gene relative to the calibrator. Thus, the relative variation in the expressed gene of interest in comparison to the control was measured to conduct statistical analyses (detailed in 3.11). We used both β-actin and RORγt as calibrators for our studies.

3.10 ELISA

ELISAs were performed on culture supernatants collected following T cell expansion for 7 days. For this, approximately 200 µL of supernatant all were collected and diluted in 3 mL PBS for a wash. After centrifugation, about 2 mL of the supernatant was stored for ELISA in cryopreserved tubes and kept at -80 ºC. The human IL-17 ELISA kit (R&D systems) was utilized for this purpose, and the procedures detailed in the protocol were followed exactly. In this kit, 100 µL supernatant was directly used. Performing a SpectraMaxPlus spectrophotometer, the absorbance values IL-17 cytokine was measured at wavelengths of 450 nm. Lastly, the concentration of the cytokine in the samples were accurately quantified using SoftMax Pro software by taken into account that the supernatants were diluted 15X from the original cultures. Using this software, the calibration curve was created from a dilution series of the protein standard provided with the kit, based on which the signal computed from each sample was converted into a concentration value.

3.11 Statistical data interpretation and graphical display

GraphPad Prism 9.5.1 was utilized for statistical analyses and to create the graphical representations of the results described in this thesis. When evaluating the effect of APS-1 and healthy control (HC) sera and APS-1 IgG on T cell subsets within CD4+ T cells isolated from healthy individuals, non-parametric t tests (Wilcoxon test) and A non-parametric, one-way ANOVA test was performed on flow cytometry data. For flow cytometry data of conditions with APS-1 and HC sera/IgG on isolated PBMCs from healthy and APS-1 individuals A non-parametric, one-way ANOVA test-Kruskal Wallis test, was performed. For the data analysis of qPCR results, effect of sera/IgG from APS-1 and controls was evaluated using non-parametric t tests (Wilcoxon test). For all statistical tests significance was characterized by a p-value of less than 0,05. For the remaining experiments where only one biological parallel was measured for each condition because of the limited number of available cells, no statistical testing was applied.

4. Results

4.1 Isolation and expansion of PBMCs

PBMCs were isolated from 70 healthy controls, and the estimated cell count (counting step detailed in 3.4) with an average of $26{,}21*10^6$ (ranged from$1{,}26*10^6$ to $61{,}45*10^6$) cells from 18 mL of blood. The cells were then frozen at -80 ºC for one to three days before being stored for a long period at -150 ºC. PBMCs were used for optimization of cell culturing procedures, preliminary qPCR experiments and preliminary flow cytometry analysis. In the experiments with PBMC cultures, PBMCs from fewer than 10 healthy controls were utilized, and the cell number was estimated manually. Cells from three APS-1 patients and three sex-matched controls were used individually for the *Candida* experiments (sections 4.6 and 4.7). For all experiments, PBMCs were expanded for 7 days *in vitro* and followed by endpoint assays using flow cytometry.

4.2 Isolation and expansion of CD4+ T cells

For every CD4+ T cell experiment, cells from 6 to10 healthy controls were magnetically isolated and merged, and the cell number was estimated with the average of $12{,}7*10^5$ (ranging between $8{,}2*10^5$ and $17{,}2*10^5$) CD4+ T cells. Harvested samples were used in optimization experiments aside from further analysis employing ABs. After 7 days of *in vitro* cell expansion, the cells were subjected to endpoint tests using flow cytometry, qPCR, and ELISA.

4.3 Flow cytometry analysis of control T cells, and patient/control PBMCs

4.3.1 Isotype controls

“Fluorescence minus one” (FMO) and isotype controls were utilized to evaluate the background fluorescence and non-specific binding in a particular channel, as stated in 3.8.2, in order to improve the specificity and accuracy of the flow assays. For the most common markers (CD3, CD4, RORγt, FoxP3, T-bet, GATA3, CCR6, CCR4, CXCR3) FMOs were used as negative controls whereas isotype controls were necessary for proper background controls for the same markers.

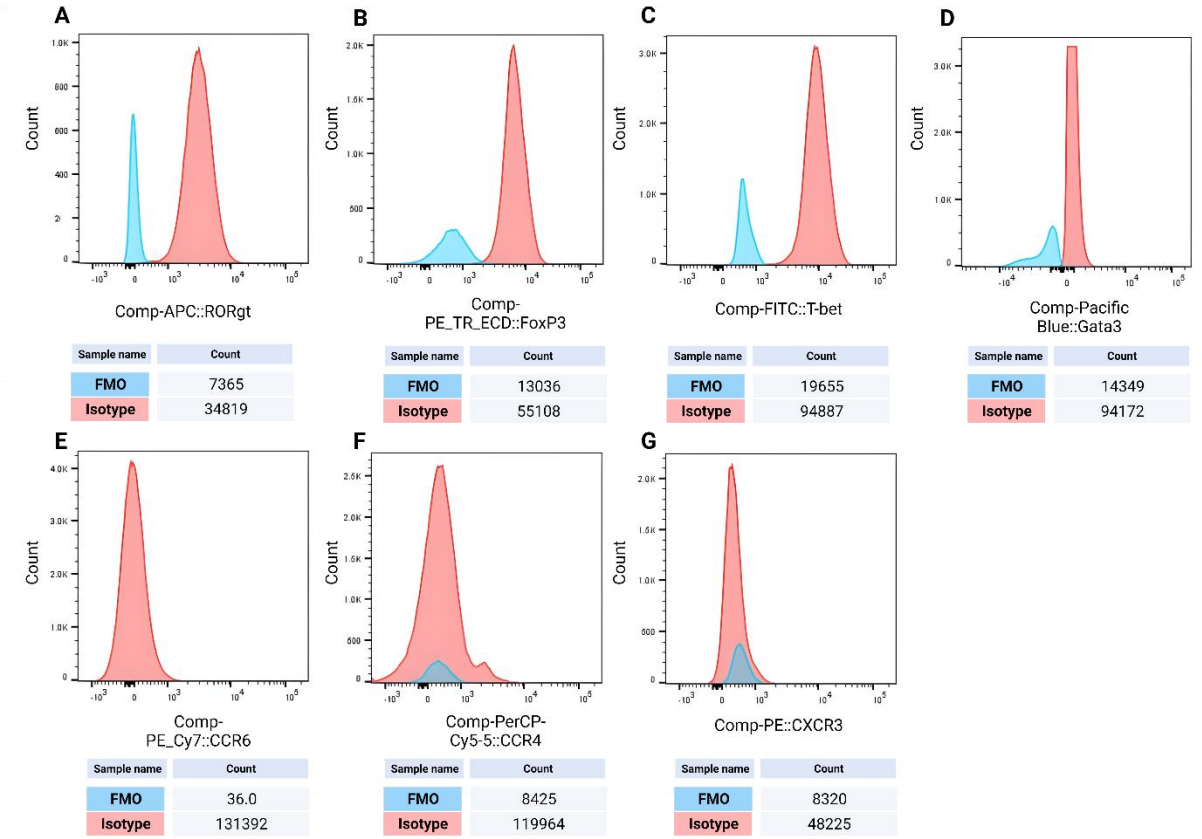

Figure 4.1 Isotype controls versus FMO controls: Comparative assessment on different channels. As depicted in the illustration, the distribution and intensity levels of the fluorescence signal from Isotype and FMO controls were compared by evaluating the number of cells in plots displaying count versus a specific channel for a particular marker**.** Cell counts indicated in boxes. The figure was created with FlowJo 10.8.1.

Figure 4.1 shows how FMOs stained compared to isotype controls, visualising that the isotype controls were needed to compensate for autofluorescence and unspecific binding for proper gating of cells post cell culturing.

4.3.2 Gating strategy for flow cytometry experiments

Subsequent to cell culture, PBMCs and isolated CD4+ T cells were collected and subjected to staining for flow cytometry analysis. This analysis aimed to evaluate the survival and proliferation of specific T cell subsets. The gating strategy used, is shown in figure 4.2.

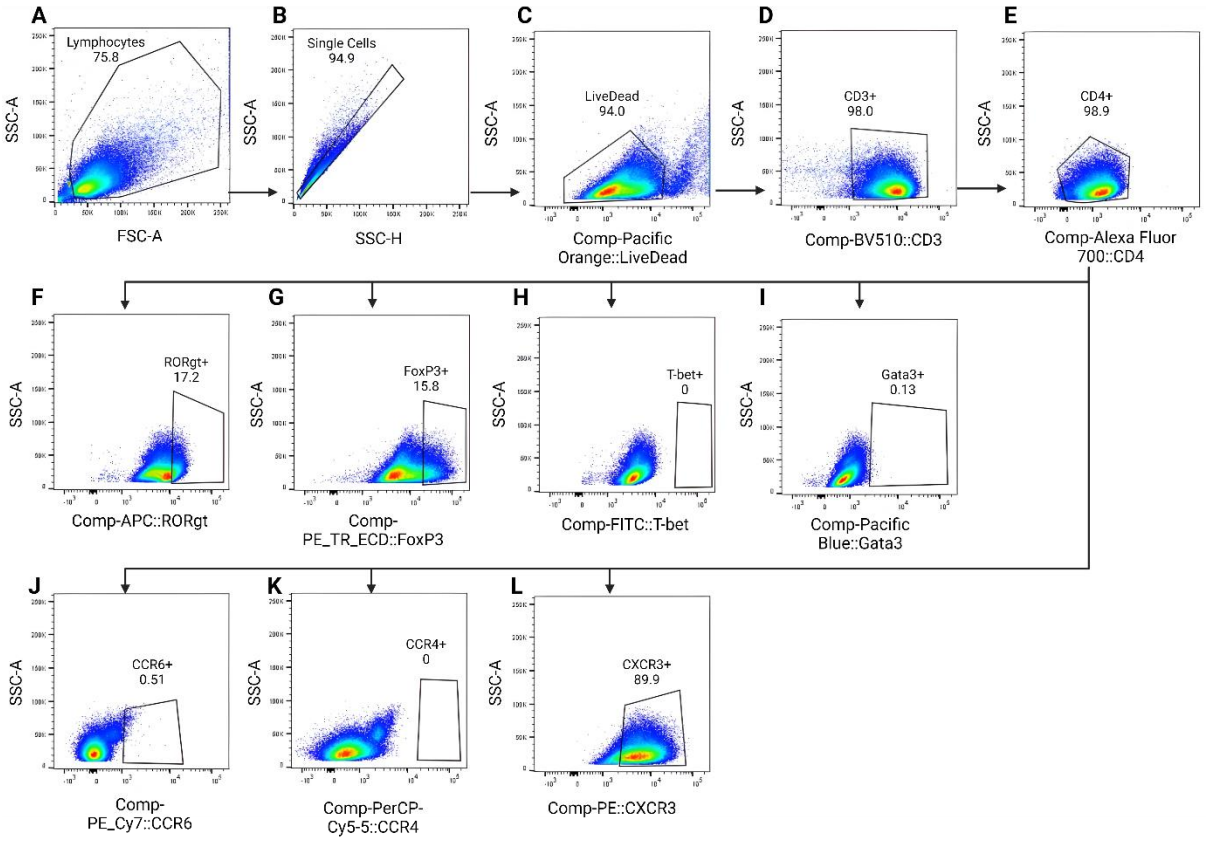

Figure 4.2 The gating strategy used for PBMCs/CD4+ cells to evaluate the quantity of viable cells and their proliferation profile after 7 days of culture in Th17 polarised conditions, Here is a representative sample from merged healthy CD4+ control cells **A**) Initially, a FSC versus SSC plot was used to identify cells with proper size and granularity **B**) Using SSC area and SSC height parameters, single-cell gating was applied to exclude cell aggregates and debris. **C**) Dead cells with damaged plasma membranes that make them susceptible to being stained by dead cell stain dye, were gated out based on a plot of SSC versus dead cell stain. Followingly, to assess the phenotypic and functional markers on cells **D**) live cells were gated for CD3+ T cells using SSC versus BV510 plot. **E**) Within the CD3+ population, CD4+ T cells were assessed using SSC versus PerCP-Cy5.5. From the CD4+ T cell population depicting SSC versus the fluorescence of the relevant fluorochrome were utilized to accurately determine the presence of these specific subsets of CD4+ T cells. The following subsets were defined based on these plots: **F**) Th17 cells, characterized by the presence of the RORγt marker. **G**) Tregs, identified by the expression of the FoxP3 marker. **H**) Th1 cells, distinguished by the presence of the T-bet marker. **I**) Th2 cells, dentified by the GATA3 marker. Moreover, the extracellular markers were also considered: **J**) CCR6, **K**) CCR4, **L**) CXCR3. The isotype controls depicted in table 3.1 was applied to set the gates right. Gating was generated using FlowJo 10.8.1.

4.3.3 Optimization of Anti-CD196 (CCR6) AB in flow cytometry experiments

The protocol for most of the markers was already available prior to this thesis, but conditions to evaluate the CCR6 expression had to be optimized. In this optimization process, the CCR6 AB dilution of 1:50 was applied to cell samples prior to the addition of any other Abs, and incubation occurred at three different conditions (Fig 4.3). The optimal incubation time of 45 minutes at 37 ºC for the anti-CCR6 AB was applied for the rest of the experiments. FMO control for this AB (described in 3.8.2) was employed to avoid any spectral overlap or interference between this fluorescence channel and the other fluorochromes in the panel.

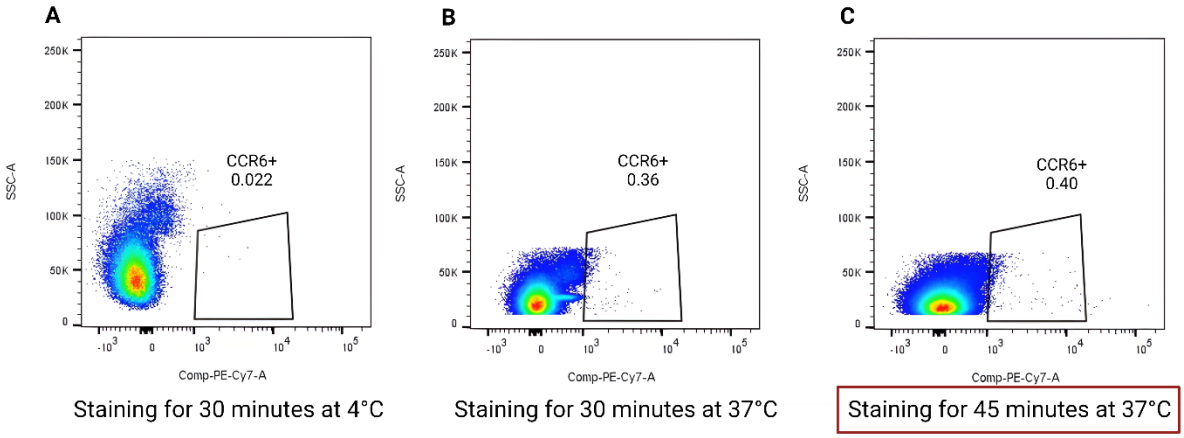

Figure 4.3 Optimizing Flow Cytometry Analysis of Anti-CD196 (CCR6) Antibody Staining. A) Anti-CCR6 AB was merged with a master mix of other extracellular markers (instructed in Appendix III) and incubated for 30 min at 4 ºC in the absence of light. **B**) & **C**) addition of anti-CCR6 AB to cell samples prior to the staining process with incubation at 37 ºC at two distinct durations B) 30 minutes, and **C**) 45 minutes. The figure was generated using FlowJo 1.8.1.

Due to difficulties in gating on the CCR6 fluorescence channel, the Anti-CCR6 AB was stained separately before other ABs. Subsequent experiments revealed that an incubation time of 45 minutes yielded the best results for staining with this particular extracellular AB.

4.3.4 Optimization of cell culturing conditions

Evaluating the impact of human AB serum addition on cell culture conditions for Th17 cell growth:

To compare conditions for Th17 differentiation and proliferation with and without AB serum, we examined two different concentrations (5 µL & 10 µL) of AB serum added to 100µL of TexMACs medium. Th17 cell signature cytokines (IL-1β, IL-6, IL-23, and TGF-β) were present during the culture in this instance.

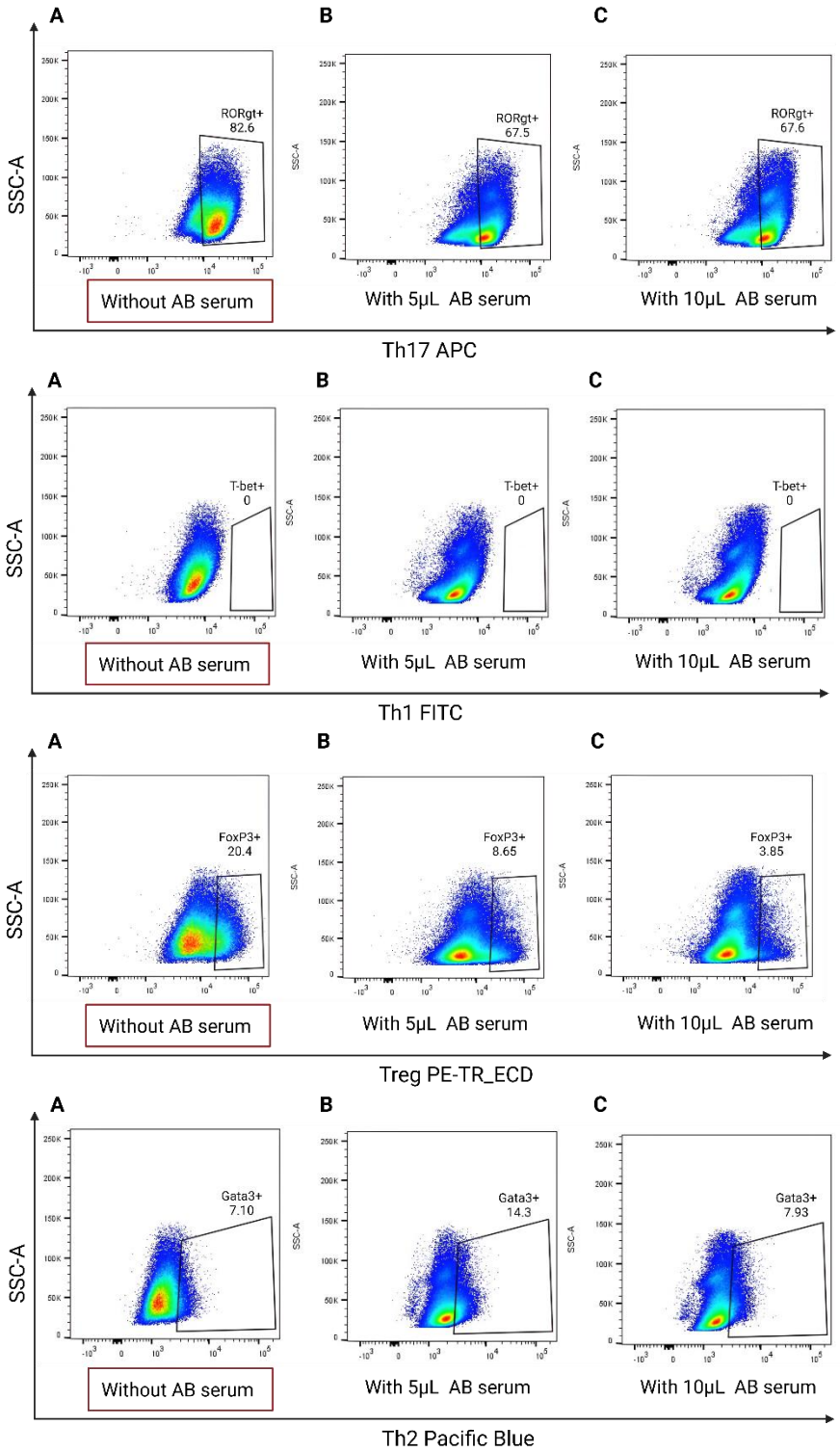

Figure 4.4 Examining the effects of AB serum addition to the culture media and how it affects development and viability of distinct T cell lineages (100 microliter cell culture; $2*10^5$ number of cells). **A**) RORγt, FoxP3, GATA3, and Tbet expression after culturing without AB serum (chosen further). **B**) RORγt, FoxP3, GATA3, and Tbet expression after culturing with 5 microliter AB serum. **C**) RORγt, FoxP3, GATA3, and T-bet expression after culturing with 5 microliter AB serum. The figure was generated using FlowJo 1.8.1.

Although no substantial differences were observed in Th17 cell proliferation within the desired concentrations of the serum during the cell culture, the addition of AB serum exhibited a slight inhibitory effect on Th17 cell development from CD4+ T cells compared to the conditions without AB serum (Fig. 4.4). Based on these findings, it was determined that using this particular serum in the culture would not be advantageous, and therefore, it was excluded from further experimentation.

Assessing the role of Anti- IFNγ and Anti-IL-4 ABs in modulating T Cell subset differentiation in cell culture:

In this optimization step, the objective was to suppress the differentiation of naïve CD4+ T cells into Th1 and Th2 cells. This was achieved by introducing ABs targeting the cytokines involved in these differentiations: anti-IFNγ (100 ng/μL) and anti-IL-4 (250 ng/2.5 μL), respectively, applied in 100 μL of TexMACs medium containing $2*10^5$ isolated CD4+ T cells (Fig 4.5).

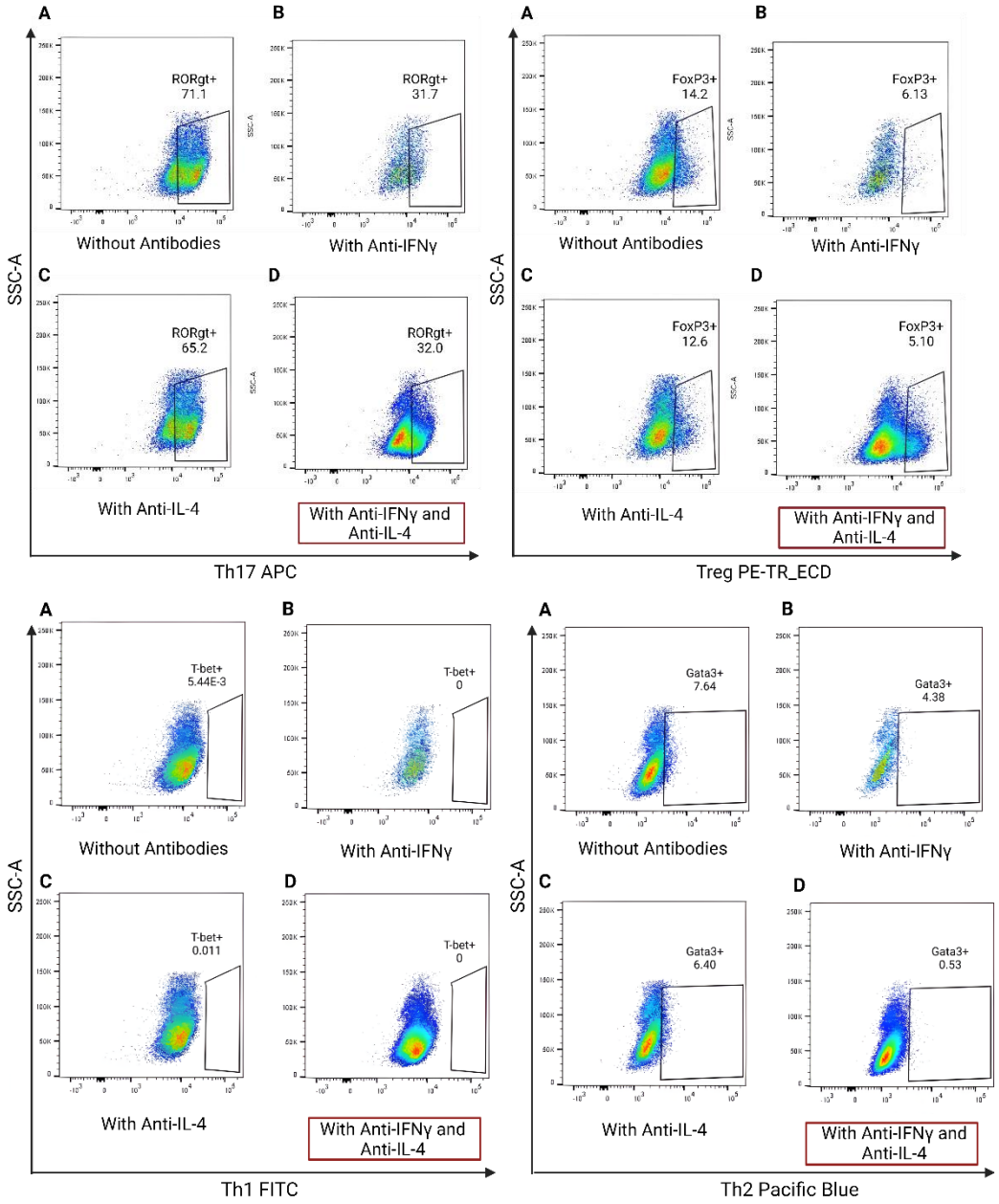

Figure 4.5. The effect of Anti-IFNγ and Anti-IL-4 ABs in modulating T cell subset differentiation *in vitro*. The figure displays four distinct cell conditions: **A**) no addition of ABs, **B**) presence of only anti-IFNγ, **C**) presence of only anti-IL4, and **D**) presence of both ABs simultaneously. The proliferation index values generated by flow cytometry analysis demonstrated less frequency of Treg and Th2 cell populations exposed to conditions with both ABs present compared to AB-free control groups (Fig 4.5D).

The observed reduction in the levels of T-bet+ and GATA3+ T cell subsets when anti-IFN-γ and anti-IL-4 ABs were introduced, in comparison to the control conditions without these ABs, prompted us to proceed with the addition of both ABs against IFN-γ and IL-4 in the cell culture. Furthermore, the addition of anti-IFN-γ demonstrated a suppressive impact on the proliferation of RORγt+ and FoxP3+ T cells. Consequently, our subsequent phase of optimization focused on examining the specific effects of different dilutions of the anti-IFN-γ on the Th17 and Treg cell differentiation, while maintaining the same Anti-IL-4 AB dilution in all culture conditions.

Assessing the influence of various Anti- IFNγ titrations on T cell subset differentiation in cell culture:

Various concentrations of Anti-IFN-γ (100ng/µL, 50ng/µL, 20ng/µL, & 5ng/µL diluted in 100µL TexMACs medium) were utilized in culture to investigate the impact of them on distinct T cell subset proliferations (Fig 4.6). This were carried out in the presence of Th17 cell signature cytokines IL-1β, IL-6, IL-23, and TGF-β, as well as anti-IL-4 (250 ng/2.5 µL) in 100µL TexMACs medium.

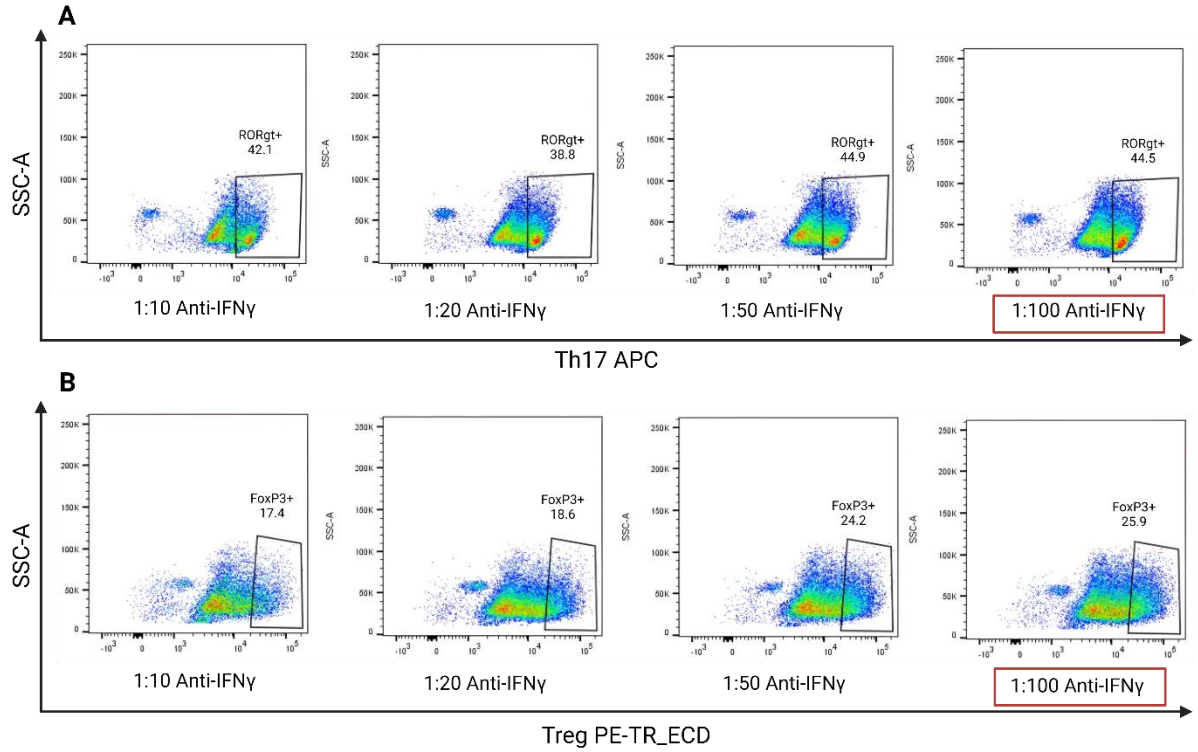

Figure 4.6. The effects of different Anti-IFNγ titrations on Treg and Th17 cell subset differentiation from isolated CD4+ T cells. A) RORγt percentage of CD4+ cells after 7 days of incubation with Th17-cell polarizing cytokines with respect to different anti-IFNγ concentrations. **B**) FoxP3 percentage of CD4+ cells after 7 days of incubation with Th17-cell polarizing cytokines with respect to different anti-IFNγ concentrations. We decided to proceed with a dilution of 5ng/µL (1:100) of this AB for the subsequent experiments.

As no notable alterations were observed in the frequency of RORγt+ T cells in response to different concentrations of the anti-IFN-γ AB, our focus shifted towards using the highest dilution of this AB (5ng/µL equal to 1:100 of stuck) in subsequent experiments.

4.4. The effect of cytokine ABs targeting IFN-Is and IL-22 on *in vitro* Th17 cell differentiation in isolated CD4+ T cell cultures

4.4.1 The influence of commercial anti-IFN-Is and anti-IL-22 ABs

As described in 1.7.4, APS-1 patients have autoABs against Type I IFNs and IL-22 cytokines, interfering with their normal activity and causing a variety of autoimmune symptoms. To study the effects of these autoABs, we initially investigate the consequences of using commercially manufactured ABs that are not derived from the patients themselves. Three experiments were conducted to achieve the stated objective. Different concentrations of anti-IFNα, anti-IFNω, and anti-IL-22 ABs (2 ng/μL, 0.2 ng/μL, 0.02 ng/μL, 0.0002 ng/μL) were separately introduced into cell cultures containing Th17-polarizing cytokines during each experiment. In experiment one and three the addition of certain AB dilutions took place at two different time points: either simultaneously (S) with or prior to (B) the addition of Th17 signature cytokines (IL-1β, IL-6, IL-23, and TGF-β) and additional ABs (anti-IL-4 and anti-IFN-γ). This experimental setup allowed for the investigation of the effects of different AB concentrations and timing on the cell cultures (Fig 4.7). Here the flow cytometry results for RORγt, FoxP3 (Tregs), and the functional markers CCR6, CCR4, and CXCR3 were presented. The evaluation of T-bet and GATA3 was not conducted due to their negligible expression levels. The impact of adding ABs against IFN-α, IFN-ω, and IL-22 cytokines was analyzed separately. The frequencies of RORγt+ T cells varied within the control CD4+ T cell population, ranging from (mean 38,9%; range 18.1-72.4%) in controls without the addition of any serum or anti-IFNα cytokine (Fig 4.10). Due to this variability, the data was not normalized or merged into a single graph. Instead, the actual percentage values of the different markers in the flow cytometry experiments were shown. No statistical tests were performed on these data.

Anti-IFNα AB

Flow cytometry analysis was performed on isolated CD4+ T cells obtained from healthy PBMCs in three separate experiments to assess the effects of adding anti-IFNα. The analysis focused on evaluating the expression of lineage markers RORγt and FoxP3, as well as extracellular markers CCR6, CCR4, and CXCR3 within the CD4+ T cell population.

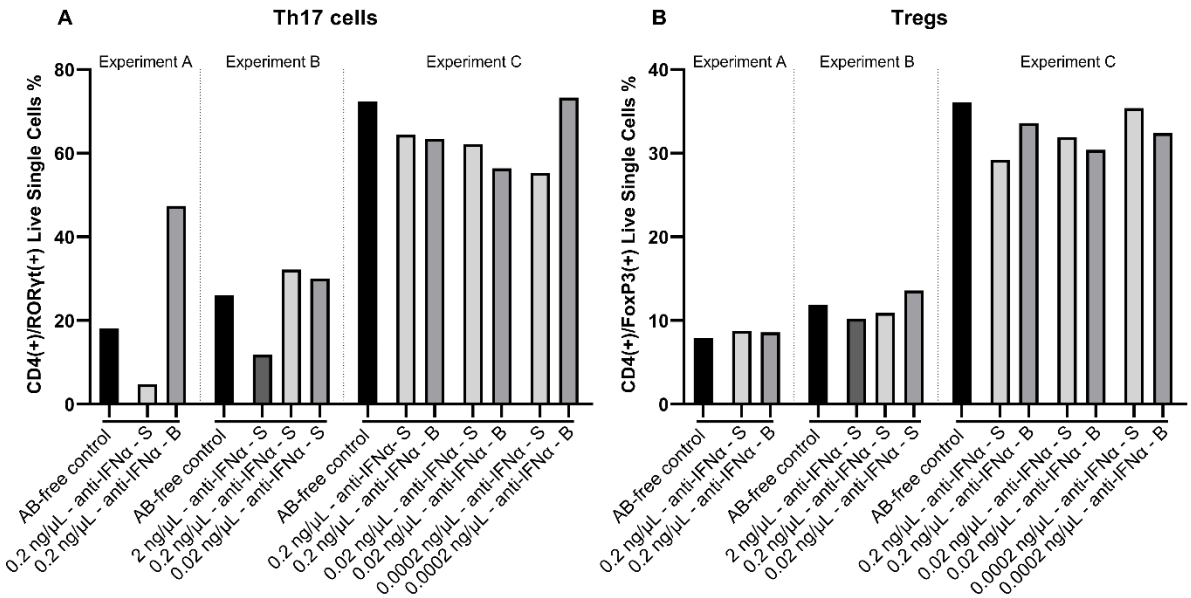

Figure 4.7. The effect of anti-IFNα in *in vitro* Th17 and Treg cell differentiation in flow cytometry. The graph shows three experiments on **A)** RORγt+ and **B)** FoxP3+ T cell differentiation after adding anti-IFNα in different concentrations and in different timepoints.**"S"**: the simultaneous addition of AB and cytokines.**"B"**: the addition of AB before the addition of cytokines**. Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. As there were only one datapoint in each bar in the histograms no statistical test was conducted.

The results presented in Figure 4.7 indicate successful generation of Th17 cells as well as Tregs from CD4+ cells. However, the assay appears to be vulnerable for either donor variability or uncontrolled conditions that might happen during cell handling, cytokine addition, or the seven-day incubation period. None of the three assays consistently showed any significant up- or downregulation of these two cell populations with different amounts or time points of anti-IFNα addition.

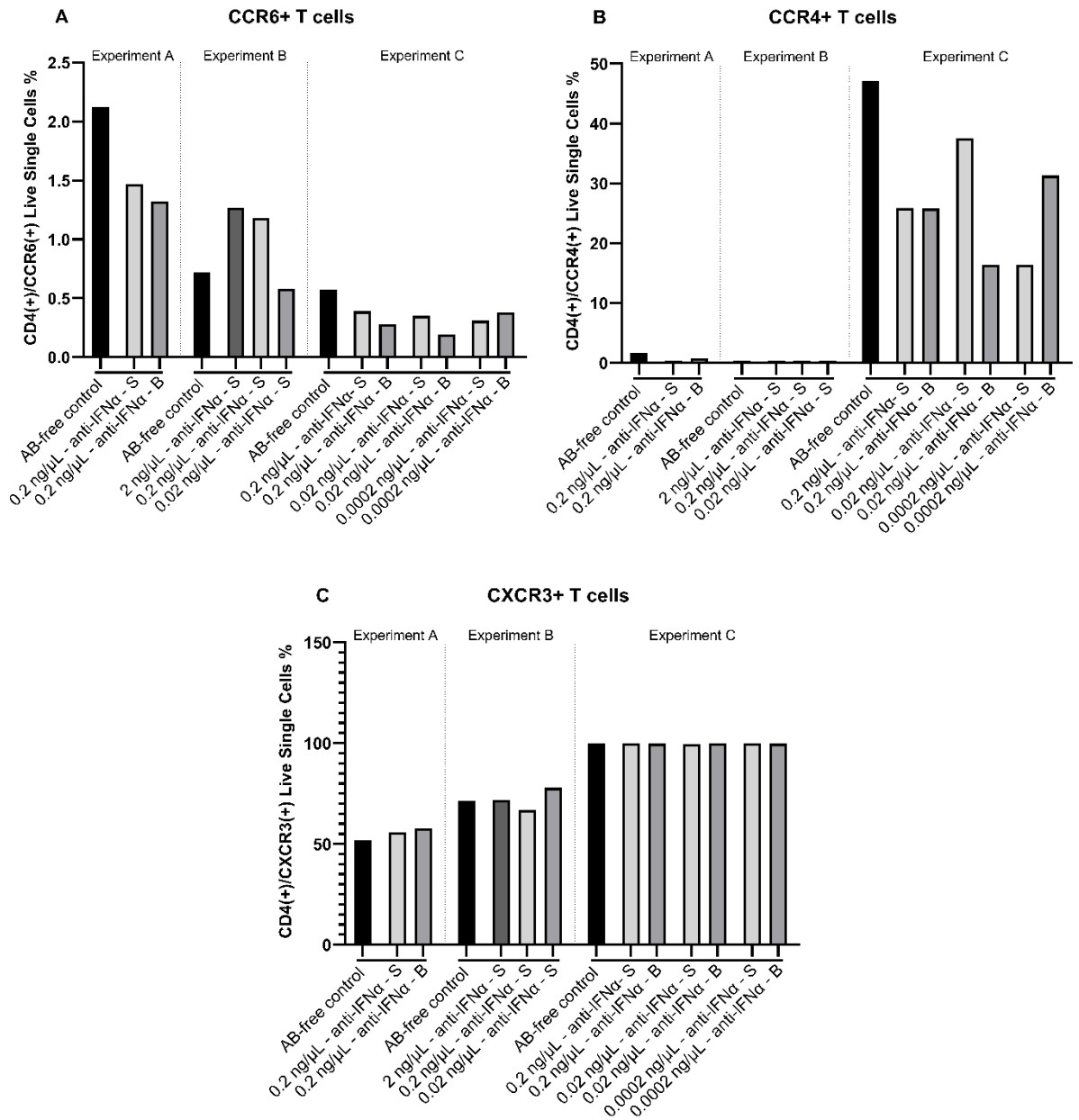

Figure 4.8. The impact of anti-IFNα in *in vitro* CCR6+, CCR4+, and CXCR3+ T cell differentiation in flow cytometry. The bar graphs illustrate the results of three experiments focusing on the differentiation of **A)** CCR6+, **B)** CCR4+, and **C)** CXCR3+ T cells after adding anti-IFNα in different concentrations and in different timepoints.**"S"**: the simultaneous addition of AB and cytokines.**"B"**: the addition of AB before the addition of cytokines**. Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. Since each bar in the histograms represents only one data point, no statistical tests were conducted.

The findings depicted in Figure 4.8 demonstrate the production of CCR6+, CCR4+, and CXCR3+ T cells derived from CD4+ cells. When alternative concentrations or timings of anti-IFNα were administered, none of the three experiments showed any discernible changes, either enhanced increase or decrease, in these three cell populations.

Anti-IFNω AB

In three separate experiments, flow cytometry analysis was conducted on isolated CD4+ T cells obtained from healthy PBMCs to investigate the impact of adding anti-IFNω. The analysis specifically examined the expression of lineage markers RORγt and FoxP3, as well as extracellular markers CCR6, CCR4, and CXCR3 within the CD4+ T cell population.

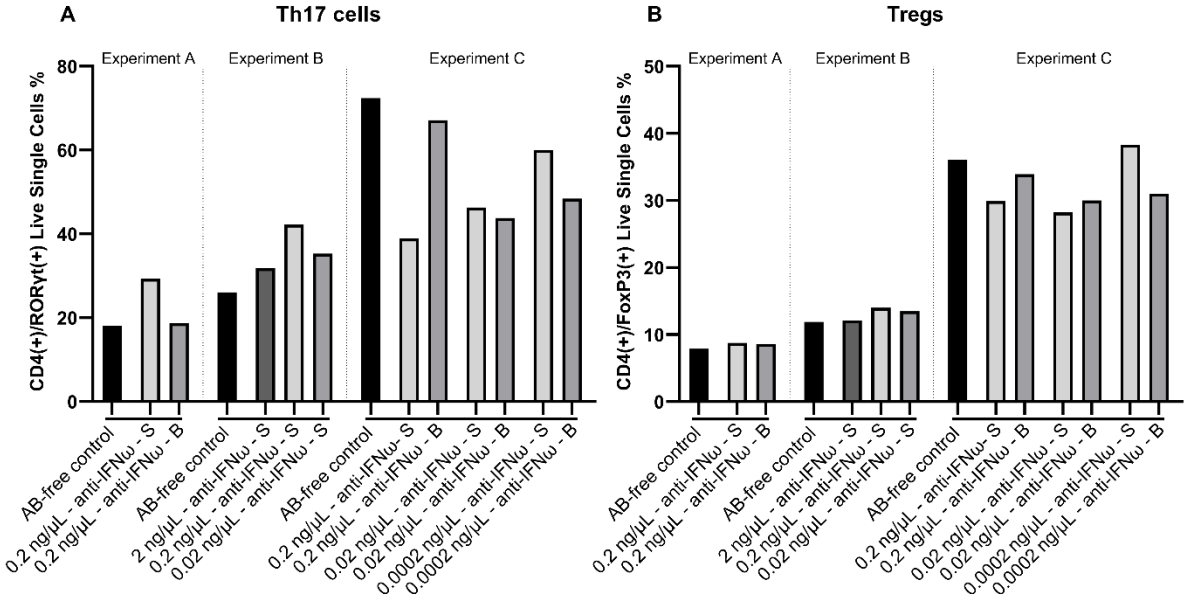

Figure 4.9. The influence of anti-IFNω in *in vitro* Th17 and Treg cell differentiation in flow cytometry. The graph presents three experiments focusing on **A)** RORγt+ and **B)** FoxP3+ T cell differentiation. Different concentrations and time points of anti-IFNα were added, with "**S**" indicating simultaneous addition of AB and Th17 cytokines, and "**B**" representing the addition of AB before Th17 cytokines. **Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. As there were only one datapoint in each bar in the histograms no statistical test was conducted.

Figure 4.9 illustrates the successful differentiation of Th17 cells as well as Tregs from CD4+ cells. The isolation and handling of cells from different donors seems to introduce variability in cell quality, viability, or functionality. However, the frequencies of RORγt+ and FoxP3+ T cells did not exhibit significant changes when exposed to different amounts or time points of anti-IFNω compared to the untreated control in the three assays that were performed.

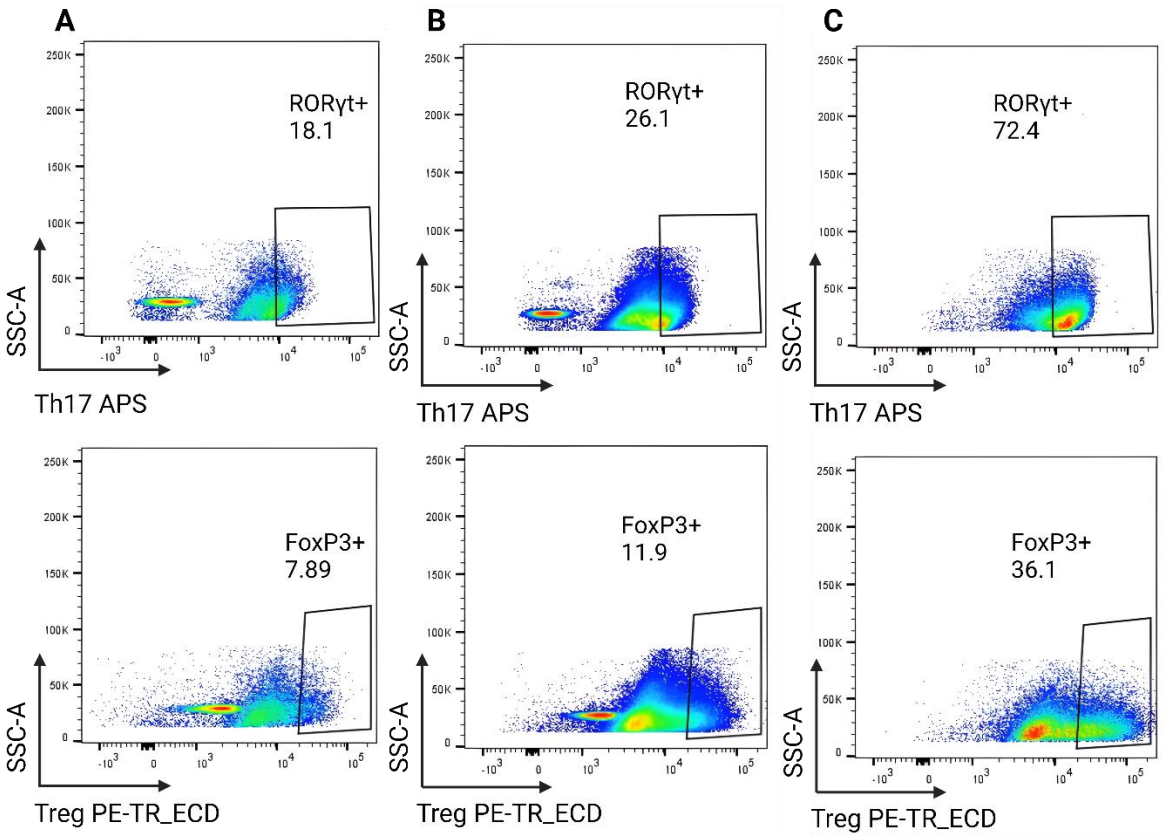

Figure 4.10. Difference in Th17 and Treg cell frequencies of control samples in three experiments with commercial ABs in flow cytometry. The first row shows RORγt while the second-row show FoxP3. **A)** experiment A, **B)** experiment B, **C)** experiment C. The figure was made by FlowJo 1.8.1.

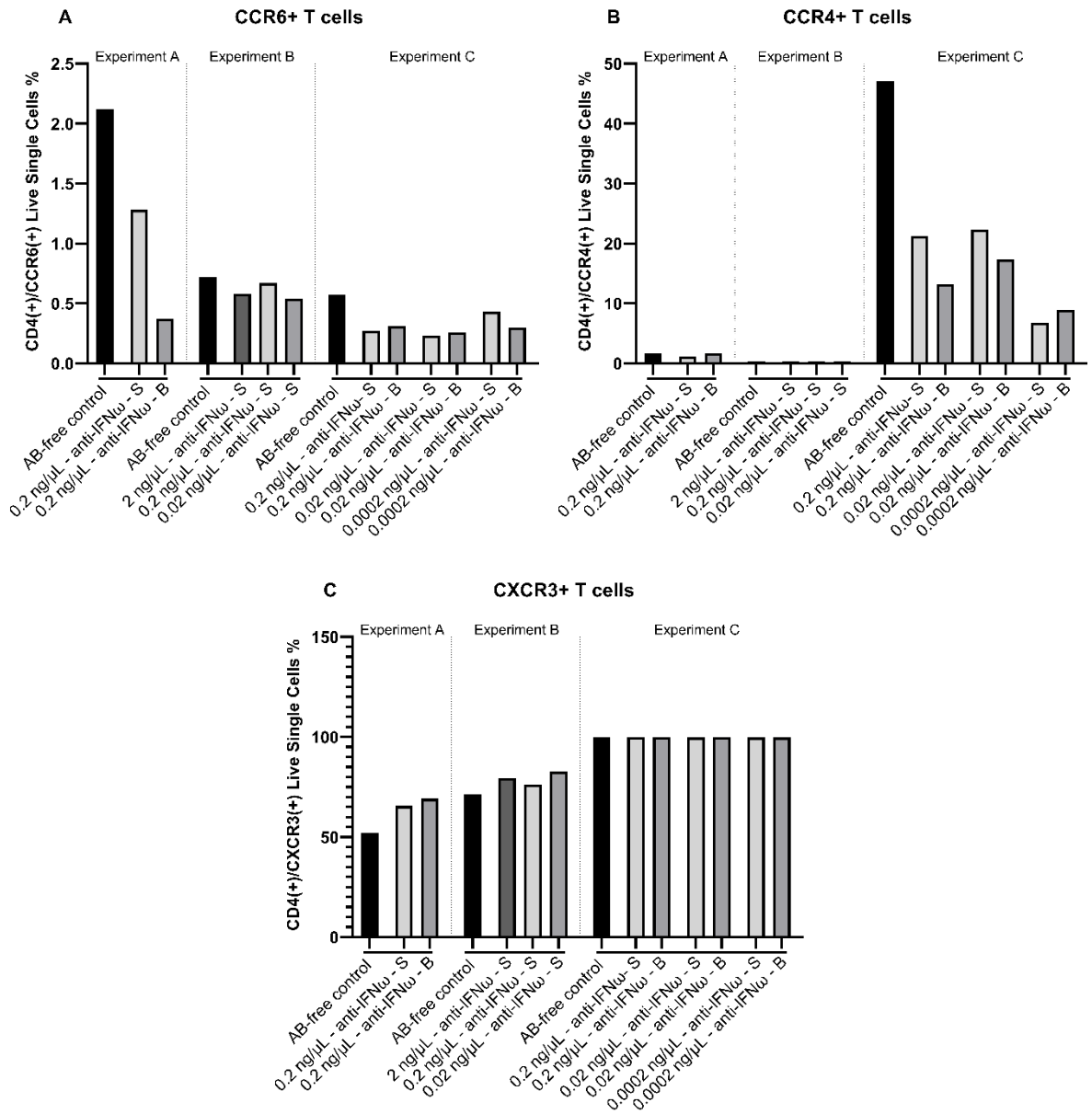

Figure 4.11. The impact of anti-IFNω in *in vitro* CCR6+, CCR4+, and CXCR3+ T cell differentiation in flow cytometry. The figures illustrate the results of three experiments focusing on the differentiation of **A)** CCR6+, **B)** CCR4+, and **C)** CXCR3+ T cells after being exposed to anti-IFNω at various doses and timepoints.**"S"**: the simultaneous addition of AB and cytokines.**"B"**: the addition of AB before the addition of cytokines**. Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. No statistical analysis was done since each bar in the histograms only shows one data point.

The results presented in Figure 4.11 indicate the existence of CCR6+, CCR4+, and CXCR3+ T cells derived from CD4+ cells. A minor decrease in CCR6+ and CCR4+ T cell populations were observed upon introduction of anti-IFNω. However, none of the three trials consistently

showed substantial changes, whether up- or downregulation, in these three cell groups when various anti-IFNω dosages or timings were used.

Anti-IL22 AB

Three independent experiments were performed using flow cytometry analysis on isolated CD4+ T cells derived from healthy PBMCs to assess the effects of introducing anti-IL22. The analysis focused on evaluating the expression levels of lineage markers RORγt and FoxP3, as well as extracellular markers CCR6, CCR4, and CXCR3 within the CD4+ T cell population.

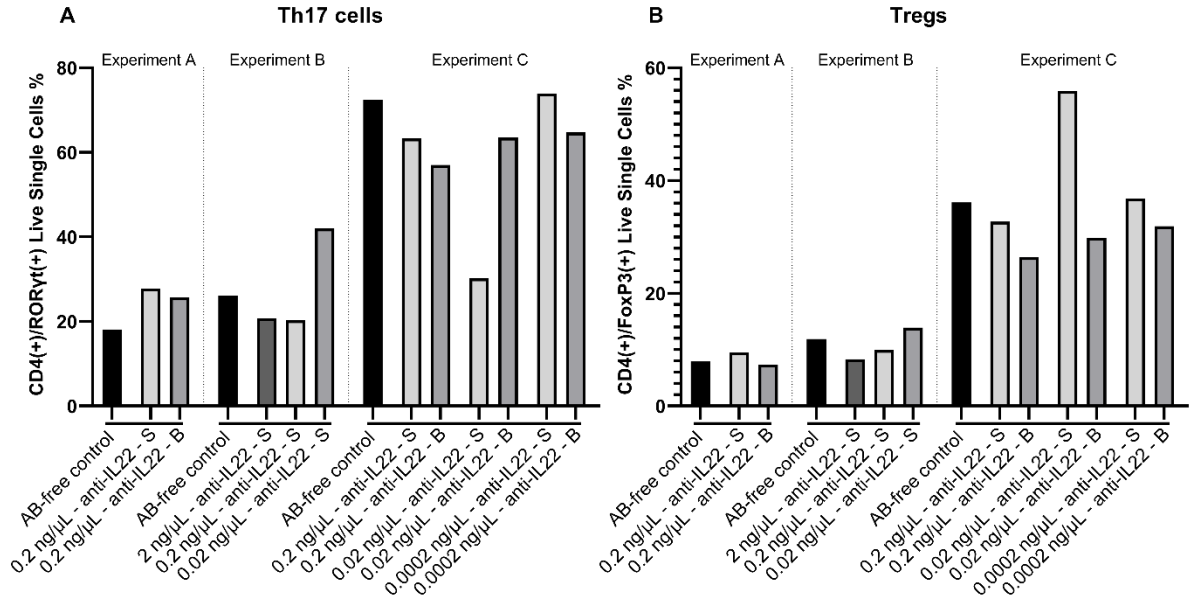

Figure 4.12. The influence of anti-IL-22 in *in vitro* Th17 and Treg cell differentiation in flow cytometry. The graph presents three experiments focusing on **A)** RORγt+ and **B)** FoxP3+ T cell differentiation. Different concentrations and time points of anti-IL-22 were added, with "**S**" indicating simultaneous addition of AB and Th17 cytokines, and "**B**" representing the addition of AB before Th17 cytokines. **Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. As there were only one datapoint in each bar in the histograms no statistical test was conducted.

The data presented in Figure 4.12 highlights the effective differentiation of Th17 cells and Tregs from CD4+ cells. However, the variations in cell frequencies observed in these assays may be attributed to donor variability and uncontrolled factors, resulting in inconsistent results. The timing and concentration of anti-IL-22 AB introduction did not show a substantial influence on the differentiation of these cell types. Therefore, based on the three conducted assays, the frequencies of RORγt+ or FoxP3+ T cells did not demonstrate significant changes when treated with different amounts or at different time points with anti-IL-22, compared to the untreated controls.

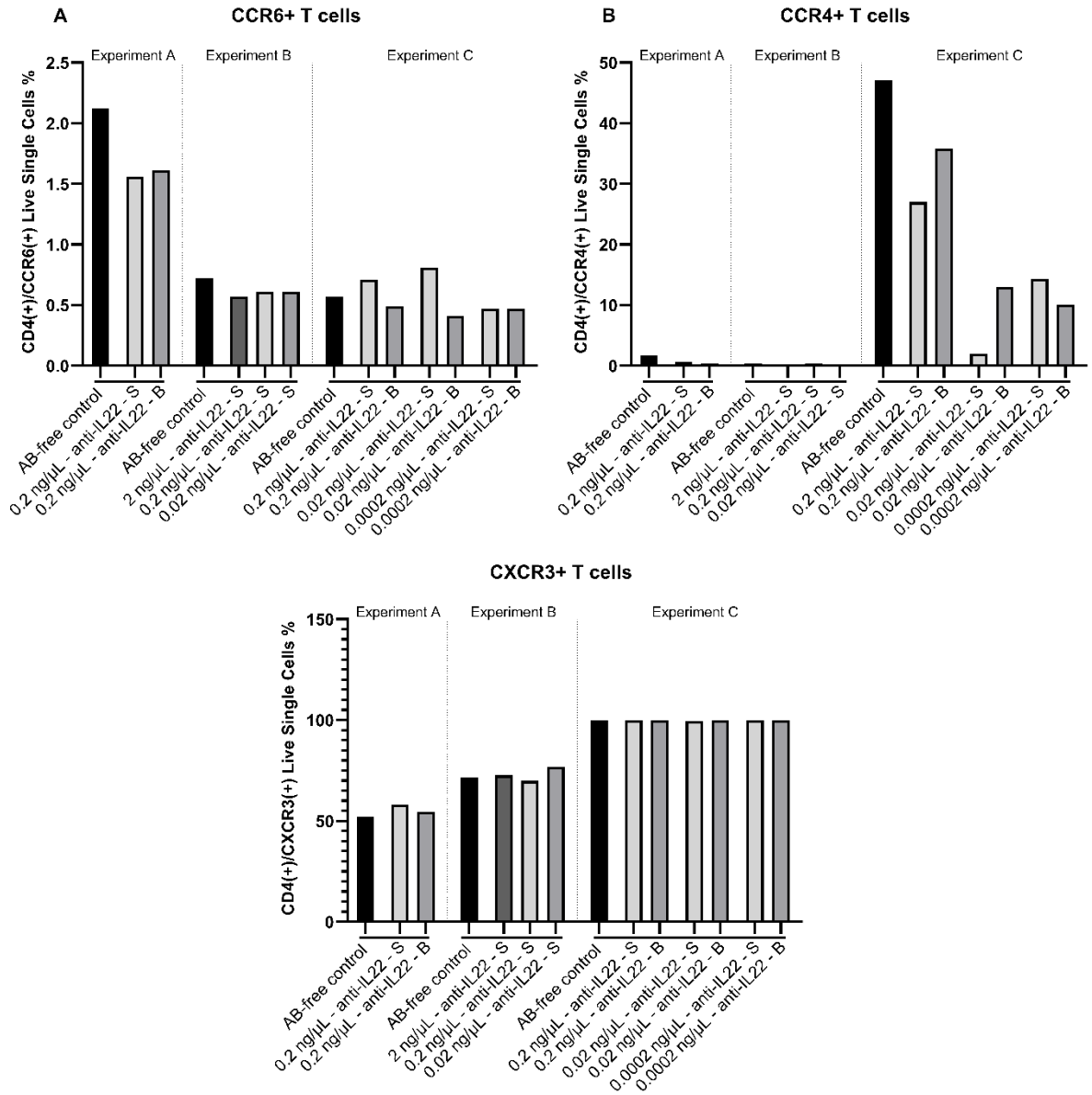

Figure 4.13. The impact of anti-IL-22 in *in vitro* CCR6+, CCR4+, and CXCR3+ T cell differentiation in flow cytometry. The figures illustrate the results of three experiments focusing on the differentiation of **A)** CCR6+, **B)** CCR4+, and **C)** CXCR3+ T cells in the presence of anti-IL-22 at various doses and timepoints.**"S"**: the simultaneous addition of AB and cytokines.**"B"**: the addition of AB before the addition of cytokines. **Experiment A**: addition of (0.2 ng/µL) of AB at the same time and before addition of Th17 cytokines. **Experiment B**: addition of (2 ng/µL, 0.2 ng/µL, 0.02 ng/µL) of AB simultaneous with the addition of Th17 cytokines. **Experiment C**: addition of (0.2 ng/µL, 0.02 ng/µL, 0.0002 ng/µL) of AB at the same time and before the addition of Th17 cytokines. Since each bar in the histograms only displays one data point, there was no statistical analysis performed.

The findings presented in Figure 4.13 show the differentiation of CCR6+, CCR4+, and CXCR3+ T cells from isolated CD4+ cells. A downward trend in CCR4+ T cell populations were observed upon addition of anti-IL-22. However, no significant alterations were observed in these cell populations when different dosages or timings of anti-IL-22 were employed.

In summary, it can be concluded that the effects of commercial AB against type I IFN and IL22 on Th17 cells and Tregs was neglectable, but that it might differ depending on the individual circumstances in the particular assays. Hence the generated assay might suffer from low robustness which lowers the power to use it for this specific purpose.

4.4.2 Gene expression analysis of Th17 cells after introduction of commercial ABs against type I IFNs and IL-22

The RNA from cultured Th17 cells was extracted, and its concentration was calculated using Nanodrop, ranging from (mean 56.8%; range 33.1-92.9%). Additionally, using spectrophotometry, the purity of the RNA was determined. The 260/280 OD ratios ranged from 1.74 to 2.01 (mean: 1.89) throughout this evaluation.

4.4.2.1 Gene expression profiling of T cell lineage markers in CD4+ T cells

The relative gene expression of T cell lineage markers in CD4+ T cells was evaluated using SYBR green-based qPCR assays. These assays were performed using cell samples from experiment A and C, where the same concentration of (0.2 ng/µL) was used for both conditions (S) and (B), following the culture with three commercial anti-IFNα, anti-IFNω, and anti-IL-22 ABs. To ensure the specificity of the primers, which targeted markers such as RORγt, FoxP3, GATA3, T-bet, CCR4, and CXCR3, melting curve analyses were performed after each run. The presence of a single peak in the melting curve analysis confirmed the specificity of the primers for the respective gene targets. The mean Ct values for six target genes ranged as follows: RORγt (23.66 to 25.21), FoxP3 (27.89 to 39.51), GATA3 (24.48 to 26.54), T-bet (25.20 to 26.35), CCR4 (20.19 to 24.59), and CXCR3 (19.07 to 21.50). Accordingly, some indicating reliable amplification of the qPCR products.

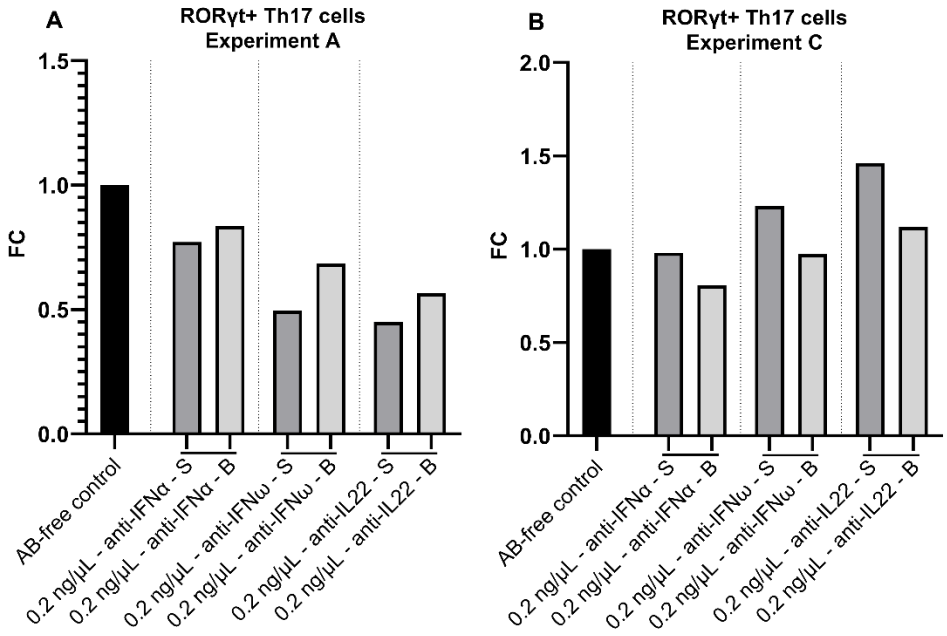

Figure 4.14. Relative changes in RORγt expression after introducing the ABs against type I IFNs and IL-22 cytokine in qPCR. Cell samples from **A)** experiment A and **B)** experiment C, were conducted to examine the impact of anti-IFNα, anti-IFNω, and anti-IL-22 ABs on the expression of RORγt+ T cells compared to a control group without ABs. In both sets of experiments, the concentrations of the ABs were consistent (0.2 ng/µL) across all conditions. The ABs were added at two different time points: before the addition of Th17 signature cytokines (labeled as "**B**") and simultaneously with the addition of these cytokines (labeled as "**S**"). Only one biological parallel was evaluated for each condition because of the limited number of available cells. Hence, no statistical testing was applied.

The relative expression of RORγt is depicted in Figure 4.14 for both experiment A and C. Being the same as the flow cytometry experiments, but where only the sample conditions with concentration (0.2 ng/µL) of the ABs were chosen for experiment C. In experiment A, there is a decreasing tendency in the proportion of RORγt-expressing cells, while experiment C shows the opposite trend. These inconsistent findings make it difficult to draw a consistent interpretation from the results.

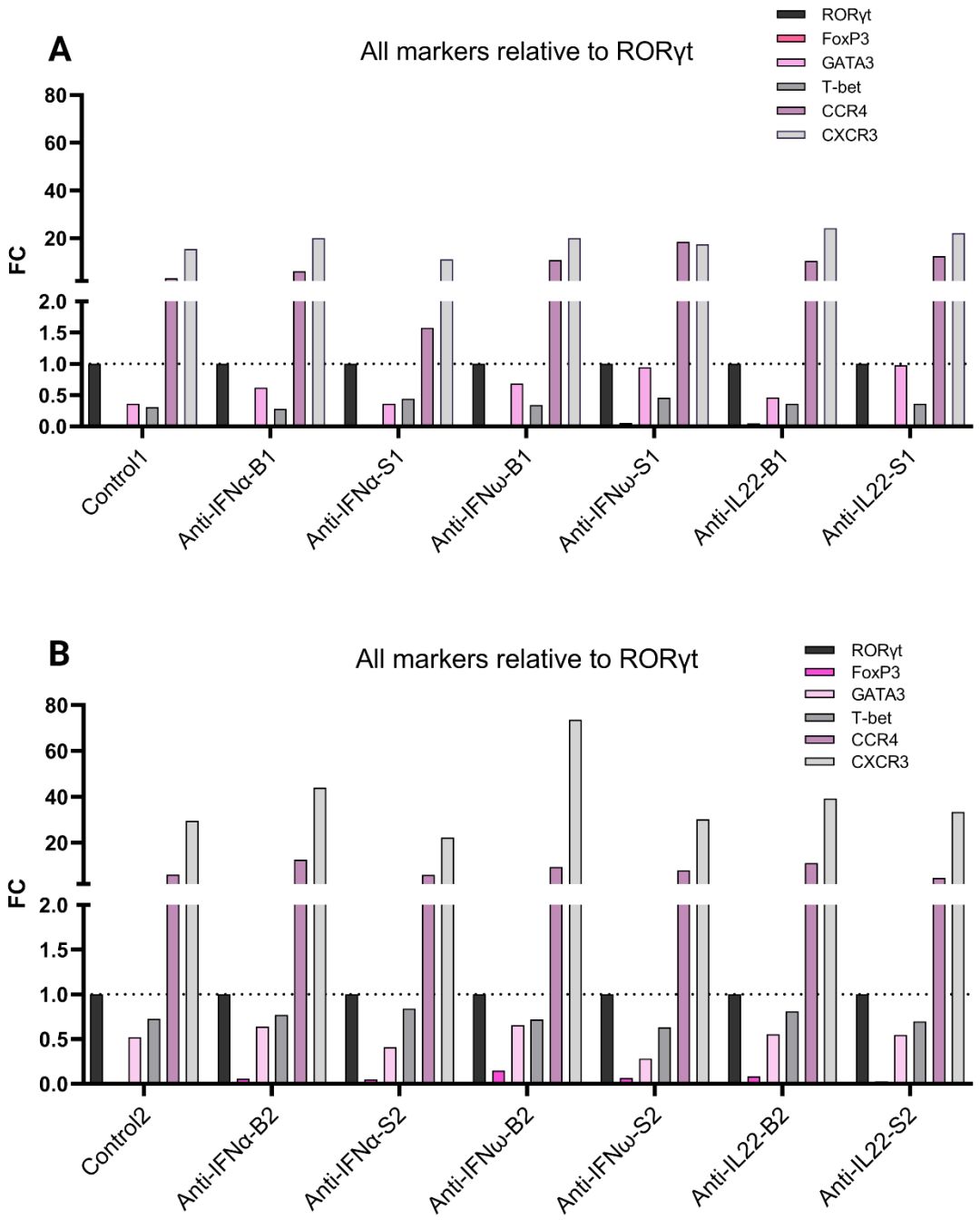

Figure 4.15 Expression of FoxP3, GATA3, T-bet, CCR4, and CXCR3 relative to RORγt expression in qPCR. Cell samples from experiment A) and C), were conducted to examine the impact of anti-IFNα, anti-IFNω, and anti-IL-22 ABs on the expression of FoxP3+, GATA3+, T-bet+, CCR4+, and CXCR3+ T cells relative to RORγt+ Th17 cell expression. In both sets of experiments, the concentrations of the ABs were consistent (0.2 ng/µL) across all conditions. The ABs were added at two different time points: before the addition of Th17 signature cytokines (labeled as "**B**") and simultaneously with the addition of these cytokines (labeled as "**S**"). Only one biological parallel was evaluated for each condition because of the limited number of available cells. Hence, no statistical testing was applied.

Figure 4.15 presents the relative expression of other markers in qPCR analysis in comparison to RORγt expression. The graphs indicate no significant differences between the conditions, but consistently demonstrate an upregulation of CXCR3 and CCR4, as well as a downregulation of FoxP3, GATA3, and Tbet relative to the Th17-marker RORγt. These findings suggest a reliable pattern of gene expression changes associated with Th17 cells.

4.4.3 Th17 signature cytokine IL-17 in cultured CD4+ T cells

Sandwich ELISA assays for IL-17 cytokine was utilized on isolated CD4+ T cell culture supernatants to assess the impact of commercial anti-IFNα, anti-IFNω, and anti-IL--22 ABs on the function of Th17 cells.

4.4.3.1 ELISA analysis of IL-17 cytokine levels after introduction of commercial ABs against type I IFNs and IL-22

After a 7-day in-vitro expansion, the differentiation and functionality of Th17 cells derived from isolated CD4+ T cells of healthy individuals were confirmed by assessing their ability to produce IL-17 cytokines in the presence of anti-IFNα, anti-IFNω, and anti-IL-22. Concentrations of IL-17 in culture supernatants in response to three different commercial ABs was as follows: for experiment with anti-IFNα (mean exp. A: 70.8 pg/mL, mean exp. B: 197.5 pg/mL, mean exp. C: 197.7 pg/mL), for experiment with anti-IFNω (mean exp. A: 178.6 pg/mL, mean exp. B: 140 pg/mL, mean exp. C: 117.5 pg/mL), and for experiment with anti-IL-22 (mean exp. A: 39.5 pg/mL, mean exp. B: 175.6 pg/mL, mean exp. C: 79.5 pg/mL). Since all expanded Th17 cells produced IL-17 cytokine, they were considered to be functional.

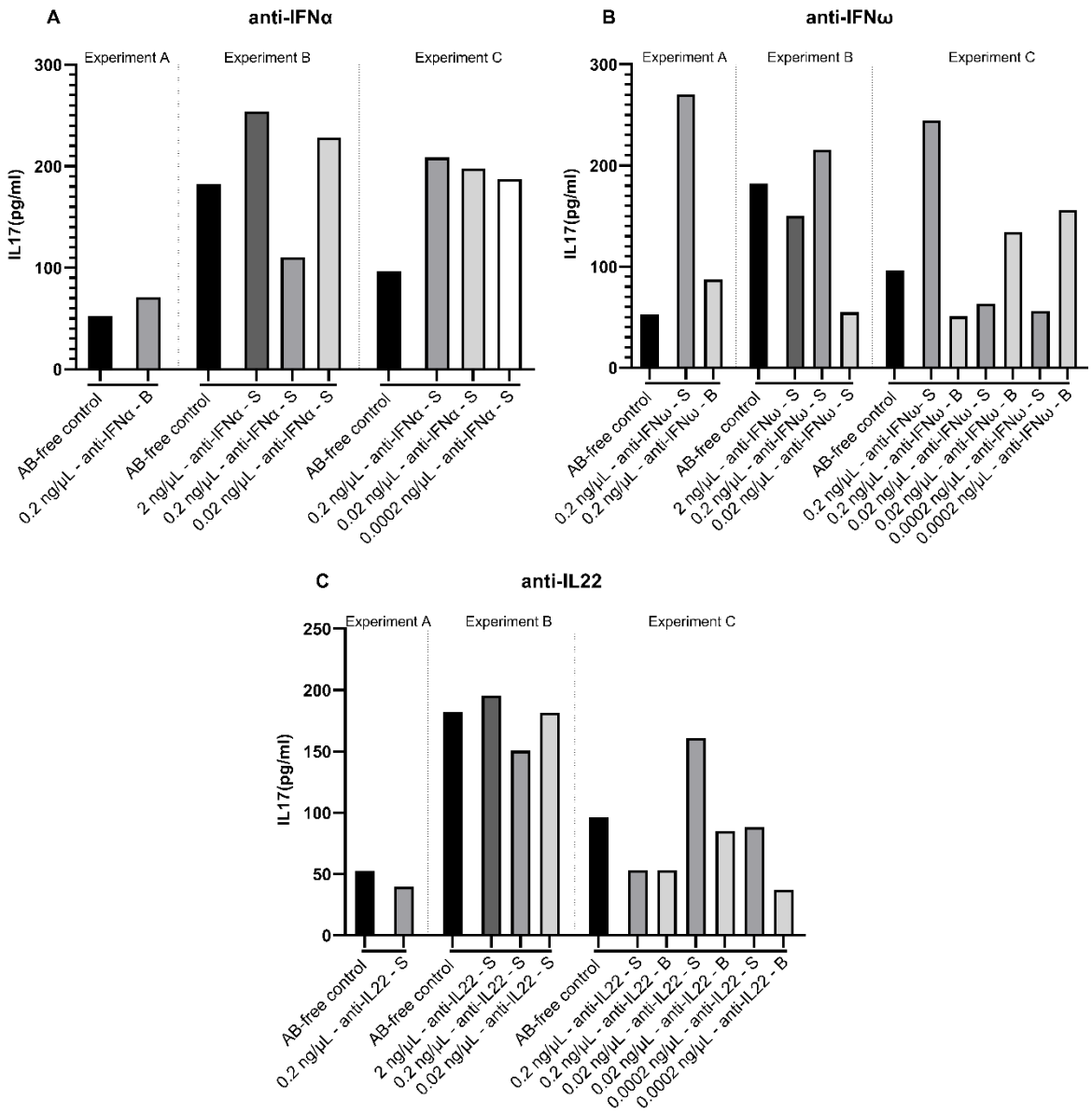

Figure 4.16 Levels of IL-17 detected in culture supernatants of CD4+ T cells treated by commercial ABs. Cell samples from experiment A and C, were conducted to examine the impact of **A)** anti-IFNα, **B)** anti-IFNω, and **C)** anti-IL-22 ABs (added to the cell culture at various doses and timepoints) on the production of IL-17 cytokine from RORγt+ Th17 cells. **"S"**: the simultaneous addition of AB and cytokines.**"B"**: the addition of AB before the addition of cytokines. Since each bar in the histograms only displays one data point, there was no statistical analysis performed.

Overall, higher concentrations of IL-17 were detected in supernatants from conditions with anti-IFNα and anti-IFNω ABs added at the same time with Th17 cytokines (Fig 4.16A/B). However, no significance was observed compared to controls and there were no clear patterns of the influences of commercial anti-cytokine Abs (Fig 4.16).

4.5 Effects of autoABs against IFN-Is and IL-22 in APS-1 patient sera on *in vitro* Th17 cell differentiation

Building upon the previous experiments that investigated the impact of commercial ABs on Th17 cell differentiation, we conducted a subsequent study, using CD4+ T cells isolated from healthy PBMCs, to examine the effects of autoABs against IFN-Is and IL-22 that were derived from APS-1 patients on *in vitro* Th17 cells. To investigate the influence on Th17 cells, the study examined the effects of sera obtained from both HC and individuals with APS-1, as well as purified IgG from three APS-1 patients. The experiments were conducted under *in vitro* conditions including IL-1β, IL-6, IL-23, TGF-β cytokines, and anti-IL-4 and anti-IFN-γ ABs. This section focuses on presenting the outcomes obtained from RORγt+ Th17 cells and FoxP3+ Tregs.

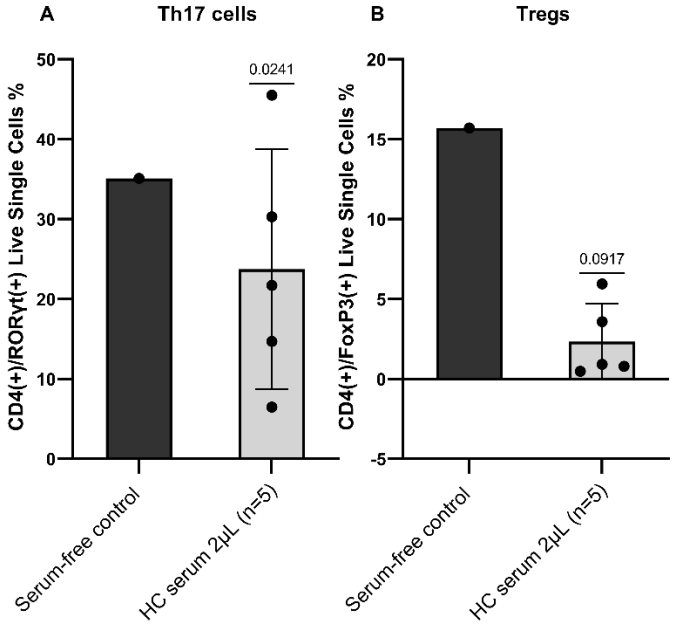

Figure 4.17. Flow cytometry data of Th17 and Treg response to HC serum versus control group without HC serum. The figure indicates the effect of HC serum on RORγt+ Th17 cells and FoxP3+ Tregs compared to the control group. Mean values are indicated by boxes and error bars show standard deviation. Cells treated with Hc serum was compared to controls using t tests (and nonparametric tests). No differential effect between the two groups was indicated by the statistical analysis performed. To assess the significance among different conditions treated with Hc serum, one-sample t-tests and Wilcoxon tests were performed. A nearly significant difference was observed for Th17 differentiation in the presence of Hc sera (P value: 0.0241) using GraphPad version 9.5.1.
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The frequency of RORγt+ Th17 cells exhibited significant variability, ranging from 6.4% to 45.5%, in response to the introduction of 2µL serum obtained from five healthy individuals. Additionally, in the same condition exposed to HC serum, the frequency of FoxP3+ Tregs ranged from 0.5% to 9.1%. With the exception of one condition that displayed the highest percentage of Th17 cells, the remaining conditions showed a suppressive response for either

Th17 or Tregs upon the addition of HC sera compared to their sera-free control. Therefore, addition of HC serum showed tendencies towards lower differentiation of these two cell subsets but without reaching statistically significant alterations.

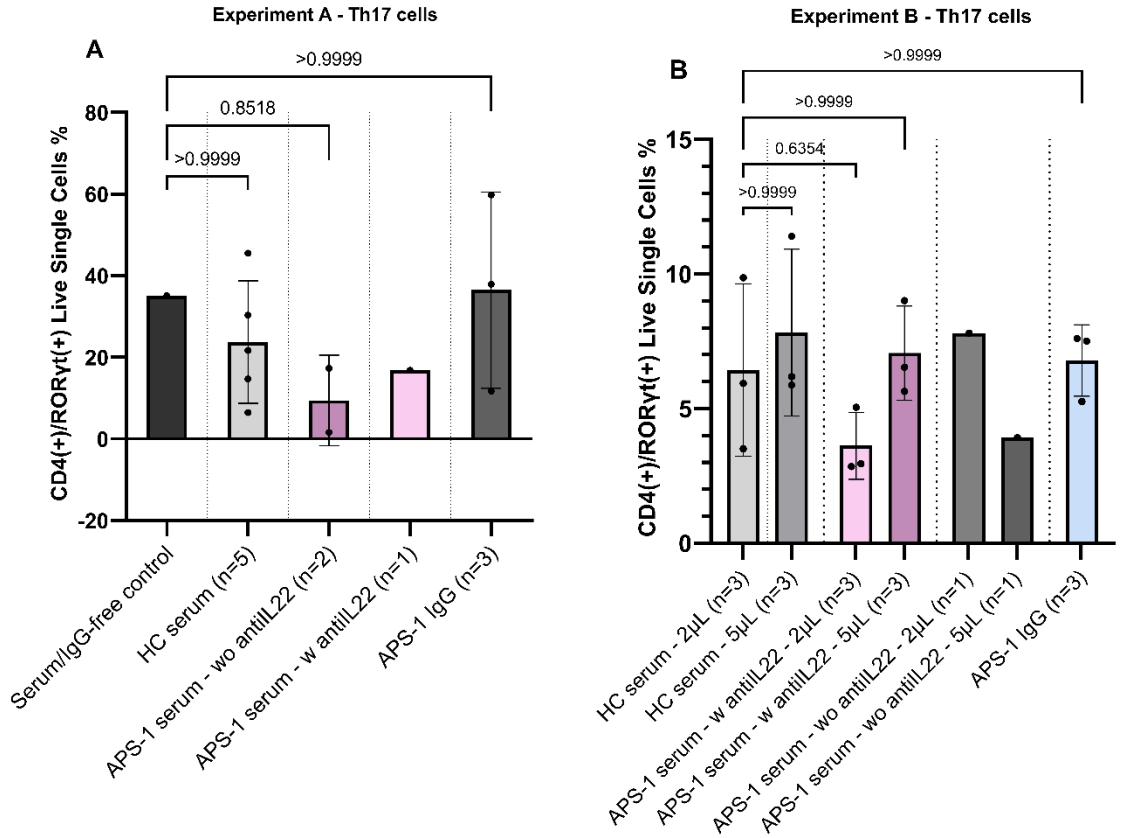

Figure 4.18. Flow cytometry data of Th17 cell response to the addition of HC serum, APS-1 patient serum, and APS-1 IgG versus control group without sera. Two sets of experiments were conducted **A)** and **B)** to investigate the effect of HC serum, APS-1 serum, and APS-1 IgG on RORγt+ Th17 cells compared to a control group. Some APS-1 sera contained type 1 IFN and IL-22 ABs (APS-1 serum w Anti-IL-22), whereas some APS-1 sera did only contain type 1 IFN ABs and not IL-22 ABs (APS-1 serum wo Anti-IL-22). **A)** Experiment A: In this experiment, three patient sera (2µL), 5 healthy control sera (2µL) and three APS-1 IgG (1µL) were included. In this experiment, various cell conditions were compared to a control condition where no serum was present. **B)** Experiment B: Addition of sera (2µL or 5µL) from APS-1 patients, IgG (1µL) or healthy control sera (2 or 5µL) were compared to sera from healthy controls (2µL). For both figs, mean values are indicated by boxes, error bars show standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test (P values are indicated on the tiles) performed by GraphPad version 9.5.1.

The addition of APS-1 sera, with or without anti-IL-22 AB, and regardless of the two different concentrations used, demonstrated inhibitory effects on Th17 differentiation but did not reach statistical significance. Moreover, the purified IgG obtained from individuals with APS-1 exhibited both stimulation and inhibition effects on Th17 cells. However, these effects were not statistically significant.

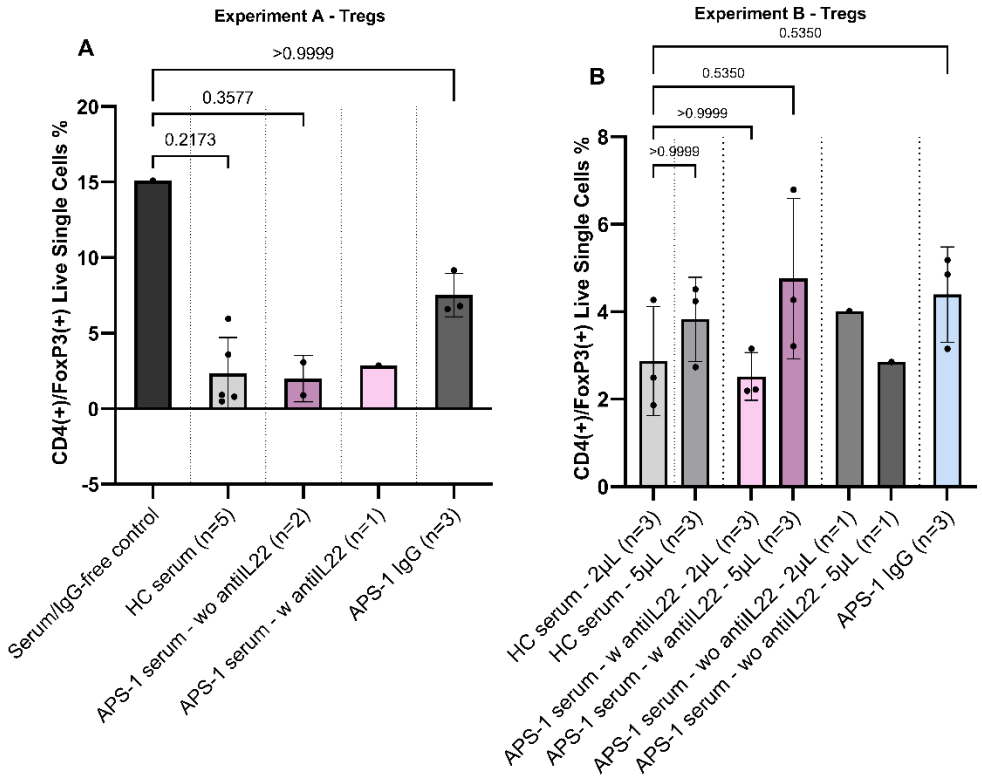

Figure 4.19. Flow cytometry data of Tregs response to the addition of HC serum, APS-1 patient serum, and APS-1 IgG versus control group without sera. Two sets of experiments were conducted **A)** and **B)** to investigate the effect of HC serum, APS-1 serum, and APS-1 IgG on FoxP3+ Tregs compared to a control group. Some APS-1 sera contained type 1 IFN and IL-22 ABs (APS-1 serum w Anti-IL-22), whereas some APS-1 sera did only contain type 1 IFN ABs and not IL-22 ABs (APS-1 serum wo Anti-IL-22). **A) Experiment A:** In this experiment, three patient sera (2µL), five healthy control sera (2µL) and three APS-1 IgG (1µL) were included. In this experiment, various cell conditions were compared to a control condition where no serum was present. **B) Experiment B:** Addition of sera (2µL or 5µL) from APS-1 patients, IgG (1µL) or healthy control sera (2 or 5µL) were compared to sera from healthy controls (2µL). For both figures, mean values are represented by boxes, and error bars indicate the standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test was used and the P values, are indicated on the figure tiles. The statistical analysis was performed using GraphPad version 9.5.1.

The inclusion of APS-1 sera, whether with or without anti-IL-22 ABs, at different concentrations, exhibited inhibitory effects on Treg differentiation compared to the control condition without serum and IgG. Furthermore, both upregulation and downregulation of this cell subset were observed when compared to the control condition containing HC serum. Additionally, the purified IgG derived from individuals with APS-1 showed both stimulating and suppressing effects on Tregs. However, these effects did not reach statistical significance.

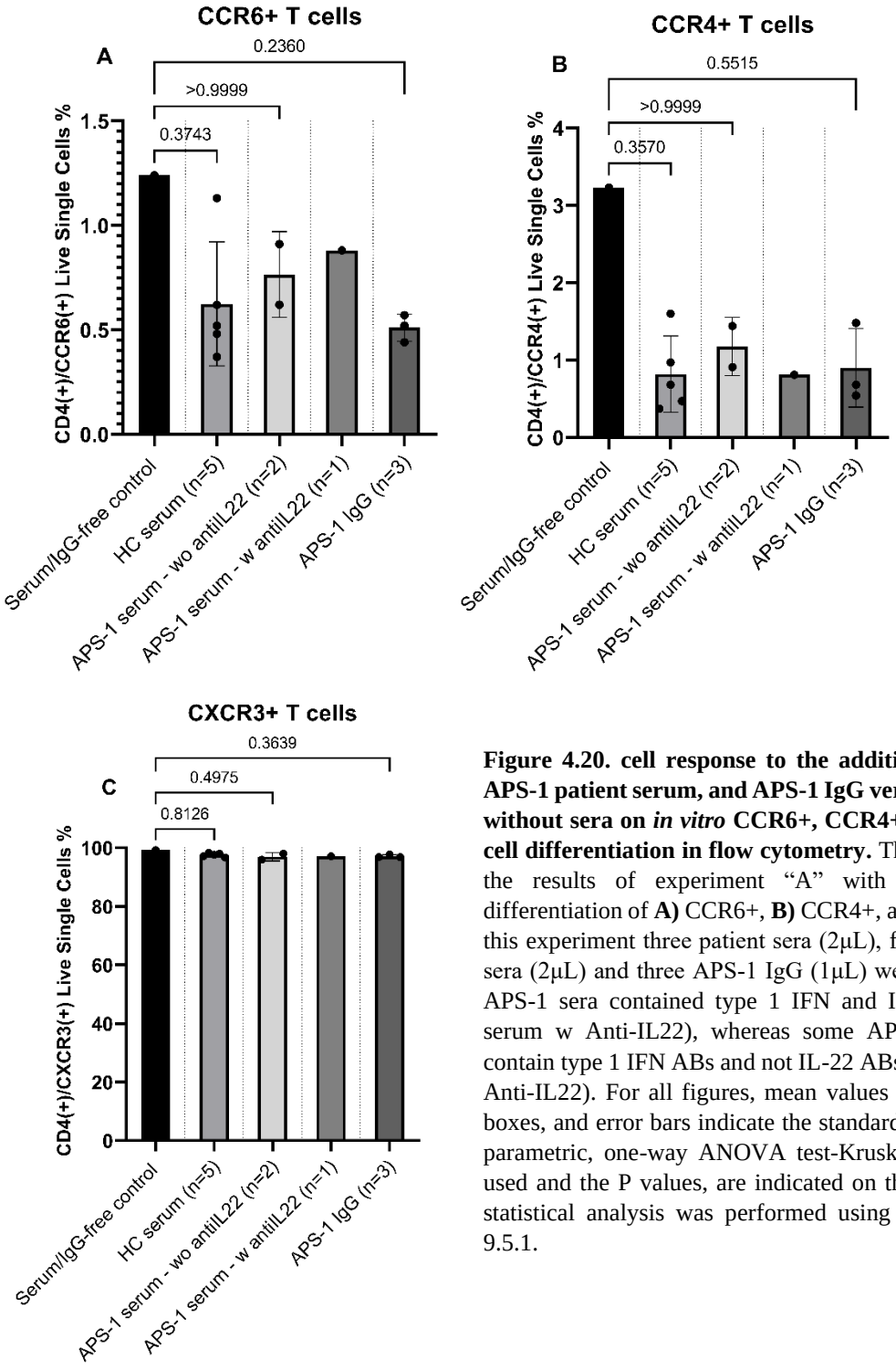

Figure 4.20. cell response to the addition of HC serum, APS-1 patient serum, and APS-1 IgG versus control group without sera on *in vitro* CCR6+, CCR4+, and CXCR3+ T cell differentiation in flow cytometry. The figures illustrate the results of experiment "A" with focusing on the differentiation of **A)** CCR6+, **B)** CCR4+, and **C)** CXCR3+. In this experiment three patient sera (2µL), five healthy control sera (2µL) and three APS-1 IgG (1µL) were included. Some APS-1 sera contained type 1 IFN and IL-22 ABs (APS-1 serum w Anti-IL22), whereas some APS-1 sera did only contain type 1 IFN ABs and not IL-22 ABs (APS-1 serum wo Anti-IL22). For all figures, mean values are represented by boxes, and error bars indicate the standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test was used and the P values, are indicated on the figure tiles. The statistical analysis was performed using GraphPad version 9.5.1.

The findings illustrated in Figure 4.20 show the differentiation of CCR6+, CCR4+, and CXCR3+ T cells from isolated CD4+ cells in experiment "A" using sera/IgG (same conditions as shown in Fig 4.19A). A trend towards a decrease was observed on the differentiation of CCR6+ and CCR4+ T cell populations, following exposure to APS-1 sera, whether with or without anti-IL-22 ABs, compared to the control condition without serum and IgG (Fig

4.20A/B). However, no differential changes were detected in the differentiation of these T cell populations in the presence of APS-1 sera/IgG.

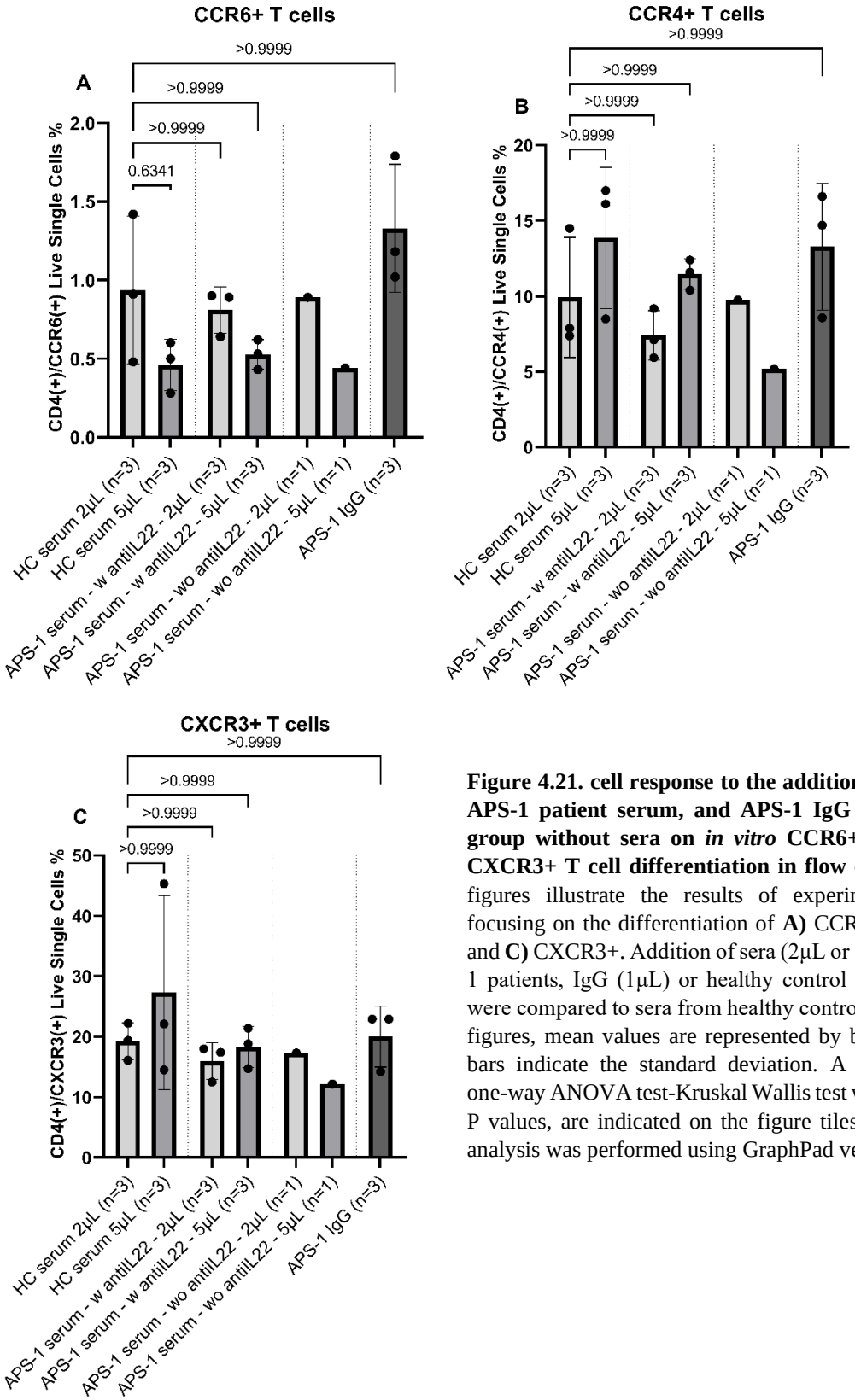

Figure 4.21. cell response to the addition of HC serum, APS-1 patient serum, and APS-1 IgG versus control group without sera on *in vitro* CCR6+, CCR4+, and CXCR3+ T cell differentiation in flow cytometry. The figures illustrate the results of experiment "B" with focusing on the differentiation of **A)** CCR6+, **B)** CCR4+, and **C)** CXCR3+. Addition of sera (2µL or 5µL) from APS-1 patients, IgG (1µL) or healthy control sera (2 or 5µL) were compared to sera from healthy controls (2µL). For all figures, mean values are represented by boxes, and error bars indicate the standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test was used and the P values, are indicated on the figure tiles. The statistical analysis was performed using GraphPad version 9.5.1.

The results presented in Figure 4.21 demonstrate the differentiation of CCR6+, CCR4+, and CXCR3+ T cells from isolated CD4+ cells in experiment "B" using sera/IgG conditions (as depicted in Figure 4.19B). The CCR6+ T cell populations showed a tendency towards a decrease when exposed to a concentration of 5μL of sera, regardless of whether it was APS-1 sera with or without anti-IL-22 ABs or healthy sera (Fig 4.21A). However, overall, no discernible effect was observed in the differentiation of these T cell populations in the presence of APS-1 sera/IgG.

4.5.1 Gene expression analysis of Th17 cells after introduction of sera from both APS-1 patients and Healthy controls

The RNA from cultured Th17 cells was extracted, and its concentration was estimated using Nanodrop, ranging from (mean 45.3%; range 12.74-110.78%). Moreover, the purity of the RNA was determined by using spectrophotometry. The 260/280 OD ratios ranged from 1.51 to 2.11 (mean: 1.87) throughout this evaluation.

4.5.1.2 Gene expression profiling of T cell lineage markers in CD4+ T cells

SYBR green-based qPCR assays were employed to assess the relative gene expression of T cell lineage markers in CD4+ T cells. These assays were conducted on cell samples obtained from experiment B, where the control group contained HC serum (2 μL). To verify the specificity of the primers targeting markers such as RORγt, FoxP3, GATA3, T-bet, CCR4, and CXCR3, melting curve analyses were performed following each run. The presence of a single peak in the melting curve analysis confirmed the specificity of the primers for their respective gene targets. According to the mean Ct values of the six target genes, some reliable and some undetermined amplifications were detected. Mean Ct values for these genes were ranging as follows: RORγt (25.76 to 30.21), FoxP3 (29.69 to 37.04 but mostly undetermined), GATA3 (23.29 to 25.68), T-bet (26.31 to 28.22), CCR4 (29.14 to 53.69 and some undetermined), and CXCR3 (18.87 to 21.52). The result from RORγt gene expression is shown in this section.

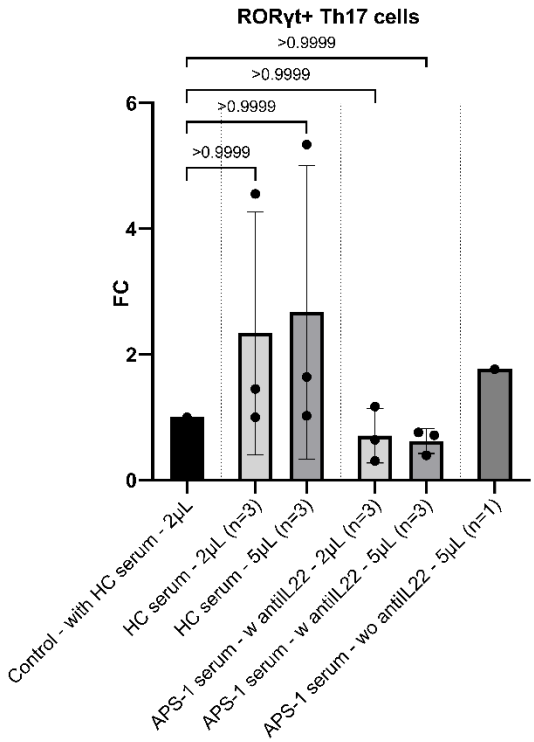

Figure 4.22. qPCR analysis of relative changes in RORγt expression after introducing APS-1 and HC sera. Cell samples from experiment B were conducted to examine the impact of APS-1 and HC sera on the expression of RORγt+ T cells compared to a control group with 2µL HC serum. Some APS-1 sera contained type 1 IFN and IL-22 ABs (APS-1 serum w Anti-IL-22), whereas some APS-1 sera did only contain type 1 IFN ABs and not IL-22 ABs (APS-1 serum wo Anti-IL-22). Sera from both HC and APS-1 individuals added at two different (2µL and 5µL) concentrations. Only one biological parallel was evaluated for the condition of APS-1 serum without anti-IL-22 because of the limited number of available cells. Hence, no statistical testing was applied. Mean values are represented by boxes, and error bars indicate the standard deviation. A non-parametric, one-way ANOVA test were performed and the P values, are indicated on the figure tiles. The statistical analysis was performed using GraphPad version 9.5.1.

Figure 4.22 presents the relative expression of RORγt in experiment B, which was carried out in the presence of APS-1 and HC sera. The data showed a decreasing trend in the proportion of cells expressing RORγt when exposed to patient sera compared to conditions with HC sera. However, the findings are inconsistent, making it challenging to derive a conclusive interpretation from the results.

4.5.2 Th17 signature cytokine IL-17 in cultured CD4+ T cells

To evaluate the effect of APS-1 and HC sera/IgG on the function of Th17 cells and to measure the levels of IL-17 cytokine, sandwich ELISA assays were performed on culture supernatants derived from isolated CD4+ T cells.

4.5.2.1 ELISA analysis of IL-17 cytokine levels after introduction of APS-1 and HC sera/IgG

After a 7-day in-vitro expansion, the differentiation and functionality of Th17 cells derived from isolated CD4+ T cells of healthy individuals were confirmed by assessing their ability to produce IL-17 cytokines in the presence of sera/IgG obtained from both HC and APS-1 individuals. It was observed that all expanded Th17 cells were capable of producing IL-17 cytokine, with mean concentrations of 46.7 pg/mL in the experimental group and 55.8 pg/mL in the control group. Therefore, these cells were considered to be functional.

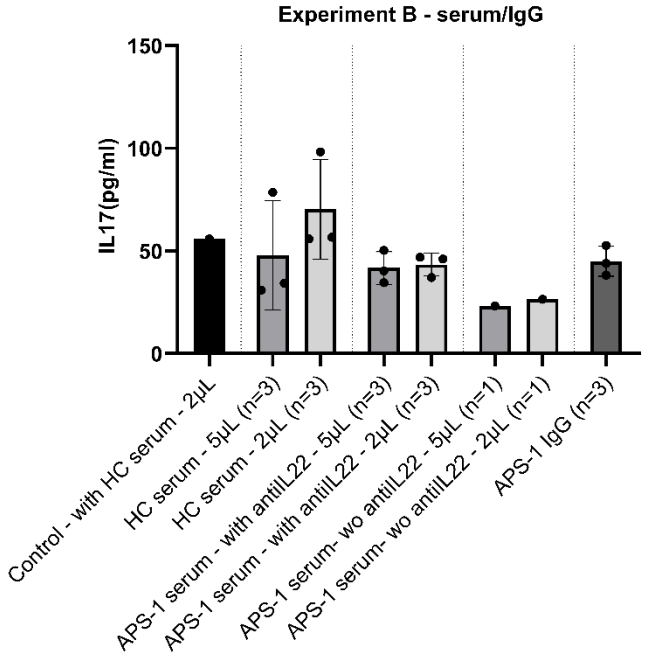

Figure 4.23. Levels of IL-17 detected in culture supernatants of CD4+ T cells treated by HC and APS-1 sera/IgG. Cell samples from experiment B, were conducted to examine the impact of APS-1 and HC sera and IgG on the production of IL-17 cytokine from RORγt+ Th17 cells compared to a control group with 2µL HC serum. Some APS-1 sera contained type 1 IFN and IL-22 ABs (APS-1 serum w Anti-IL22), whereas some APS-1 sera did only contain type 1 IFN ABs and not IL-22 ABs (APS-1 serum wo Anti-IL-22). Sera from both HC and APS-1 individuals added at two different (2µL and 5µL) concentrations. Only one biological parallel was evaluated for the conditions of APS-1 serum without anti-IL-22 with two distinct concentrations. Hence, no statistical testing was applied. Mean values are represented by boxes, and error bars indicate the standard deviation. A non-parametric, one-way ANOVA test was conducted using GraphPad version 9.5.1, from which no significant changes in IL-17 levels compare to the control with 2µL HC serum.

In general, a slight decrease in the levels of IL-17 cytokine was observed when the Th17 cells were exposed to patient serum without anti-IL-22 autoABs. However, no significant difference was seen when comparing the impact of APS-1 sera/IgG to the controls with HC serum on the levels of IL-17 cytokine.

4.6 Effects of autoABs against IFN-Is and IL-22 in APS-1 patient sera and IgG on *in vitro* PBMCs from healthy donors

We performed a follow-up investigation to evaluate the impact of autoABs against type I IFNs and IL-22, sourced from APS-1 patients, on healthy PBMCs in an *in vitro* setting. The study also included the presence of HKCA and utilized Lipopolysaccharide (LPS) as a control to assess the cellular response to HKCA. To accomplish this, the study assessed the impact of sera obtained from both HC individuals and APS-1 patients, as well as HC and APS-1 purified IgGs. The experiment was carried out under *in vitro* conditions including the presence of IL-1β, IL-6, IL-23, TGF-β cytokines, and anti-IL-4 and anti-IFN-γ ABs. This section presents the results obtained from the analysis of RORγt+ Th17 cells and FoxP3+ Tregs.

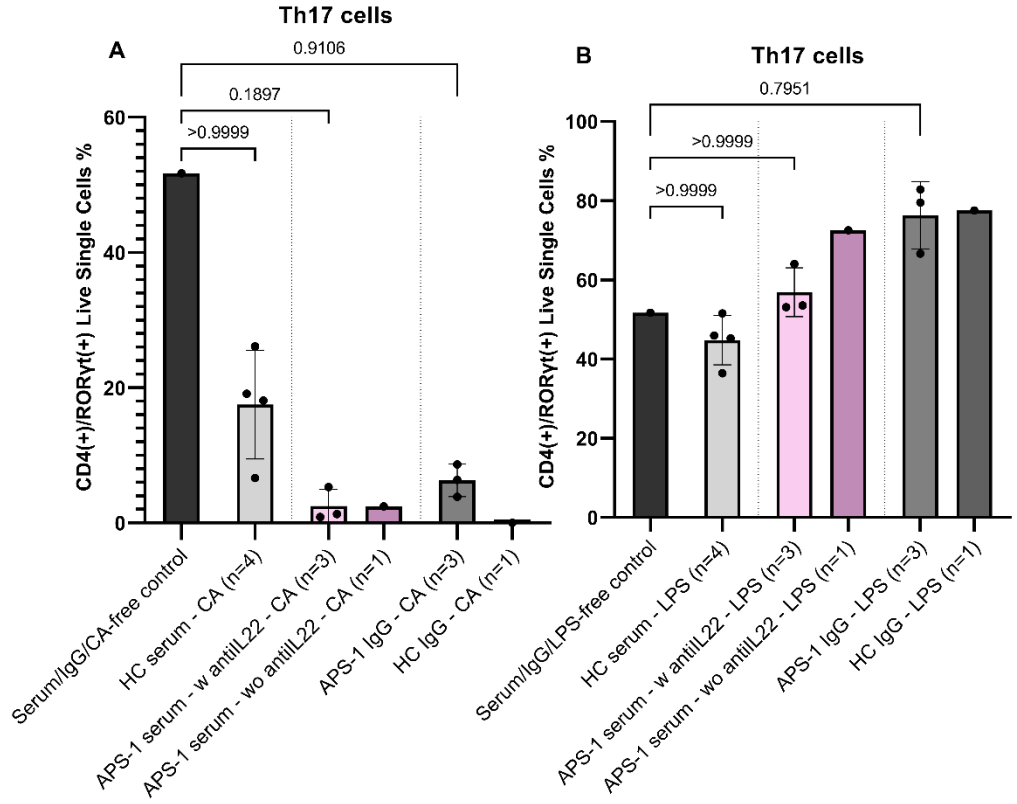

Figure 4.24. Flow cytometry analysis of the impact of HC/APS-1 Sera, and HC/APS-1 IgG on Th17 cells from *in vitro* HC PBMCs in the Presence of A) HKCA; B) LPS. The experiment was performed to assess the effect of serum and IgG from both (APS-1) patients and healthy individuals (HC) on Th17 cells within CD4+ T cell population from healthy PBMCs compared to the serum/IgG-free control group. Every condition, except for the control, had HKCA (CA) (for A) or LPS (for B). Among APS-1 sera, one did not contain (without = wo) autoABs against IL-22 cytokine, but all have autoABs against type I IFNs. The rest contained all three autoABs (with = w). In this experiment, various cell conditions were compared to a control condition with no serum/IgG. Mean values are indicated by boxes, error bars indicate standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test (P value are indicated on the tiles) using GraphPad version 9.5.1.

Compared to the control group, a noticeable trend towards a decrease in Th17 cells within the CD4+ T cell population, although without reaching statistical significance in any case, was observed when HKCA was introduced along with an even larger decrease when sera and IgG from both HC individuals and APS-1 patients were added (Fig. 4.24A). LPS was employed in this experiment as a means to induce an inflammatory response in cell cultures, serving as a control. This allowed for a comparison of its effects on cells to those of HKCA under identical conditions with the presence of sera and IgG from both HC and APS-1 patients. No similar decreasing in Th17 cell tendencies as for addition of HKCA with/without sera/IgG were revealed when adding LPS instead of HKCA (Fig. 4.24B).

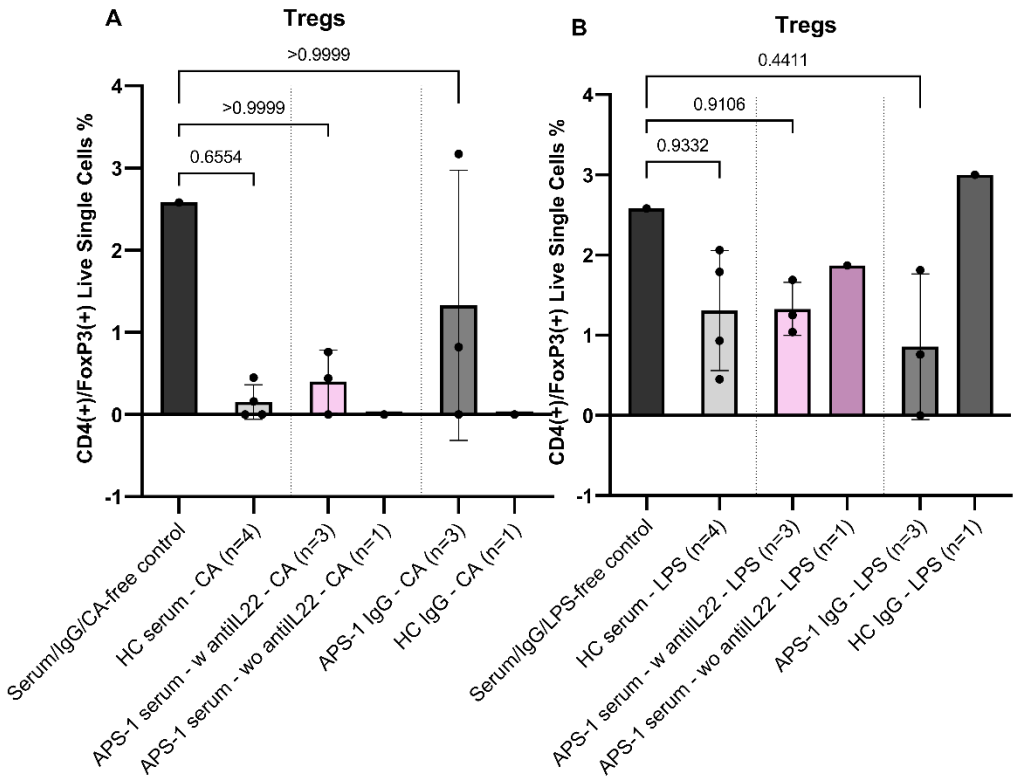

Figure 4.25. Flow cytometry analysis of the impact of HC/APS-1 Sera, and HC/APS-1 IgG on Tregs from *in vitro* HC PBMCs in the Presence of A) HKCA; B) LPS. The experiment was performed to assess the effect of serum and IgG from both (APS-1) patients and healthy individuals (HC) on Tregs within CD4+ T cell population from healthy PBMCs compared to the serum/IgG-free control group. Every condition, except for the control, had HKCA (CA) (for A) or LPS (for B). Among APS-1 sera, one did not contain (without = wo) autoABs against IL-22 cytokine, but all have autoABs against type I IFNs. The rest contained all three autoABs (with = w). In this experiment, various cell conditions were compared to a control condition with no serum/IgG. Mean values are indicated by boxes, error bars indicate standard deviation. A non-parametric, one-way ANOVA test-Kruskal Wallis test (P value are indicated on the tiles) using GraphPad version 9.5.1.

A trend towards a decrease in the frequency of Tregs within the CD4+ T cell population was observed when HKCA was added, although this decrease was not statistically significant. Furthermore, a larger decrease in Tregs was observed when sera and IgG from both healthy control individuals and APS-1 patients were introduced (Fig. 4.25A). However, it is worth

noting that there was one condition involving cells exposed to patient IgG where this trend was not observed. In this experiment, LPS was used as a control to induce an inflammatory response in cell cultures. This allowed for a comparison of the effects of LPS on cells to those of HKCA under identical conditions with the presence of sera and IgG from both HC individuals and APS-1 patients. It was observed that the addition of LPS did not lead to a similar decrease in Treg frequencies as observed with the addition of HKCA, regardless of the presence of sera and IgG (Fig. 4.25B)

4.7. *In vitro* assessment of autoABs against IFN-Is and IL-22 in APS-1 patient Sera on PBMCs from APS-1 patients and healthy controls

In the latest experiment, PBMCs were obtained from three APS-1 patients (as described in Appendix I). Additionally, PBMCs from three healthy controls were isolated and utilized as sex-matching controls (Appendix II), as previously explained in section 4.1. Similar to the previous study involving PBMCs, this experiment incorporated the use of HKCA and LPS. The effects of sera derived from both HC individuals and APS-1 patients, as well as HC and APS-1 purified IgGs, were evaluated in the presence of HKCA. The experiment was conducted under consistent *in vitro* conditions, including the presence of IL-1β, IL-6, IL-23, TGF-β cytokines, as well as anti-IL-4 and anti-IFN-γ ABs. The results of the study of RORt+ Th17 cells and FoxP3+ Tregs are presented in this section.

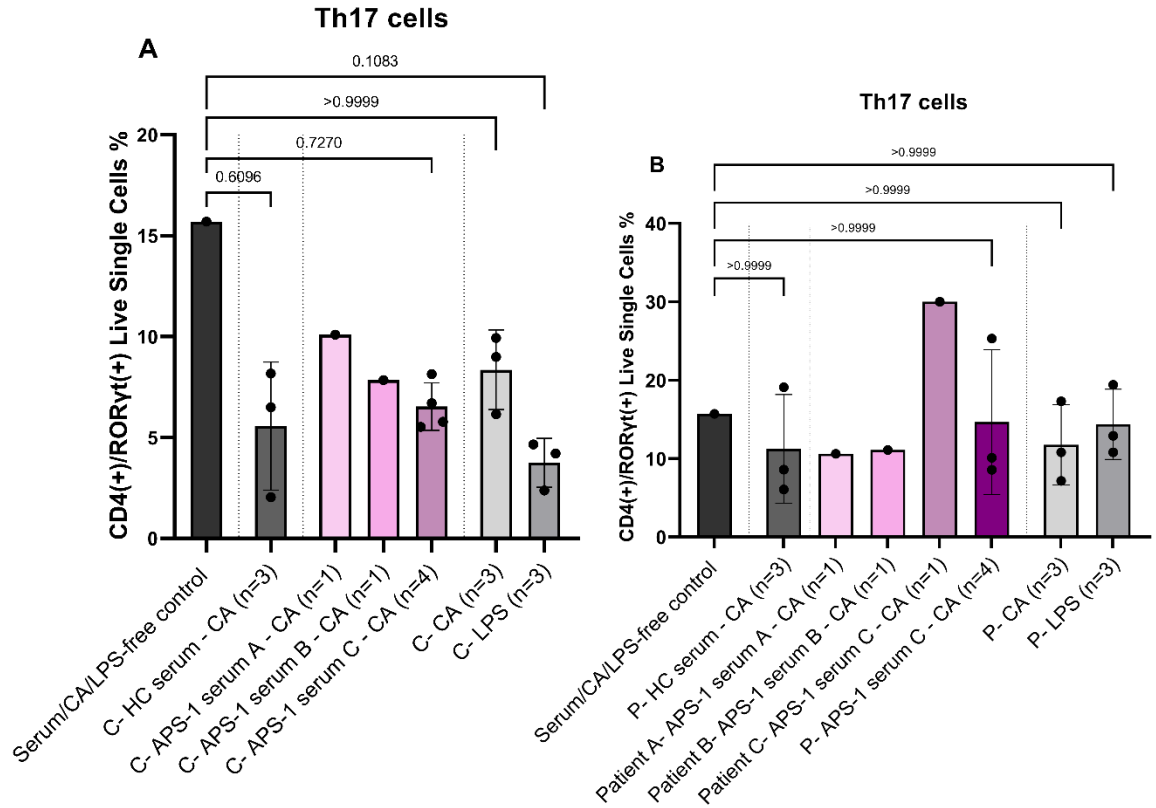

Figure 4.26. *In vitro* impact of sera on Th17 cells within PBMCs in the presence of HKCA in flow cytometry. A) Cells from healthy controls (HC); **B)** Cells from APS-1 patients (APS-1). The objective of the experiment was to evaluate the impact of serum obtained from both patients and healthy individuals on Th17 cells within healthy/patient PBMCs compared to the HKCA/serum-free control group. Two other control groups, one contained HKCA and the other with LPS, which both lacked serum, were also included. All APS-1 sera included in the study had presence of both ABs against type I IFNs and IL-22 cytokine. Specifically, APS-1 serum A, B, and C were derived from the corresponding APS-1 patients A, B, and C, whose PBMCs were utilized in the experimental culture (Fig 4.26). Consequently, to assess the effect of each serum, they were added separately in three distinct conditions. Serum C from one of the patients was selected to be added in all control conditions to comparing its effect among three distinct HC PBMCs. Mean values are indicated by boxes, the error bars represent the standard deviation. Statistical comparisons were conducted (P value are indicated on the tiles) using a non-parametric, one-way ANOVA test (Kruskal Wallis test) using GraphPad version 9.5.1.

When HKCA was combined with sera from both HC individuals and APS-1 patients, a clear trend towards a reduction in Th17 cells within the CD4+ T cell population was observed, in comparison to the control group without HKCA. This applies to both APS-1 cells and healthy controls cells (A and B in Fig 4.26). This small suppressive effect on Th17 cell development was also detected in the presence of LPS. However, no statistically significant differences in Th17 frequencies were observed when compared to controls.

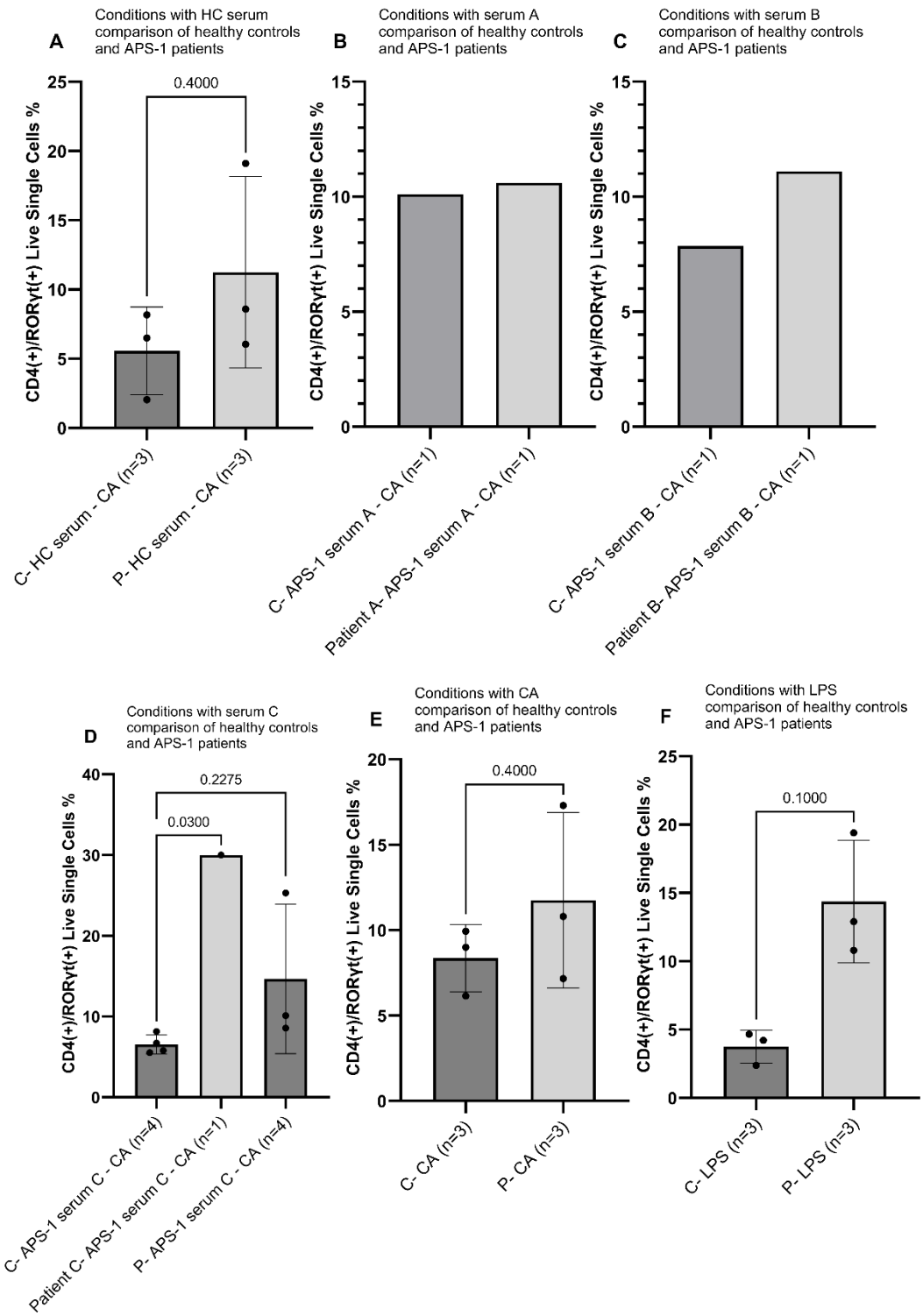

Figure 4.27. Comparison of the Th17 cell frequencies under identical culture conditions within PBMCs from APS-1 and HC individuals in flow cytometry. Cells from healthy controls (C) in comparison to cells from APS-1 patients (P/Patient). All APS-1 sera included in the study had presence of both ABs against type I IFNs and IL-22 cytokine. Specifically, APS-1 serum A, B, and C were derived from the corresponding APS-1 patients A, B, and C, whose PBMCs were utilized in the experimental culture (Fig 4.26). Comparing patient and control Th17 cells exposed to **A)** HC sera, **B)** APS-1 serum A, **C)** APS-1 serum B, **D)** APS-1 serum C, **E)** HKCA (CA), **F)** LPS. E) and F) both lacked sera. Mean values are indicated by boxes, the error bars represent the standard deviation. Statistical comparisons were conducted (P value are indicated on the tiles) performing t tests (and non-parametric tests) in GraphPad version 9.5.1.

With the exception of one condition (Fig 4.27D) where Th17 cells from patient C, exposed to their corresponding serum, showed a significant effect with an increase in Th17 cell differentiation compared to the same condition in PBMCs from a healthy control (P value: 0.0300). The remaining conditions did not show any significant difference when compared to their respective control conditions.

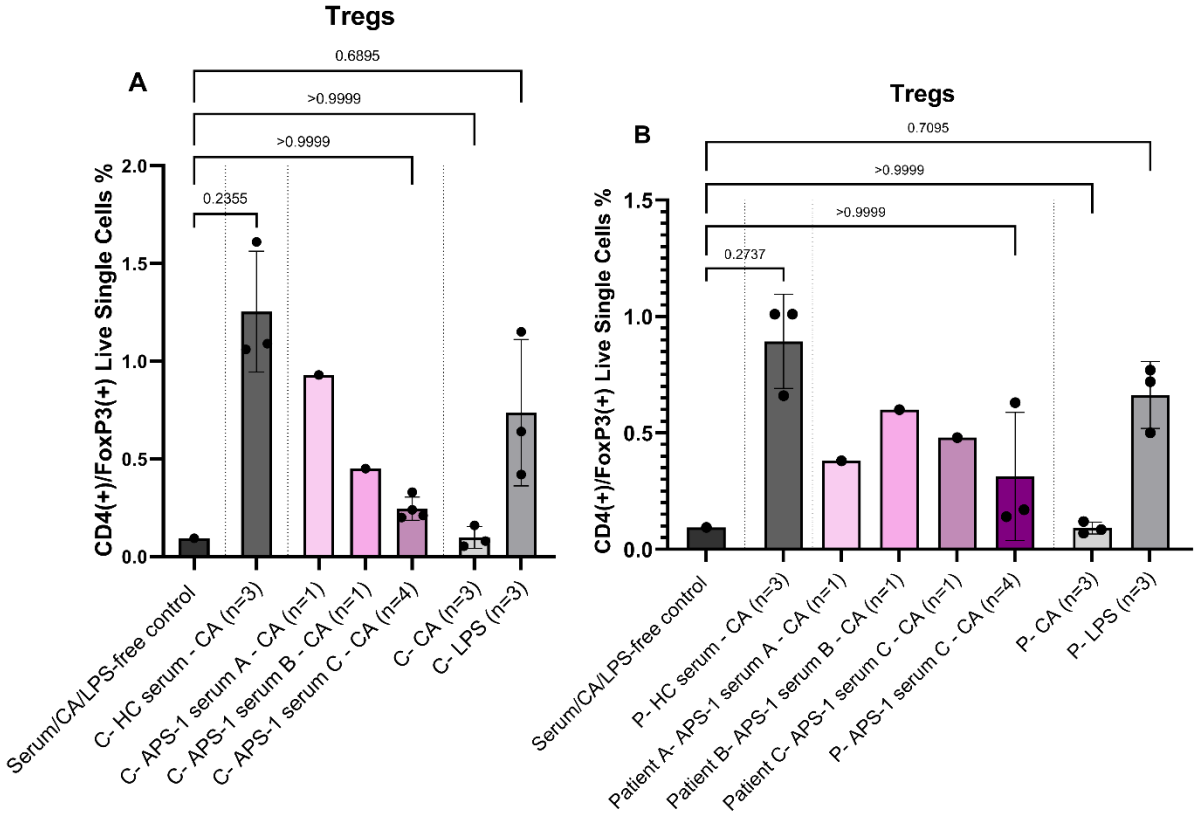

Figure 4.28. *In vitro* impact of sera on Tregs within PBMCs in the presence of HKCA in flow cytometry. A) Cells from healthy controls (HC); **B)** Cells from APS-1 patients (APS-1). The objective of the experiment was to evaluate the impact of serum obtained from both patients and healthy individuals on Tregs within healthy/patient PBMCs compared to the HKCA/serum-free control group. Two other control groups, one contained HKCA and the other with LPS, which both lacked serum, were also included. All APS-1 sera included in the study had presence of both ABs against type I IFNs and IL-22 cytokine. Specifically, APS-1 serum A, B, and C were derived from the corresponding APS-1 patients A, B, and C, whose PBMCs were utilized in the experimental culture (Fig 4.28). Consequently, to assess the effect of each serum, they were added separately in three distinct conditions. Serum C from one of the patients was selected to be added in all control conditions to comparing its effect among three distinct HC PBMCs. Mean values are indicated by boxes, the error bars represent the standard deviation. Statistical comparisons were conducted (P value are indicated on the tiles) using a non-parametric, one-way ANOVA test (Kruskal Wallis test) using GraphPad version 9.5.1.

In contrast to the effect of HKCA on Th17 cell differentiation, a tendency towards an increase in Tregs within the CD4+ T cell population was observed when compared to the control group without HKCA (Fig 4.28A). Similarly, a slight stimulatory effect on Treg development was detected in the presence of LPS (Fig 4.28B). However, no statistically significant differences in Treg frequencies were detected compared to controls.

5. Discussion

Due to its rarity and clinical variability, APS-1 remains a challenging disease to study and treat, posing difficulties in the field of medicine and research. The worldwide distribution, genetic variants, and clinical symptoms of APS-1 are all the subject of ongoing research. These initiatives seek to advance diagnosis, create focused therapeutics, and enhance support for people throughout the world who suffer from this rare autoimmune condition. Importantly, as APS-1 is a model disorder for endocrine autoimmunity based on its monogenic cause and relevant impact of the disease gene *AIRE* for immune tolerance, knowledge that comes out from such studies can be informative for a range of autoimmune and immune deficiency disorders and can provide new knowledge on central and peripheral tolerance.

It is known that patients with APS-1 commonly suffers from opportunistic candida infections, and several studies have pinpointed mechanism that could be implicated in this immune deficiency manifestation of APS-1. First and foremost, Th17 cells are very important in *Candida albicans* clearance, and it is known that patients with mutations in genes in the IL-17-pathway are prone to candidiasis (Puel 2020; Puel et al. 2010). APS-1 patients have been found to harbour neutralizing autoABs against Th17-mediators (IL-22, IL-17A and IL-17F) (Kisand et al. 2010), and this can for sure be implicated in the pathogenesis but has never been fully established. Secondly, cells (PBMCs) from patients with APS-1 have been found to express less IL-22 than controls, and they produce significantly less IL-23, a regulator of Th17 cells upstream in the signalling pathway, compared to healthy controls (Bruserud, Oftedal, Landegren, et al. 2016). Thirdly, APS-1 patients have autoABs against type I interferons (Meager et al. 2006), which may not directly be involved in Th17-pathways but might still have an effect on Th17/other T cell balances. However, there is still unanswered questions in relation to how these cytokine autoABs influence Th17-cell differentiation. This is what this project wanted to find out.

To this end, we first optimised the methodology for conducting *in vitro* studies on IFN and Th17 cytokine autoABs found in APS-1 individuals, with a specific emphasis on T cells and the balance between Th17 cells and other T cell subsets. Accordingly, cells obtained from both APS-1 patients and healthy controls were utilized to investigate the potential impact of these autoABs on Th17 cells. This investigation involved employing multiple endpoint assays including both protein levels and RNA transcript levels. The overall findings we provide supports no major effect for the APS-1 cytokine autoABs for *in vitro* cultures of PBMCs or

expanded Th17 cells. Testing on isolated CD4+ T cells, no consistent differential expression for *in vitro* Th17 cells in respond to commercial autoABs against anti-IFNα, anti-IFNω, and anti-IL-22 were observed in flow cytometry experiments. However, these changes were not deemed significant in terms of Th17 development. Furthermore, the impact of these commercially available cytokine ABs did not result in significant alterations in the expression of the RORγt gene compared to its expression in AB-free control group using qPCR. Subsequently, minor negative effects of APS-1 patients' sera and IgG harboring the same autoABs on Th17 cells were observed. A noticeable trend towards decreased development of Th17 cells was also seen when exposed to HC sera in the same experiments. However, statistically no discernible effect of APS-1 sera/IgG and HC sera was detected on the differentiation of RORγt+ T cells from CD4+ T cells *in vitro*, nor on the RORγt gene expression compared to the control group.

CMC, a common clinical manifestation observed in APS-1 patients, affects the mucous membranes and skin and is primarily caused by *Candida albicans*. The development of CMC in APS-1 is believed to be associated with immunological dysregulation and impaired function of T cells, specifically a deficiency in the production or activity of Th17 cells, which play a critical role in fungal defence (Philippot, Casanova, and Puel 2021). Accordingly, in this study, we investigated the impact of HKCA in the presence of HC and APS-1 sera/IgG on PBMCs obtained from healthy individuals. The results showed a drop in Th17 cell proliferation within the CD4+ population of healthy control PBMCs when HKCA was present, compared to the conditions exposed to LPS. However, this difference was not statistically significant based on the analysis conducted. Similarly, Th17 cells within the CD4+ population of APS-1 patient PBMCs showed a non-significant effect of sera/IgG when exposed to HKCA. It is important to note that this is a preliminary study with a small sample size of only three patients, so care must be taken when interpreting these findings**.**

In this project, a variety of assays were utilized in combination, allowing us to comprehensively evaluate the effects of cytokine autoABs on various aspects of T cell behavior and functionality referring to cytokine receptor activity and production of IL-17. While parts of the qPCR techniques were already in use in the lab (optimization described in 3.9.3) and the ELISA-assays were commercial ready-to-use kits, the differentiating *in vitro* RORγt+ Th17 cells from isolated CD4+ T cell population was a novel approach for the lab, necessitating the optimization and validation of the methodology. Therefore, the flow cytometry panel for

accurately characterizing and gating on this population of T cells required optimization and validation as well.

5.1 *In vitro* cell culture for Th17 cells

In vitro cell culturing of T cells offered us the ability to manipulate and control various aspects of the experimental conditions. This included having control over the composition of the culture media and selectively including or excluding specific factors or stimuli, allowing us to optimize the nutritional and growth factor requirements for ideal differentiation, function, and maintenance of Th17 cells. By studying T cells in isolation in an *in vitro* setting, we were able to minimize the potential interference of other immune cells and the variability of cytokine levels that exist in the complex in vivo environment. This targeted approach enabled us to specifically investigate and gain insights into the effects and responses of T cells, resulting in increased efficiency in screening and testing of various conditions to study cellular behavior and functions. Moreover, investigating T cells in an *in vitro* setting reduces reliance on animal models, thereby resolving ethical issues raised by animal experiments.

However, it is essential to acknowledge the limitations of *in vitro* cell culturing. Cell culture techniques cannot adequately replicate the complex interactions that occur within tissues and organs, including the intricate interplay between different cell types, the extracellular matrix, and physiological gradients. The conditions used for cell culturing themselves, may introduce errors or biases that can influence the results. *In vitro* experiments in simplified conditions, without the presence of other immune cell types and systemic factors, including hormones, growth factors, and immune mediators, may exhibit altered behavior, signalling, gene expression, and functionality compared to their *in vivo* counterparts. Therefore, it is crucial to validate and complement findings from *in vitro* studies with in vivo experiments to obtain a comprehensive understanding of T cell biology and function.

5.2 Th17 cells and Tregs share TGF-β-mediated signalling pathway

Th17 cells are frequently implicated in the development of autoimmune disorders due to their involvement in the immune response (Korn et al. 2009). Conversely, autoimmune conditions often demonstrate reduced quantities or impaired functioning of Tregs (Crispin, Martínez, and Alcocer-Varela 2003; Yasuda, Takeuchi, and Hirota 2019). Notably, the TGF-β-mediated signalling pathway has a dual function in promoting the differentiation of both Th17 cells and Tregs from naive CD4+ T cells. In an inflammatory milieu, the combination of TGF-β and IL-6 cytokines promotes the differentiation of Th17 cells while suppressing the development of

Tregs (Korn et al. 2009). At lower concentrations, TGF-β works together with IL-6 to enhance the expression of the IL-23 receptor and facilitate the differentiation of Th17 cells (Zhou et al. 2008). Accordingly, during the *in vitro* culturing of isolated naïve CD4+ T cells we included concentration of IL-6 (30 ng/mL) with TGF-β1 (5 ng/mL) of Th17-polarizing cytokines as suggested by a standardised protocol by Miltenyi.com. Assessing distinct T cell fractions harvested from CD4+ T cell cultures by flow cytometry, both RORγt+ Th17 cells and FoxP3+ Tregs population within the CD4+ T cells were generated with a presence for the Th17 cell population. To achieve optimal differentiation of Th17 cells while suppressing Treg differentiation in the presence of TGF-β cytokine, one approach could involve adjusting the concentrations of Th17 cytokines in the culture media. This could be explored by either increasing the concentrations of IL-6, IL-23, and IL-1β while keeping the TGF-β concentration low, or by decreasing the TGF-β concentration while maintaining a constant level of other cytokines involved in Th17 cell differentiation. Since the differentiation of Th17 cells from naïve CD4+ T cells required a 7-day expansion period, the timing and duration of cytokine exposure could influence the differentiation outcomes. This could be possible by providing TGF-β together with other Th17 cytokines during the early stages of culture, then removing TGF-β completely by changing the media at later stages. This approach might allow for the initial induction of Th17 cell differentiation while minimizing the potential for Treg development as the culture progresses. Other cytokines could maybe also been tried. IL-21, as an example, can act synergistically with other cytokines, such as IL-6 and TGF-β, to drive Th17 cell differentiation and expansion, and later involve in the survival, maintenance and long-term functionality of mature Th17 cell (Martinez et al. 2008; Yang, Anderson, et al. 2008).

As long as we generated Tregs out of CD4+ T cells along with Th17 cells, this project was not solely focused on investigating the effects of APS-1-related cytokine autoABs on Th17 cells, it, therefore, encompassed both Th17 cells and Tregs, aiming to understand the impact of APS-1-related cytokine autoABs on these two distinct T cell populations.

5.3 Inhibition of *in vitro* Th1 and Th2 cell differentiations from isolated naïve CD4+ T cells

Signaling through IFNγ cytokine, mainly produced by activated T cells, triggers the differentiation of naïve CD4+ T cells into Th1 cells. This process involves the activation of multiple factors, including the upregulation of IFNγ production itself, which collectively enhance the commitment and function of Th1 cells (Schoenborn and Wilson 2007), while inhibiting the Th17 lineage-specific transcription factor RORγt (Manetti et al. 1994).

Additionally, the IL-4 cytokine plays a crucial role in the differentiation of naive CD4+ T cells into Th2 cells. It promotes the expression of GATA3 lineage-specific transcription factor, as well as other Th2-associated cytokines (Walker and McKenzie 2018), while inhibits the differentiation of other T cell lineages, such as Th17 cells (Harrington et al. 2005). According to this, ABs against IFNγ and IL-4 were included in the culture conditions to neutralize the effect of them on Th1 and Th2 cell differentiation, respectively, thereby favouring the development and maintenance of Th17 cell population. Through flow cytometry analysis and comparison of the proliferative capacity of distinct T cell subsets in the conditions with and without ABs, the findings showed that blocking the signalling of these specific cytokines using their corresponding ABs resulted in the successfully inhibition of Th1 and Th2 cell differentiation and expansion. Interestingly, the inclusion of anti-IFNγ AB demonstrated a suppressive effect on all four T cell subsets examined. It is plausible that the anti-IFNγ ABs could engage in non-specific interactions, such as interactions with Fc receptors, and cross-reactivity with alternative cell subsets or cytokines, in the absence or low abundance of the targeted Th1 cell population and its specific cytokine signalling in the culture conditions. Thus, to achieve optimal proportion of Th17 cell subset differentiation from CD4+ T cells, the highest titration (5ng/μL) of the anti-IFNγ AB in the culture conditions. By doing so, we sought to maximize the inhibitory effect on the differentiation of other T cell subsets while minimizing potential off-target effects on Th17 cells. However, selective targeting and suppressing the differentiation of Th1 and Th2 cells in an *in vivo* environment could pose significant challenges and might be potentially impractical due to the complexities of the immune system and the interdependencies between different cell populations. Additionally, there could be a risk of unintended consequences, including the disruption of vital physiological processes or impacting the function of other cell populations. Moreover, the ability to precisely regulate numerous experimental parameters, such as the timing, duration, and concentration of ABs, could be a constraint of *in vivo* experiments. These limitations could make it challenging to achieve immediate, consistent, and reproducible results when attempting to selectively suppress cell lineage differentiation.

5.4 Effect of sera on T cell lineages

The findings of multiple studies have demonstrated that human serum, including samples obtained from patients, healthy individuals, AB serum, can have diverse effects on T cell differentiation outcomes. For example, sera from patients with AIDS-related complex (ARC) or acquired immune deficiency syndrome (AIDS) demonstrated inhibitory effects on the

proliferative response of an interleukin-2 (IL-2)-dependent cytotoxic T-cell line (Donnelly et al. 1986). It has further been shown that sera obtained from pregnant women contain factors that inhibit the specific functions of T cells (Salméron et al. 1991). T cells that were stimulated with PHA/feeder cells and autologous serum from two different donors exhibited a significantly longer proliferative lifespan compared to T cells stimulated with PHA/feeder cells and AB serum (Roeth et al. 2005). Culturing mesenchymal stem cells with human serum resulted in a notable reduction in CD3/CD28-induced T-cell activation, which consequently led to impaired T cell differentiation (Pérez-Ilzarbe et al. 2009). Elevated levels of IL-10 in the sera of patients with systemic lupus erythematosus (SLE) was found to induce the expression of Fas and FasL on the surface of CD4+ T cells, leading to the promotion of apoptosis within this specific subset of cells. (Yang et al. 2015). CAR T cells exposed to human platelet lysate cultured sera demonstrated a less differentiated T cell phenotype and gene signature. This altered phenotype was correlated with improved proliferative capacity and enhanced anti-tumor effects when subjected to long-term *in vitro* coculture experiments (Torres Chavez et al. 2019).

In our experimental study, we consistently observed that the addition of AB serum, HC serum, and sera from APS-1 patients resulted in a consistent suppression of T cell development in both T-cell and PBMC cultures. These observed effects could be attributed to the unique composition and properties of the serum utilized in the experiments. The presence of specific ABs or other factors in the serum could bind to cell surface receptors or other substances involved in T cell signalling, thereby influencing the differentiation outcomes. Moreover, the serum might contain growth factors and cytokines that provide additional signals capable of modulating T cell differentiation. While the AB serum is heat inactivated prior to use , this was not done with the healthy control or APS-1 serum. This could have been done and the results would maybe then be different.

5.5 Proliferative capacity of Th17 cells

The proliferative capacity of Th17 cells can vary from experiment to experiment due to various factors. One such factor is aging of the subject of the study, which has been shown to impact CD4 T cell differentiation. As individuals age, there is a decline in the differentiation and functional capabilities of CD4 T cells. This age-associated decline can result in an altered distribution of T cell subsets, and is often associated with diminished cellular and humoral immune responses (Lefebvre et al. 2016). In this study, the analysis of proliferation index

values using flow cytometry revealed variations in the proportion of Th17 cells generated in each *in vitro* experiment. Given that the composition of the media, concentration of activating stimuli (such as anti-CD3/CD28 ABs and cytokines), and other reagents used in the culture conditions were consistent across the experiments involving CD4+ T cell cultures, the observed variations in Th17 cell proliferation could likely be attributed to inter-donor variability or that the assay is not robust enough to handle slight variations in day-to-day experiment conditions. As peripheral blood derived CD4+ T cells were isolated, merged, and utilized from approximately 10 healthy blood donors for use in each experiment in order to get enough cells, this could be beneficial or non-optimal for the experiments. On the positive side, merging of samples will counteract that one odd donor's immune system result in strange findings. However, inter-variability between donors could still pose problems for the reliability of the assays. These variations could be influenced by prior activation or exposure to different stimuli in the donors' circulation or for example gender, age, and genetic disturbances. Eventually, it could affect the responsiveness and proliferative capacity of CD4+ T cells, resulting in divergent responses in terms of Th17 cell proliferation. Therefore, due to our current limited knowledge regarding *in vitro* Th17 cell differentiation, which exhibits significant variability depending on the specific culture conditions (Veldhoen et al. 2009), and the observed differences in transcriptional profiles between *in vitro*-generated Th17 cells and those found in vivo (Zhou, Chong, and Littman 2009), it is crucial to interpret the results with caution. In *in vitro* settings, these potentially confounder effects for the outcomes will always pose challenges but could potentially be counteracted by having a really large pool of donor cells which has been checked previously, and then use this pool for all experiments. This was not possible for this study.

5.6 Proteomic versus transcriptomic results

To gain a thorough understanding of T cell differentiation, researchers can conduct proteomic analysis to comprehensively characterize the proteome of different T cell subsets. This approach involves identifying and quantifying proteins present in the sample, which provides valuable insights into protein expression levels, post-translational modifications, and interactions between proteins. By analyzing the proteomic data, researchers can uncover specific proteins and pathways that play a role in the function of distinct T cell lineages. Additionally, proteomic analysis can provide information about the functional state of T cells, reveal protein networks, and potentially identify biomarkers associated with specific T cell lines (Apte and Mehendale 2005). Transcriptomic analysis, on the other hand, focuses on

measuring gene expression levels, particularly the presence of mRNA molecules in a sample, allowing for the identification of genes that are differentially expressed in T cell subsets and subsequent investigation of the molecular mechanisms underlying T cell lines through the examination of gene expression patterns (Supplitt et al. 2021).

In this study, the proteomic data was generated from T cell subsets that were cultured under different conditions using commercial ABs, HC sera, and sera from patients with APS-1. This data was obtained by analyzing the samples through flow cytometry, which enabled the identification and characterization of different T cell subsets based on specific extracellular and lineage markers. After successfully generating Th17 cells as well as the protein expression of CCR6 as the main surface marker characterizing the Th17 lineage (Acosta-Rodriguez et al. 2007; Annunziato, Cosmi, Santarlasci, Maggi, Liotta, Mazzinghi, Parente, Filì, et al. 2007; Singh et al. 2008) in the *in vitro* T cell culture conditions based on the flow results, we conducted an additional proteomic analysis to provide further evidence and confirmation of the effective production of Th17 cells from CD4+ T cells. Accordingly, ELISA was employed as part of the proteomic analysis to provide more precise and quantitative assessment of IL-17 cytokine expression in the cell culture supernatants of various samples. The outcomes indicated that the IL-17 cytokine was detected in the majority of T-cell samples, confirming the efficient differentiation of Th17 cells from CD4+ T cells.

With the intention of integrating proteomic and transcriptomic data, we employed qPCR analysis to gain a more holistic understanding of T cell subsets, effectively connecting gene expression with protein function. Through this analysis, we successfully detected the expression of the RORγt gene in all samples derived from isolated CD4+ T cell cultures, confirming the successful *in vitro* differentiation of Th17 cell lineage from naïve CD4+ T cells. Following that, a comparison was made between the gene and protein expression levels of the CXCR3 extracellular marker, which is predominantly expressed on Th1 cells (Langenkamp et al. 2003; Groom and Luster 2011). Although flow cytometry data did not show protein expression for T-bet+ Th1 cells in all experiments involving T cell and PBMC cultures, both proteomic and transcriptomic analyses consistently indicated high expression of the CXCR3 receptor. Furthermore, qPCR analysis from T cell cultures revealed the presence of the T-bet gene, although its expression level was lower compared to the expression level of the RORγt gene.

There could be several reasons why proteomic and transcriptomic results can differ in experiments. This is indeed seen quite often in molecular biology studies and can have both methodology or functional reasons (Koussounadis et al. 2015; Vogel and Marcotte 2012; Fukao 2015). According to the biological dogma, RNA is transcribed from DNA, and this is then translated to amino acids and proteins, all very regulated processes. Thus, the relationship between gene expression and protein expression is complex and not always linear. Various regulatory mechanisms, such as signalling pathways, and protein interactions, could independently influence protein abundance, decoupling it from mRNA levels, highlighting the need to consider these additional layers of regulation beyond transcription. Moreover, rapid changes in gene expression at the transcriptional level could happen, while the synthesis and accumulation of proteins could require more time. This delay might lead to disparities between mRNA and protein levels at a specific time point. Additionally, proteins could exhibit diverse turnover rates, meaning that their abundance might not directly correspond to mRNA levels. These factors introduce differences between mRNA and protein expression, emphasizing the necessity of taking time lag and protein turnover into account when analyzing gene expression and protein abundance. Furthermore, the sensitivity, accuracy, and coverage of the techniques employed to detect and measure proteins and mRNA could differ. Discrepancies between proteomic and transcriptomic analyses could arise due to variations in experimental conditions, such as differences in sample preparation, culture conditions, and cellular states. These factors might influence the outcomes and contribute to divergent results between the two analyses(Koussounadis et al. 2015; Vogel and Marcotte 2012; Maier, Güell, and Serrano 2009).

However, CCR4 and CXCR3 were here shown to be produced in the CD4+ cells both be the protein and transcript measurement methodologies. Multiple research studies have indicated that distinct Th17 subsets can be distinguished based on the differential expression patterns of CCR4, CCR6, and CXCR3. For instance, the simultaneous expression of CCR6 and CCR4 identifies a specific subset of human Th17 cells that exhibit the ability to produce IL-17A and undergo proliferation when exposed to *Candida albicans* and Staphylococcus aureus (Acosta-Rodriguez et al. 2007; Zielinski et al. 2012; Melé et al. 2015). On the other hand, the co-expression of CCR6 and CXCR3 characterizes a heterogeneous population referred to as Th1-Th17, which is capable of producing both IL-17A and IFN-γ (Acosta-Rodriguez et al. 2007). The small induction of CCR6, but high levels of CCR4 and CXCR3 described here indicates that our artificial made Th17-cells might be mixture of Th17 and Th1 cells, or most are Th17-Th1 cells.

5.7 Influence of APS-1 related cytokine autoABs and HKCA on Th17 cells

Th17 cells play a role in promoting the production of various cytokines such as IL-17A/F, IL-22, IL-21, and IL-26. These cytokines contribute to the recruitment and activation of neutrophils, as well as induce the production of proinflammatory cytokines, chemokines, and antimicrobial peptides in different cell types (Liang et al. 2006; Wolk et al. 2006; Kisand et al. 2010; Korn et al. 2009). Studies provided evidence that autoABs against Th17-associated cytokines are implicated in the pathogenesis of CMC in APS-1 patients (Kisand et al. 2010) (Ahlgren et al. 2011). A study revealed that patients with CMC exhibited significant and consistent reductions in IL-17F and, particularly, IL-22 responses. However, these responses remained unaltered in rare cases without CMC (Kisand et al. 2010). Also, it has been observed that the Th17 pathway is activated and upregulated in response to C. albicans infections, while the secretion of IL-22 is reduced. It was also shown that both APS-1 patients and control PBMCs exhibit a robust Th1 response to C. albicans (Ahlgren et al. 2011).

One of the main hypotheses in our study was that the presence of neutralizing cytokine autoAbs in individuals with APS-1, specifically high-titer autoABs against type 1 IFNs, IL-22 cytokines, might have an impact on Th17 cells. We postulated that these autoABs could potentially influence the differentiation and development of Th17 cells, leading to either a negative or positive effect on this specific cell lineage. For this we employed commercially available anti-IL-22, anti-IFNα, and anti-IFNω ABs, as well as APS-1 patient sera and IgG containing same cytokine ABs in *in vitro* cell cultures. We performed these experiment in different culture set ups using isolated healthy CD4+ T cells and PBMCs, as well as patient PBMCs. Furthermore, we examined the effect of these ABs in APS-1 sera and IgG samples in the presence of HKCA stimulation. A comparative analysis was performed using PBMCs from three sex-matched controls and three APS-1 patients. Accordingly, we observed a minor reduction in the differentiation of RORγt+ Th17 cells from isolated CD4+ T cells and a decrease in the frequency of RORγt-expressing Th17 cells within the CD4+ T cell population in PBMCs. However, these changes did not reach statistical significance. Furthermore, our results indicated a reduction in Th17 expression in PBMC cultures from both control individuals and APS-1 patients when subjected to HKCA stimulation. Additionally, the absence of T-bet+ Th1 cells was observed in all analyzed samples, indicating a divergence from the previously observed Th1 response in APS-1 and control subjects (Ahlgren et al. 2011).

When comparing previous studies conducted in vivo and *in vitro*, several examples illustrate the observed differences. For instance, in vivo studies conducted in mice have provided evidence for the potential importance of IL-17 cytokines and IL-22, in the defence against *Candida* infections (Conti et al. 2009; van de Veerdonk et al. 2009). However, evaluating the immune response to *Candida* in mucocutaneous tissues of mice poses challenges (Netea et al. 2008) due to the broader roles of IL-17 cytokines in controlling various pathogens, particularly in the lungs and gastrointestinal tract (Khader, Gaffen, and Kolls 2009; Dubin and Kolls 2008). In contrast, in vitro experiments using human cells stimulated with *Candida* have shown a selective induction of IL-17A and IL-22 production in Th17 cells (Acosta-Rodriguez et al. 2007; Liu et al. 2009). Moreover, individuals with STAT3 deficiency, characterized by a lack of IL-17-producing Th17 cells, display an increased susceptibility to CMC and staphylococcal infections (de Beaucoudrey et al. 2008; Ma et al. 2008; Milner et al. 2008; Minegishi et al. 2009). According to these findings, variability between in vivo and in vitro induction of Th17 differentiation by APS-1 autoABs can be attributed to multiple factors, such as the complexity of the in vivo environment, which encompasses an intricate network of interactions may not be fully replicated in simplified in vitro systems, leading to differences in the outcomes of Th17 cell differentiation. in vitro experiments offer a controlled and simplified environment, which may not fully represent the intricate complexity of in vivo conditions.

5.8 Conclusions

In summary, we have optimized the cell culture conditions for Th17 cell differentiation from isolated healthy CD4+ T cells, repeated the same procedure for PBMC cultures, and subsequently used it with end-point assays for preliminary effect of cytokine autoABs on T cells from controls and APS-1 patients. Overall, our experiments detected no statistically significant findings which supports that cytokine AB from APS-1 patients impacts on Th17 differentiation in vitro.

5.9 Limitations

Due to the time-consuming nature of the assays used in this study, the number of patients and healthy controls that could be included was constrained, thereby reducing the statistical power of the analyses conducted. PBMCs isolated from healthy individuals was found to vary in sample size. In addition, the cryopreservation step of isolated PBMCs during sample processing led to some cell loss, and the number of isolated CD4+ T cells after magnetic cell

collection was insufficient for both flow cytometry and qPCR analyses. Based on the limited yield of CD4+ T cells isolated from PBMCs of each individual healthy donor, we estimated that a sufficient number of CD4+ T cells could be obtained by isolating PBMCs from approximately 10 donors for each experiment. The calculation aimed to ensure an adequate sample size for the experimental analysis. Variability in the quantity and viability of isolated PBMCs could arise from differences in sample processing techniques, such as the time elapsed between blood collection and PBMC isolation. The limited number of isolated CD4+ T cells, could be attributed to the freezing, thawing, and washing steps involved in the sample processing, which could introduce external stressors that have the potential to affect the viability of the cells. It could also be attributed to the use of magnetic (LS) columns in the cell isolation process. These columns had a restricted capacity to handle a large quantity of cells, which limited their flexibility in accommodating varying sample sizes. In cases where the cell population was high, multiple columns were utilized for this procedure. This itself was time-consuming and increased the likelihood of cell loss. Moreover, the rarity of APS-1 and the limited volume of blood that can be sampled from patients posed challenges in obtaining an adequate number of PBMCs for the study. Therefore, it was not possible to isolate and culture CD4+ T cells from patient PBMCs. Additionally, due to the limited availability of APS-1 cell samples, the focus was primarily on culturing cells for flow cytometry analysis. Another constraint in performing relative gene expression analysis of different T cell lineage markers on PBMCs was the potential lack of strict restriction of these transcription factors to CD4+ T cells. This limitation arises from the heterogeneous nature of PBMCs, which consists of various immune cell populations. As a result, the gene expression signals obtained might not solely reflect the expression patterns within CD4+ T cells.

Regarding the commercial AB which were employed; these may not reflect the APS-1 autoABs at all. They may react towards different epitopes than the patient ABs, and we have no control of the concentration of the ABs.

Notably, this is an *in vitro* study with very artificial conditions for cells to be in. Although it is clear that we managed to generate cells that express the lineage marker RORγt, this might not reflect real Th17 cells that would be seen in *in vitro* circumstances. Nevertheless, due to different biology between mice and men making the use of animal models non-optimal for detailed immune responses, restricted access to patient materials and restricted access to native in vivo cells, in vitro studies on human blood are important to give clues on immunology mechanisms.

5.10 Future perspectives

By addressing the knowledge gap and gaining a deeper understanding of the intricate immune dysregulation in APS-1, one can pave the way for more effective treatments and interventions in the future. To optimize the culture conditions further and make the assay robust for future investigations, several factors can be considered, such as testing different combinations, range of concentrations, or timing of additional cytokines and growth factors to determine the optimal milieu for promoting desired cell responses. Examining the effects of different culture durations, such as shorter or longer incubation times, or evaluating time-dependent responses by analyzing samples at multiple timepoints. Evaluating different culture media formulations and supplements can further optimize the assay. Additionally, creating a reference cell sample by collecting a large number of cells from a single source, such as a buffy coat, is a practical strategy to promote consistency and facilitate future experiments. A substantial amount of blood from a few APS-1 patients and isolating Th17 cells directly from their samples as well as expanding the use of different patient sera, including those with only anti-IFNω ABs or IFNα ABs, can provide valuable insights into the effects of specific cytokine autoABs in APS-1. Moreover, by incorporating additional functional markers for both flow cytometry, and qPCR analysis, a more comprehensive understanding of T cell subtypes in APS-1 can be achieved. The inclusion of specific ABs and markers facilitates a broader assessment of immune dysregulation in APS-1, shedding light on the potential impact of cytokine autoABs on other particular T cell populations such as Th1, Tregs, and Th2 cells. Furthermore, functional assays that can track the activation of signalling pathways such as phospho-flow cytometry, which involves the inclusion of ABs targeting phosphorylated intracellular markers, such as STAT proteins can be involved for unraveling the mechanisms underlying this autoimmune disorder. Future research investigating the effects of APS-1 cytokine autoABs on Th17 cells can hold great promise for expanding our understanding of APS-1 pathogenesis and identifying the intricate interactions and mechanisms involved, these studies may ultimately contribute to the development of personalized and effective treatments for APS-1 patients.

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6. Appendix

The Appendix has been removed for BORA-submission, but can be available through direct contact with the master student or supervisors.