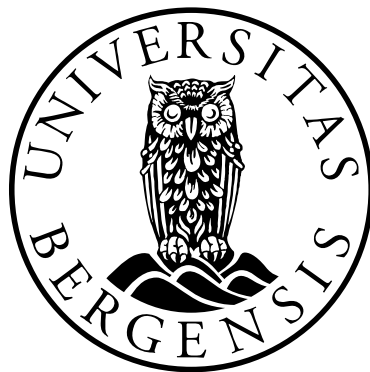# Autoimmunity and viral immunity in Addison's disease

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Dissertation for the degree of philosophiae doctor (PhD)
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Scientific environment

The research included in this thesis was conducted from 2012 to 2015 at the Department of Clinical Science, Section for Endocrinology, Faculty of Medicine and Dentistry, University of Bergen, with Dr. Eirik Bratland and Professor Eystein S. Husebye as supervisors.

Acknowledgements

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Abstract

Autoimmune Addison's disease (AAD) is caused by an immunological destruction of the steroid producing cells of the adrenal cortex. Both genetic and environmental factors are involved in disease development, and while multiple studies have highlighted several genes linked to the disease, far less is known about possible environmental factors and the role played by the adrenal tissue itself.

By studying the well-established human adrenocortical carcinoma cell line NCI-H295R as a model of the adrenal cortex, adrenal cells were shown to respond to environmental factors in the form of virus induced type I and III interferons (IFNs) by potentiating chemokine production and by upregulation of MHC class I and the tissue specific enzyme 21-hydroxylase (21OH). This can be important traits in the T cell-mediated adrenal tissue destruction, recruiting potentially 21OH-specific autoreactive T cells to the tissue. In addition, the same IFNs were shown to have a direct cytotoxic effect on the adrenocortical cells.

From a large clinical material we have found that AAD patients have elevated levels of the chemokines CXCL10 and CXCL9 in their sera, while producing significantly lower levels of the same chemokines after stimulation of PBMC with type I and II IFNs and the TLR3 ligand poly (I:C). This is also the case when investigating the relative mRNA expression of selected IFN stimulated genes (ISGs) after IFN or poly (I:C) stimulation.

Cytomegalovirus (CMV) has been implicated in autoimmune disease development, including AAD. Nevertheless, we found that AAD patients in general have normal humoral and cellular immunity towards CMV, with no differences in $CD8^+$ T cell specific responses. However, the AAD patients were found to have significantly lower levels of total circulating $CD8^+$ T cells. While CMV infections do not appear to be linked to AAD disease development in general, individual patients showed signs supporting CMV as a possible perpetrator. One patient had serological signs suggesting a reactivating CMV infection, while having extremely low levels of CMV

specific CD8$^+$ T cells. The same patient was also part of the chemokine study, where she had low chemokine production after IFN stimuli in addition to an upregulation of three ISGs in her peripheral blood. Intriguingly, the daughter of this patient also had AAD and, despite of being anti-CMV IgG positive, had virtually no CMV specific CD8$^+$ T cells. These findings suggest that an inheritable immunological phenotype may increase individuals' susceptibility to develop AAD, but also impair their ability to control viral infections.

Taken together, the work included in this thesis provides important insight into AAD development. We have shown using adrenocortical cells that the adrenal cortex could play a part in its own destruction in response to interferons induced by viruses. Furthermore, while having a normal cellular and humoral immunity towards the common virus CMV, the innate immune system of AAD patients does not appear to function optimally. Thus for individual patients, CMV infection could be a precipitating event in disease development.

Table of contents

Abbreviations

AAD	Autoimmune Addison's disease
ACTH	Adrenocorticotropic hormone
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
APC	Antigen presenting cell
APS	Autoimmune Polyendocrine Syndrome
BcR	B cell Receptor
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CRH	Corticotrophin-releasing hormone
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte Associated protein 4
CXCL	CXC chemokine Ligand
CXCR3	C-X-C chemokine Receptor type 3
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GC	Glucocorticoid
GD	Graves' disease

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPA	Hypothalamic-pituitary-adrenal
HT	Hashimoto's thyroiditis
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK	Janus kinase
LDH	Lactate dehydrogenase
MHC	Major Histocompatibility complex
MIC-A	MHC class I related chain A
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymic epithelial cell
NK	Natural killer
NO	Nitric oxide
nTregs	Natural regulatory T cells
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
Poly (I:C)	Polyinosinic-polycytidylic acid
PTPN22	Protein-tyrosine phosphatase non-receptor type 22
PRR	Pattern recognition receptor
pTregs	Peripherally-derived regulatory T cells
qRT-PCR	Quantitative real-time polymerase chain reaction

RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROAS	Registry of Organ specific Autoimmune Diseases
SEAP	Secreted embryonic alkaline phosphatase
SFU	Spot forming units
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Sjögren's syndrome
STAT	Signal transducer and activation of transcription
TcR	T cell Receptor
Tfh	Follicular helper T cells
TGFβ-1	Transforming growth factor beta 1
Th	T helper
TNF	Tumor necrosis factor
Tregs	Regulatory T cell
tTregs	Thymus-derived regulatory T cell
T1D	Type 1 diabetes
USP18	Ubiquitin specific peptidase 18
21OH	21-hydroxylase

List of Publications

The thesis is based on the following papers, and will be referred to in the text by Roman numerals:

Paper I

A. Hellesen, <u>K. Edvardsen</u>, L. Breivik, E. S. Husebye and E. Bratland (2014)

The effect of types I and III interferons on adrenocortical cells and its possible implications for autoimmune Addison's disease

Clinical and Experimental Immunology 2014 June 176(3): 351-362

Paper II

<u>K. Edvardsen,</u> T. Bjånesøy, A. Hellesen, L. Breivik, M. Bakke, E. S. Husebye and E. Bratland (2015)

Peripheral blood cells from patients with autoimmune Addison's disease poorly respond to interferons *in vitro*, despite elevated serum levels of interferon-inducible chemokines

Journal of Interferon & Cytokine Research 2015 May 15. [Epub ahead of print]

Paper III

<u>K. Edvardsen,</u> A. Hellesen, E. S. Husebye and E. Bratland (2015)

Analysis of cellular and humoral immune responses against cytomegalovirus in patients with autoimmune Addison's disease.

Manuscript

1. Introduction

Autoimmune Addison's disease, or primary adrenocortical insufficiency, is a prototypic autoimmune endocrine disorder characterized by the destruction of the cells in the adrenal cortex. Understanding the term autoimmunity comes from knowledge of the term immunity. Immunity is the ability to resist and fight attacks from possible intruders. The host, or in this case, the human body, is equipped with an immune system consisting of several specific immune cells and molecular components that help the body escape and eradicate infections. These infections could be a result of microbes entering through the skin, airways or gastrointestinal tract.

We are dependent on our immune system for survival, but the mechanisms of the immune system could potentially also cause significant morbidity or even death. One way this might happen is when the cells of the immune system lose their ability to discriminate foreign (non-self) antigens from self-antigens. This response to self is a phenomenon known as autoimmunity, which in turn could lead to cells of the immune system attacking and destroying normal tissues within the body, an autoimmune disease.

First this thesis gives a brief introduction of the immune system and its components, followed by antiviral immunity and autoimmune diseases. The main focus will then be on Addison's disease and how viral infections and antiviral immunity could be important mediators in the development of autoimmune Addison's disease.

1.1 Innate immunity

The immune system is divided into two different compartments with highly specialized immune cells. The innate immune response is the host's first line defense and is essential for an early response against intruding pathogens. This early immune response is orchestrated by immune cells such as macrophages, neutrophils, mast

cells, dendritic cells (DCs) and natural killer (NK) cells [1]. These cells ingest (phagocytose) and destroy microbes, activate the complement system and produce cytokines and chemokines, signal molecules that activate and recruit additional cells to the affected area [1]. The complement system is a set of plasma proteins that ultimately may lead to lysis of bacteria and infected cells [2].

The components of the innate immune system are ancient, and have evolved to recognize highly conserved pathogen associated molecular patterns (PAMP) on the intruders by the use of pattern recognition receptors (PRR) [3]. The best described PRR are the Toll like receptors (TLRs) recognizing bacterial cell wall components and nucleic acids of virus and bacteria. They were first discovered in *Drosophila melanogaster*, and a few years later they were also identified in humans [4, 5]. TLRs are located in different compartments of the cell, such as within the cytosol, on the cell surface, or inside endosomes which are exposed to different kinds of microbes [5]. The innate immune system is also responsible for the activation and initial stimulation of the adaptive immune response, as described below.

1.2 Adaptive immunity

Adaptive immunity is the ability to recognize and respond specifically to a first time infection, and to learn and adapt with a quicker response the next time around. Adaptive immunity is dependent on antigen recognition, leading to lymphocyte activation and migration to infected tissue. The antigen recognition is highly specific, and only a few initial clones of lymphocytes will share receptors specific for the same antigen. The lymphocytes of the adaptive immune system are the T (Thymus derived) lymphocytes which carry the T cell receptor (TcR) and the B (Bone-marrow derived) lymphocytes carrying the B cell receptor (BcR). These two types of lymphocytes have very different actions of antigen binding and cellular activation, but similar for both is the vast array of possible receptors displayed on the cells surfaces. The antigen-binding site is made up by variable regions, encoded by three classes of different gene segments which is brought together by a unique recombinatory process

that theoretically could give rise to about 10^{15} different antigen specificities for B- and T cells [6].

1.2.1 Antigen presentation, TcR binding and activation

The secondary lymphoid organs, such as the spleen and lymph nodes play a very important role in the immune system. Here the T cells encounter professional antigen presenting cells (APC), for instance DCs and macrophages, bringing antigens to these lymphoid organs from different sites of the body. These antigens are portrayed as peptides bound to major histocompatibility complex (MHC) molecules, also known as human leucocyte antigen (HLA) in humans. The antigen is processed from intracellular or extracellular microbes and presented by MHC class I or MHC class II respectively. MHC Class II is expressed primarily by the professional APCs macrophages, DCs, B Cells, monocytes and endothelial cells while MHC class I is expressed, at varying degrees, on most cells of the body [7]. Antigens encountered extracellularly are ingested through the acidic environment in vesicles in the cell, these vesicles then fuse with others carrying the MHC class II molecules, which bind antigen peptides varying from 13-17 amino acids in length and present them on the cell surface to T cells [7, 8]. When a cell is infected by a microbe the antigen may be found in the cytosol, where it is degraded and transferred to the endoplasmic reticulum (ER). In the ER, peptides consisting of 8-10 amino acids encounters the MHC class I and the receptor-peptide complex is transferred to the cell surface to be presented for T cells [9].

In addition to being responsible for transferring antigens to sites of recognition and presenting these antigens in a manner enabling identification by specific lymphocytes, APC also needs to provide additional "signals" to activate T cells. This is part of a three-step process of T cell activation that also underlines the importance of the innate immune system in the activation of adaptive immunity. The first step is the actual binding of the TcR to the MHC complex bearing the peptide. In addition, there is also co-stimulatory binding of a second receptor on the T cell, the Cluster of Differentiation (CD) 28 receptor bound to the B7 ligand (CD80/CD86) on the APC

[10]. Binding of microbial PAMPs to PRR enhances expression of B7 on the APC and leads to secretion of inflammatory signal molecules such as cytokines and chemokines [11]. These secreted molecules contribute the third signal in the activation process of naïve T cells.
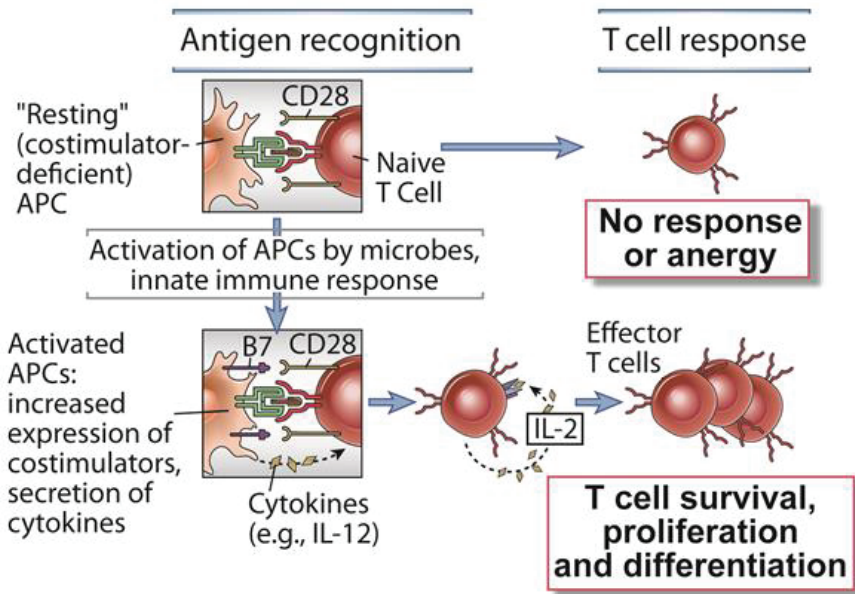
Abbas, Lichtman and Pillai. *Cellular and Molecular Immunology*, 7th edition, 2011 © Elsevier

Figure 1: Co-stimulation in T cell activation. T cells are dependent on expression and secretion of co-stimulators for activation. If the APC does not express the B7 ligand or secrete stimulatory cytokines the T cell will not respond to MHC binding, but rather enter a state of unresponsiveness (a mechanism termed anergy). When APCs are activated as part of an innate immune response to microbes, co-stimulators will be expressed and the T cells activated. This ensures that T cells will not respond to antigens that inflict no danger to the body.

The same co-stimulatory process explained in Figure 1, is also used by the immune system in an inhibitory fashion. While CD28-B7 binding is important in initial T cell activation, activated T cells also start to express a down-regulatory receptor, cytotoxic T-lymphocyte-associated protein 4 (CTLA4), that binds to B7 on APCs and inhibits the T cell response by diminishing T cell proliferation and production of cytokines [12].

1.2.2 T cell subsets and phenotypes

The T cells are divided into two main subsets based on whether they carry the co-receptor CD4 or CD8, and are then further classified into subtypes. Co-receptor CD4 is responsible for MHC class II binding, while CD8 recognize MHC class I. Once an antigen is encountered by the antigen-specific naïve T cell (as described above), a process of activation and proliferation of that T lymphocyte is initiated. The $CD4^+$ T cells differentiate into different effector (helper) and regulatory T (Tregs) cell subsets.

The cytokines produced by the APC during antigen recognition drives the activation of transcription factors and the differentiation of the T cell into the main effector subtypes (Th1, Th2 and Th17) [13]; the differentiation into Th1 cells are driven by a combination of IFN-γ and IL-12, and they are important effector cells in the immune response against intracellular pathogens. Another subtype is the Th2 cells, their differentiation is mediated by IL-4 and IL-2, and these cells are important in the defense against extracellular parasites. Th2 cells are often associated with allergy and asthma [14]. The differentiation into a third subtype of helper cells, the Th17, is driven by TGFβ-1, IL-6, IL-21 and IL-23. These cells mediate the immune response against extracellular bacteria and fungi [15]. The $CD4^+$ T helper (Th) cells are responsible for activation of macrophages, $CD8^+$ T cells and B cells and produce a range of cytokines that differentiate and fine-tunes the immune response [11], as shown in Figure 2. Follicular helper T cells (Tfh) enter the follicles of lymphoid organs and are the most powerful activator of B cells which stimulates the production of antibodies [16].
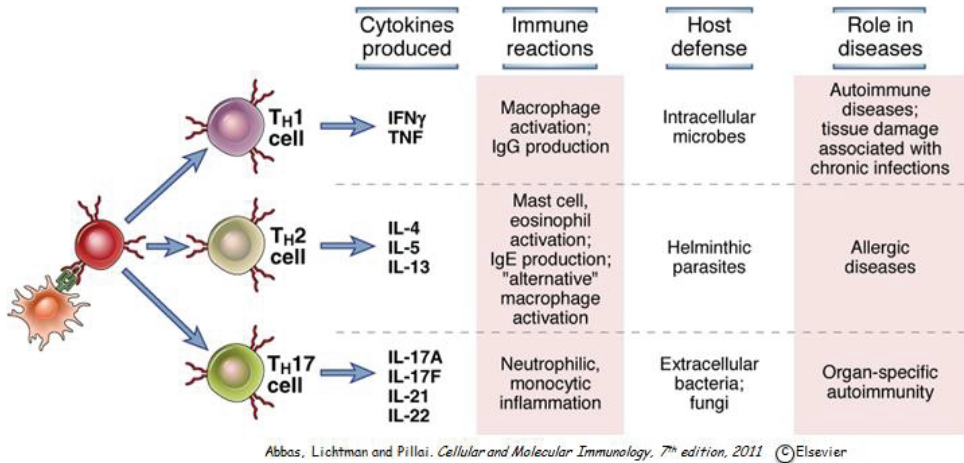
	Cytokines produced	Immune reactions	Host defense	Role in diseases
T_H1 cell	IFNγ TNF	Macrophage activation; IgG production	Intracellular microbes	Autoimmune diseases; tissue damage associated with chronic infections
T_H2 cell	IL-4 IL-5 IL-13	Mast cell, eosinophil activation; IgE production; "alternative" macrophage activation	Helminthic parasites	Allergic diseases
T_H17 cell	IL-17A IL-17F IL-21 IL-22	Neutrophilic, monocytic inflammation	Extracellular bacteria; fungi	Organ-specific autoimmunity

Abbas, Lichtman and Pillai. *Cellular and Molecular Immunology*, 7th edition, 2011 ©Elsevier

Figure 2: Development of different CD4+ helper T cells. The naïve CD4+ T cell give rise to several T helper cell subtypes specific for different infectious pathways. These subsets produce a variation of cytokines essential for activation of the different immune cells. The different subsets could also present as possible perpetrators in various diseases.

While the different Th cell subsets are responsible for actions that are pro-inflammatory, the Tregs are important contributors in dampening of inflammation and for inhibiting the effector cascade. These cells are either thymus-derived Tregs (tTregs) (also known as natural (nTregs)) that are presented to different self-antigens in the thymus, or peripherally derived Tregs (pTregs) generated in the periphery when naïve T cells are introduced to persistent self-antigens and IL-2 during T cell maturation [17-19].

Activation of naïve CD8+ T cells leads to differentiation into cytotoxic T lymphocytes (CTLs), which are responsible for killing infected cells, typically virus infected cells that present the antigen bound to MHC class I molecules [10]. These cells will be described further below in relation to antiviral immunity.

1.2.3 B cells and antibodies

While T cells only recognize the peptides presented in the context of MHC on other cells, B cells recognize native antigens. The B cells are responsible for the humoral adaptive immune response of the host. This response is initiated when antigen

interacts with the BcR on the B cell membrane and, given that the B cell receives the appropriate help from Th cells, antibodies of the same antigen specificity are secreted as a soluble form of the BcR from the cell. The B cell antibodies are immunoglobulins of five different isotypes, IgA, IgD, IgE, IgG and IgM [6]. The antibodies have several effector functions, including neutralization of toxins, marking microbes for destruction, activation of complement and inflammation among others [20]. The activated B cell undergoes clonal expansion and differentiates into short and long-lived plasma cells and memory B cells [6].

1.2.4 Immunological tolerance

Immunological tolerance is the ability of a lymphocyte not to respond to certain antigens. This is the immune systems way of ensuring that the lymphoid cells do not react towards self-tissue or cells by a complex process that eliminate lymphocytes responding to self [11]. Immunological tolerance is divided into central and peripheral, where the central tolerance takes place in the generative (primary) lymphoid organs, and peripheral tolerance in peripheral tissues and compartments.

The central tolerance of T cells consists of a positive and a negative selection which takes place in the thymus, where the immature T cells migrate from the bone marrow. The positive selection takes place in the outer thymic cortex, where double positive $CD4^+CD8^+$ cells are generated and interacts with MHC class I/II presented by cortical thymic epithelial cells through their TcR. The cells with no affinity to the MHC will undergo apoptosis or death by neglect, while cells able to interact will continue the migration to the medulla as either $CD4^+$ or $CD8^+$ T cells [21]. In the medulla the T cells and their receptors are presented to MHC class I/II presenting self-antigens on intrathymic APCs, such as DCs, medullary thymic epithelial cells (mTECs) and B cells [22]. If the TcR have low affinity to the presented MHC/peptide complex, it is assumed that they will not respond to self and therefore the naïve T cell are allowed to migrate further to peripheral tissues. However, T cells carrying receptors with high affinity for the self-peptides will be marked for destruction (apoptosis) in the negative selection process [23]. Another fate for the immature T cells binding to self-antigens

is development into Tregs [24]. Although central tolerance exists to avoid self-reactive cells to enter the periphery, this is not always the case. All self-reactive T cells that bind with low and intermediate affinity to the self-peptides cannot be eliminated, in part since not all self-antigens are expressed in the thymus [25]. Peripheral tolerance is another layer of regulation to restrain self-reactive T cells in the periphery with three different outcomes. (I) The T cell could become anergic, functionally inactivated, when recognizing the peptide without proper co-stimulation, (II) the APCs or DCs in particular could send signals of deletion (apoptosis) or (III) there could be a suppression of the T cells in cooperation with Tregs [25, 26].

Central tolerance development for B cells takes place in the bone marrow. A BcR that binds self-antigen with high affinity will undergo receptor editing, exchanging the autoreactive part of the immunoglobulin receptor through the process of gene rearrangement [27]. If the receptor editing is unsuccessful, the B cell will die by apoptosis [28]. In the periphery, the fate of the B cells recognizing self-antigens without proper T cell co-stimulation is anergy [29]. In the spleen and lymph nodes helper T cells can contribute to deletion of autoreactive B cells by apoptosis in a Fas (CD95) dependent matter [30, 31].

1.3 Antiviral immunity

When a virus invades the host it has a relatively short lifespan outside the cells. However, if the virus manages to infect the cells of the host, it replicates within the cell and the infection can then spread throughout the body. Detection of the infection first triggers an innate immune response, followed by a cascade of actions that activate adaptive immunity, CTL in particular, as a result of antigen presentation and cytokine secretion [32]. Antibodies are also important in detecting and neutralizing viruses outside the cells [33]. Interferons (IFNs) are a class of cytokines important both in the innate and adaptive immune responses having antiviral, antiproliferative and immunomodulatory effects. They consist of three families: The type I interferons,

the type II interferon or IFN-γ, and the type III interferons [34]. NK cells and T cells are the predominant producers of IFN-γ, acting on several cell types, macrophages and monocytes in particular, carrying the IFN-γ receptor [35]. Virus infected cells secrete type I interferons and other cytokines such as IL-12 that bind directly to their receptors on NK cells and T cells and function as costimulatory molecules activating signaling pathways leading to production of IFN-γ [36-38]. The most recent discovered IFN family are the type III interferons, including IFNλ1, IFNλ2, IFNλ3 and IFNλ4 [39-41]. The type III interferons have similar properties as the type I interferons and they are produced by most cell types, DCs in particular, but they have a more restricted potential since their receptor is mainly expressed on epithelial cells [42].

1.3.1 Type I Interferons

Type I IFNs were the first cytokines discovered and today several different variants are known [43]. IFN-α (13 subtypes), IFN-β, IFN-ε, IFN-κ and IFN-ω are all different variants of type I IFNs identified in humans [44]. IFN-α and –β are the most common and best studied subtypes, and are essential to the immune system in clearing viral infections. When pathogens enter the body and stimulate PRR, most cells are capable of producing IFN-α and –β as a response, but the major producers are the plasmacytoid dendritic cells (pDC) [45]. In viral infections the TLRs of the endosomes, such as TLR3, 7, 8 and 9 expressed in different subsets of IFN-producing cells, play an important role in binding to double-stranded RNA, single-stranded RNA and microbial DNA [46]. Type I IFNs also play an important part in the activation of the adaptive immune response through innate immunity, by inducing the expression of the co-stimulatory molecules and MHC class I on the APCs needed to activate $CD8^+$ T cells [47].

During a viral infection the type I interferons interfere with the viral replication by induction of interferon stimulated genes (ISGs). This induction is the result of a signaling cascade starting with IFN-α/β binding to their common receptor, a complex of IFNAR1 and IFNAR2 subunits expressed on a wide variety of cell types, leading

to the stimulation of the JAK-STAT pathway following induction and expression of hundreds of ISGs [34, 48, 49]. These genes are involved in several steps of the process of inhibiting and clearing the infection, from enhancing production of pro-inflammatory cytokines to dampening the response by anti-inflammatory cytokines and mediators. The chemokine CXC chemokine ligand 10 (CXCL10) is a classic example of an ISG, which is upregulated both in response to IFN-α/β and IFN-γ [50, 51]. The CXCL10 binds to its chemokine ligand CXCR3 on lymphocytes in order to recruit them to the sites of infection [52].
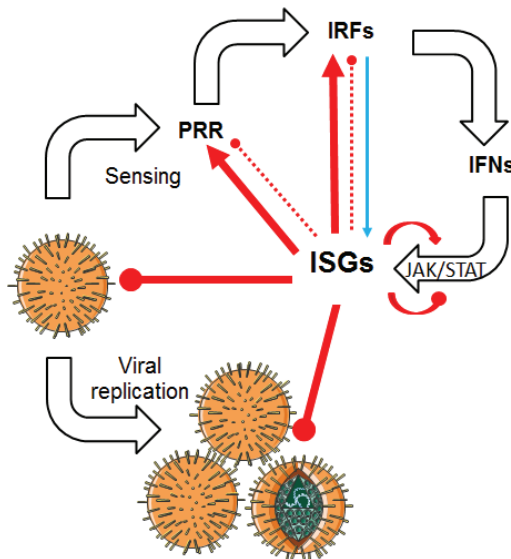
Figure 3: Diverse roles for interferon stimulated genes (ISGs) in the IFN antiviral pathway. During a viral infection the pathogen associated molecular patters on the virus is sensed by the pattern recognition receptors (PRR). This in turn leads to activation of interferon regulatory factors (IRFs) and transcriptional induction of IFNs. The IFNs signal through the Janus kinase and Signal transducer and activator of transcription (JAK/STAT) pathway and induces production of ISGs. This main process is illustrated with the large white arrows. The IRFs can also induce some ISGs directly without induction of IFNs (thin blue arrow). Some ISGs block viral replication (blocked red line). The red arrows are the ISG that is part of the IFN signaling pathway or leads to production of more IFNs. The negative regulators of the cascade are illustrated by the dotted lines, targeting the cascade at various time points. Adapted from Schoggins and Rice, 2011, produced using Servier Medical Art (http://www.servier.com/Powerpoint-image-bank)

1.3.2 Cytotoxic T cells

Once naïve CD8^{+} T cells are activated in the lymph node or spleen, they can differentiate into effector CD8^{+} T cells, or cytotoxic T lymphocytes (CTLs). Type I and II IFNs are important mediators in the CD8^{+} T cell response by functioning as co-stimulators that augments CD8^{+} T cell activation and proliferation, by inducing ISGs affecting the T cells and also by increasing expression of MHC class I on APCs (DCs especially) [48, 53]. As mentioned earlier, the MHC class I molecules are expressed on almost all nucleated cells of the body, making the CTL able to respond against intracellular pathogens in most tissues and thereby assist in viral clearance. The activated T cells upregulate the expression of the inflammatory chemokine receptors such as CXCR3 which allow them to bind to IFN induced chemokines released by APCs in response to viral infections (ISGs) and guide them from the site of recognition to the site of infection in the peripheral tissues [54].

CTL are very efficient in clearing of infections. By inducing apoptosis of the infected cells they destroy the components the virus needs to replicate and survive [32]. The main effector mechanism of CTLs is calcium dependent release of cytotoxic granules containing proteins specific for CTLs, such as perforin and granzymes [55, 56]. The perforin assists in the entering of granzymes through the membrane of the infected cell, where the granzymes induce apoptosis through various actions [56, 57]. CTL may also kill target cells by upregulation of the Fas ligand (CD95L) and interacting with Fas (CD95) on the target cell. The target can also be other lymphocytes in the process of terminating the lymphocyte proliferation after viral infection is cleared [58]. The activated CTL produces high amounts of IFN-γ, sustaining the inflamed environment by further stimulation of cells located in the inflamed tissue [35].

1.4 Autoimmunity and autoimmune diseases

During their normal development, the cells of the adaptive immune system, or rather their receptors are introduced to antigens that are part of the body to evade

immunological actions to self. Autoimmunity as by definition is failure of immunological tolerance. In this situation adaptive immune cells mistake self-antigens for foreign and destroys the cells that express them or produce antibodies against self (autoantibodies). Some degree of autoimmunity is probably beneficial for the organism, for instance in the context of the central nervous system where self-specific T cells has been shown to aid in tissue repair [59]. But when a large scale immunological response leading to destruction of vital organs and tissues is activated, it turns into an autoimmune disease. Autoimmune diseases have a huge impact on society in the western world, affecting 5-10% of the population, ranking as the third most prevalent cause of disease and the tenth leading cause of death in American females [60-62]. Autoimmune diseases are either systemic, targeting several organs or tissues, or organ specific, where especially the endocrine organs seems to be at risk [63].

Today's knowledge of what actually cause autoimmune disease is limited, but three different components are believed to contribute: A genetic predisposition, environmental factors and failure of immune regulation. There are rare examples where mutations in a single gene cause autoimmune disease, for instance in the autoimmune regulator (*AIRE*) gene which leads to autoimmune polyendocrine syndrome type I (APS-1) [64], but in most autoimmune diseases several different genes and gene combinations are believed to contribute. The genes encoding antigen presenting molecules, HLA (MHC) class I and class II, have a strong link to many autoimmune diseases. These genes are highly polymorphic, giving rise to a great variation in the shape, size and charge of the peptide binding pocket of the protein they encode. One amino acid change could regulate the degree of interaction between the HLA peptide binding grove and the antigen or autoantigen [65]. Some HLA variants are shown to convey protection for autoimmune diseases, while others make an individual more susceptible. The HLA molecules are responsible for antigen presentation at two critical time points; in the thymus during establishment of central tolerance and in the periphery during initiation of immune responses [66, 67]. There are also variants in several other genes with links to autoimmune diseases. These are typically genes encoding different parts of the immune regulation such as co-

stimulatory molecules, cytokines, contributors of the different signaling cascades, regulation of apoptosis and phagocytosis [68].

Disregarding the monogenetic autoimmune diseases, genes alone are not enough to disturb the normal immune regulation. Different environmental factors have been suggested as possible disease triggers. In the case of an infection, the pathogen activates both the adaptive response through presentation of antigen and also stimulates the innate secretion of cytokines and expression of co-receptors. If this happens in the presence of autoreactive cells it might be the initiating factor of the autoimmune process, known as bystander activation [69, 70]. This issue will be discussed further in the following sections. Tissue damage could also provide adjuvant for the autoreactive T cells as damage-associated molecular patterns (DAMPs), as well as increasing the presence of self-antigens. [71]. The process of molecular mimicry is another suggestion of how infections can induce an autoimmune reaction. This occurs when foreign antigens share structural similarities, charges and sequences as antigens from self, and the activated T cells or antibodies cross-react with self [72, 73]. Other environmental factors, such as smoking, exposure to toxins, estrogens and silicone implants have also been discussed in relation to autoimmune diseases [74-76].

1.4.1 Viral infections, type I IFN production and autoimmunity

When addressing the role of viral infections in autoimmune diseases, we must consider the antiviral immunity and interferon production explained previously. If a virus infects a host cell, ISGs are induced leading to production of interferons and ultimately viral clearance. However, in recent years it has been shown that IFN-α/β also can be harmful during a viral infection by activating an inflammatory reaction leading to tissue damage that worsens the disease picture [77]. It's been shown that IFN-α can induce increased expressions of autoantigens, such as Ro52, seen in Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) [78] and that autoantibodies in combination with material released by apoptotic cells can increase production of type I IFNs by pDC [79]. Increased IFN serum levels have been

reported in patients with SLE [80] suggesting IFN involvement in disease pathology, and recently type I IFN activity was also shown to be increased in the organ specific disorders Hashimoto's thyroiditis (HT), Graves' disease (GD) and type 1 diabetes (T1D) [81, 82]. In addition, there has also been several reports were treatment of chronic viral infections using interferon therapy has induced autoimmune disease development or worsening of existing disease [83, 84]

1.5 Autoimmune Addison's disease

Autoimmune Addison's disease (AAD) is a classic example of an organ specific autoimmune disease. The organ affected is the adrenal glands, and the disease and clinical symptoms was first described in 1855 by Thomas Addison after investigating the adrenals of 11 deceased patients [85]. At that time tuberculous adrenalitis was a major cause of Addison's disease, as it still is in the developing countries today [86, 87]. However, presently in the western world, autoimmune adrenalitis accounts for 80-90% of the cases [88-90]. The prevalence of Addison's disease in Norway is estimated to be 144 per million [88], the same number varying from 93-117 in other developed countries [91-93] making it one of the more rare autoimmune disorders. The incidence in the Norwegian study was found to be 4.4 per million [88]. Like most autoimmune diseases it has a female preponderance (from 55 to 77%) and the clinical symptoms normally presents between 30 and 50 years of age, although it can occur at all ages [91, 92, 94, 95].

1.5.1 The adrenal glands

The adrenal glands are one of several endocrine organs of the body. They are located on top of the left and right kidney, and consist of the adrenal cortex and the adrenal medulla, separated both in function and structural appearance. The medulla is the core of the gland (Figure 4) and its chromaffin cells produces catecholamine hormones that are released as a result of stimulation of the sympathetic nervous system, a stress response. The specific hormones released are adrenaline

(epinephrine) and noradrenaline (norepinephrine), responding to stress by increasing the heart rate and blood pressure [96]. The medulla is surrounded by the adrenal cortex, which is the affected tissue in AAD.

The adrenal cortex is divided into three zones based on their histological picture (Figure 4). The three zones produce different groups of steroid hormones, all derived from cholesterol. The layer closest to the medulla is entitled zona reticularis, the middle layer is zona fasiculata and the outermost layer is zona glomerulosa [97]. Zona golmerulosa secretes the mineralocorticoid aldosterone, which affects the kidney through the mineralocorticoid receptor and plays an important part in regulating the salt and water balance, and thereby the blood pressure. The cells of zona fasciculata secrete the glucocorticoids, the most important being cortisol, as a direct response to adrenocorticotropic hormone (ACTH) secreted by the pituitary gland. The release of ACTH is again regulated by corticotropin-releasing hormone (CRH) secreted from the hypothalamus. The cortisol then signals the hypothalamus to shut down production in a negative feedback manner and the whole process together is entitled the hypothalamic-pituitary-adrenal (HPA)-axis [98]. Cortisol is a vital hormone with diverse effects on almost all cells and tissues of the body regulating and assisting in metabolism, cardiovascular function, blood pressure and immune responses. Zona reticularis produce adrenal androgens, such as androstenedione, dehydroepiandrosterone (DHEA) and 17-hydroxyprogesterone. Little is known about their physiological role, but they are believed to function as prohormones for production of active androgens and/or estrogens in other tissues [99].
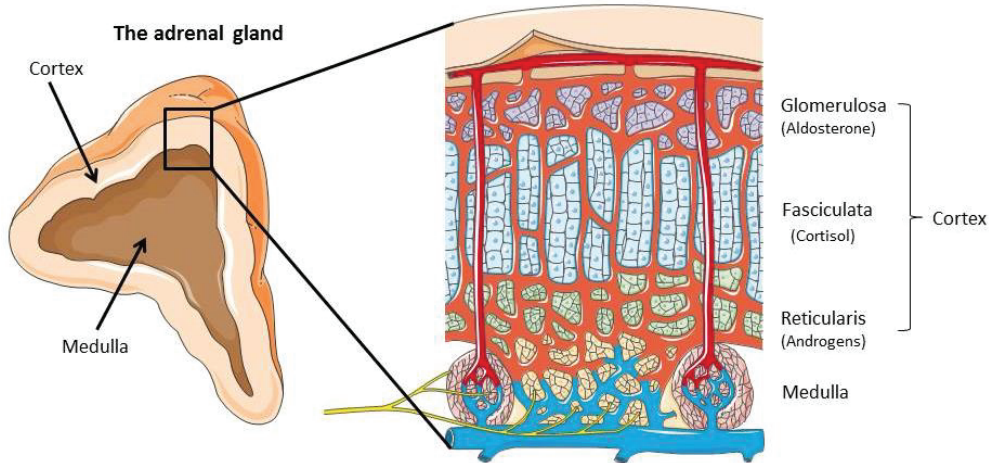
Figure 4: Schematic overview of the adrenal gland. The adrenal gland is divided into the adrenal medulla and the adrenal cortex. The cells of the medulla produce and secrete catecholamines, while the cells of the adrenal cortex produce vital steroid hormones. The adrenal cortex is separated in three different layers; furthest out is the zona glomerulosa, followed by the zona fasciculata and the zona reticularis which is located closest to the medulla. Figure produced using Servier Medical Art (http://www.servier.com/Powerpoint-image-bank).

1.5.2 Clinical and histological picture in AAD

After initiation of the disease the three layers of the adrenal cortex is gradually destroyed by immune responses, leaving only a fibrous tissue. A normal healthy adrenal is around 4-6 cm long, 2-3 cm wide and 3-6 mm thick [100], while they shrink in size in diseased individuals. Histopathological surveys of deceased AAD patients reveal a widespread infiltrate of mononuclear cells including lymphocytes, plasma cells and macrophages [101, 102]. AAD has a subclinical disease development, the properties of the adrenals allows them to produce adequate amounts of hormones up until 10% of functional adrenocortical cells remain [103], and then the clinical symptoms will start to show. These patients have a variety of clinical symptoms, such as fatigue, weight loss, nausea and vomiting, which are vague and hard to diagnose. Some clinical symptoms are much more specific and characteristic of Addison's, for instance hyperpigmentation of the skin and mucosal surfaces caused by the elevated levels of ACTH and salt cravings [90]. It wasn't until the late 40's

that a sustainable treatment option for Addison's, synthetic glucocorticoids, was introduced in medical care, before that time the diagnosis was fatal [104].

1.5.3 AAD in autoimmune polyendocrine syndromes

AAD may occur isolated, or it could present alongside other known autoimmune diseases in an autoimmune polyendocrine syndrome (APS) seen in about 50-60% of the cases [88, 90, 105]. APS-1 has already been mentioned as an example of rare a monogenic autoimmune disease, and is defined by the presence of at least two of the following; AAD, primary hypoparathyroidism or mucocutaneous candiasis [106, 107]. AAD is the least common of the three, even though it occurs in up to 80% of adult APS-1 patients [108]. The disease onset is usually during childhood, much earlier than for most patients with isolated AAD [109]. APS-2 is a more common autoimmune polyendocrine syndrome, defined as having AAD in combination with autoimmune thyroid disease and/or T1D [110, 111]. However, the presence of other autoimmune diseases such as gonadal failure, vitiligo, pernicious anemia and alopecia also occurs [112]. A third syndrome with AAD is also classified, APS-4, including AAD and other autoimmune diseases, while excluding thyroid disease and T1D [112].

1.5.4 Steroid cytochrome P450 21-hydroxylase (21OH) and AAD

Autoantibodies against steroid cytochrome P450 21-hydroxylase (21OH) are present in more than 90% of recently diagnosed patients with AAD, declining somewhat in patients with longstanding disease [113, 114]. The presence of circulating antibodies against the adrenal cortex has been known since the late 50's, but the identity of 21OH was not known until 1992 [115]. 21OH is an intracellular enzyme specifically expressed in the adrenal cortex. It is the dominant autoantigen in AAD, and today autoantibodies against it are used for diagnostic purposes [114]. Autoantibodies against 21OH may be detected in individuals with other autoimmune diseases like hypoparathyroidism, in members of families with an accumulation of autoimmune diseases, and also in less than 0.5% of the healthy population [116, 117]. Therefore, having 21OH autoantibodies does not mean that you will develop AAD for certain,

however the levels of autoantibodies correlates with the risk of disease development [118] and it is a valuable disease predictive marker if AAD is suspected.

It is not known whether the antibodies against 21OH play a role in the development of AAD, or if they are just products of the immune mediated adrenal destruction. One study measured the IFN-γ production in cells from AAD patients stimulated with 21OH, and found that it was enhanced in the presence of 21OH-specific autoantibodies, indicating that the antibodies could work as adjuvants augmenting the immune reaction against the adrenals [119]. Also, it was recently shown that a woman with newly diagnosed Addison's disease and high 21OH antibody titers experienced sustained improvement and were able to discontinue steroid treatment after Rituximab treatment for B cell depletion [120].

1.5.5 Genetic predisposition

As with autoimmune diseases in general, there are genes that are associated with AAD. The HLA associations have been intensively studied, and certain class II alleles, such as HLA-DR3 and DR4, have been linked to AAD [121]. The more specific haplotypes are HLA-DRB1*0301-DQA1*0501-DQB1*0201 (DR3/DQ2) and DRB1*04-04-DQA1*0301-DQB1*0302 (DR4.4/DQ8) [88, 113, 122]. An association between AAD and MHC class I have also been reported, more specifically to HLA-B*08 [123] and HLA-B*08 in combination with DR3 [124]. The MHC region is located on chromosome 6 as well as two other genes that are mentioned in connection with AAD, MHC class I related chain A *(MIC-A)* and the gene encoding 21OH (*CYP21A2*). Variants in both convey increased susceptibility when they are present together with DR3/DQ2 and DR4.4/DQ8, but MIC-A has also been described as an independent risk factor in some studies [123, 125, 126].

Several non-MHC susceptibility genes have also been reported for AAD, of which protein-tyrosine phosphatase non-receptor type 22 (*PTPN22*) is a well-studied example [127, 128]. This gene and its specific single nucleotide polymorphism, 1858C>T (rs2476601) substituting arginine with tryptophan in position 620, has been described for several autoimmune diseases, including rheumatoid arthritis (RA), T1D,

SLE and GD [129-132]. This is an enzyme expressed in hematopoietic cells, specifically T cells, B cells, DCs and macrophages which regulate immune signaling through antigen receptors (the TcR) and pattern recognition receptors [133, 134]. It's been debated whether the disease variant is a gain of function or not, and it is likely involved in several stages of autoimmune disease development. The disease variant has been shown to alter the responsiveness of mature T cells and to cause failure in upregulation of type I IFN production after TLR signaling [134, 135].

1.5.6 Viral infections as a trigger of AAD

As mentioned previously, viral infections and the interferons induced by infections have been implicated for several autoimmune diseases. There are also reports regarding viral infections, interferons and AAD, suggesting that this could be important factors in the disease development. Adrenalitis as a result of a viral infection by herpes simplex [136], hepatitis B [137], Epstein-Barr [138], cytomegalovirus [139] and human herpesvirus-6 [140] have been reported. These are all examples of persistent viruses giving constant presence of viral antigens. This could drive a long term immune response resulting in autoimmunity [70]. Alternatively, they could be initial triggers of autoimmunity, involved in the early phase. Viral association to AAD have also been shown indirectly in cases where recombinant IFN-α is used as treatment for chronic viral infections and have led to induction of autoantibodies against 21OH [141, 142], and have resulted in short periods of transient adrenal insufficiency [141]. IFN-α therapy have also led to a worsening of symptoms in a patient already diagnosed with AAD [143]. A case report links hepatitis C viral infection to subclinical adrenal insufficiency where the condition actually worsened after recombinant IFN-α therapy [144].

1.5.7 Cytomegalovirus and Addison's disease

Since several different viruses have been reported in relation to AAD, it doesn't seem likely that there is just one single perpetrator. However, cytomegalovirus (CMV) is a good candidate due to its high infection rate, the great variation in CMV antigens that could be presented, and the broad CD8^{+} T cell response it induces [145, 146]. Human

CMV is a herpes DNA virus which after primary infection will establish a lifelong latent infection, with the possibility for periodical reactivation and shedding of infectious virus [147]. Antibodies against CMV as a result of a previous infection are present in around 50-60% of the adult population in developed countries [148-150]. CMV infects various parts of the body, and is also able to infect and replicate in adrenocortical cells [151].

There have been several reports of Addison's disease development in patients with a human immunodeficiency virus (HIV) infection or acquired immunodeficiency syndrome (AIDS) that are infected by CMV, however this is not considered of autoimmune ethology but rather a direct cytopathic effect by CMV on the adrenal cells [152]. Nevertheless, there also exist reports were CMV infection alone is proposed as the initiating factor of AAD development [153].

1.5.8 Proposed pathogenesis of AAD induced by virus infection

Several factors are involved during development of an autoimmune disease, and since AAD is an organ specific disease, it is reasonable to assume that the tissue itself is important, and also has a role to play. The cells of the adrenal cortex have several properties that could support viral infections as triggers or inducers of autoimmune disease in predisposed individuals. For instance, they express TLR3, as well as several other TLRs and produce important inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF-α [154-156]. In resolving a potential viral infection of the adrenals lymphocytes need to enter the affected tissue. This is achieved through secretion of chemokines such as CXCL10 and adhesion molecules by tissue resident cells [157]. CXCL10 is reported to be elevated in sera from AAD patients [158-160]. This chemokine could be produced by activated immune cells, or potentially by cells of the adrenal cortex. This has been shown in a study of autoimmune thyroid disorders, where the thyroid follicular cells of the target tissue produced CXCL10 [161]. This was also demonstrated for primary adrenal zona fasiculata cells which secreted CXCL10 after stimulation with the cytokines IFN-γ and TNF-α [162] and in an

adrenocortical carcinoma cell line stimulated with a viral dsRNA substitute both alone and in synergy with IFN-γ and TNF-α [159].

However, recruiting lymphocytes to the adrenal during a viral infection is probably not enough to sustain an inflammatory environment for so long that more than 90% of the adrenocortical cells are destroyed during that period. The lymphocytes recruited could potentially be autoreactive, and their activity could be affected by interacting with the adrenal cells. An experiment investigating the autoimmune development in non-obese diabetic (NOD) mice showed a selective avidity maturation of the infiltrating T-lymphocyte population, suggesting a preferential expansion of T cells binding strongly to self, versus T cells with low-affinity TcRs [163]. The main antigen recognized by autoreactive T cells in AAD is believed to be 21OH, since it's exclusively expressed in the adrenal cortex and because of the presence of 21OH antibodies in these patients. Several studies support this notion, showing that T cells from AAD patients proliferate and produce IFN-γ after stimulation with 21OH peptides and full length protein, and demonstrating specific CD8- and CD4-restricted epitopes [119, 164, 165].

With these properties in mind, the proposed mechanism of tissue destruction and disease development as a result of an acute or latent viral infection (adapted from Bratland and Husebye, Groom and Luster, and Fujinami *et al.* [54, 70, 166]) is as follows;

Viral infection and initial immune activation in the adrenal cortex:
In a genetically predisposed individual infected by a virus able to infect and replicate in the adrenocortical cells, TLR3 senses the infection through binding of double stranded RNA (dsRNA) produced by most viruses during their replication cycle [167]. TLR3 activation could in turn lead to IFN production and activation of ISGs such as CXCL10 and upregulation of MHC class I, as explained previously. Destruction of infected cells, either by the infection itself or by the innate immune response, releases cellular components (including 21OH) into the extracellular environment. The cellular content is ingested by DCs or macrophages shown to be

abundant in the adrenal cortex [168], and presented as antigens bound to MHC class II. If the virus is intact it could then infect the DC and be presented bound to MHC class I, or exogenous antigens could be cross-presented by MHC class I [169, 170].

Antigen presentation and activation of lymphocytes in the lymph node:

The DCs migrates to the nearest lymph node were they activate potentially autoreactive naïve CD4$^+$ and CD8$^+$ T cells. IFN-γ is produced as a result of T cell activation, which further activates the APC to release cytokines important for differentiation into CD4$^+$ effector T cells (Th1), such as IL-12. Activated CD4$^+$ Th1 cells are important helpers in the activation and clonal expansion of potentially autoreactive B cells and CTLs. The B cells then produce 21OH autoantibodies that can augment the inflammatory reaction. The activated effector cells upregulate expression of the CXCR3 receptor, and migrates to the infected tissue in response to the elevated levels of CXCL10.

Autoimmune destruction of the cells in the adrenal cortex:

Autoreactive CTLs exert a cytotoxic effect on the adrenal cells by recognizing 21OH-derived peptides in the context of MHC class I presented on the cell surface. This has been shown *in vitro* by co-culturing 21OH-specific CD8$^+$ T cells with the human adrenocortical carcinoma cell line NCI-H295R, resulting in increased levels of the degranulation marker CD107 and excessive production of granzyme B [164]. The CD4$^+$ Th1 cells secrete IFN-γ and TNF, which can stimulate macrophages to release inflammatory cytokines (TNF and IL-1β), nitric oxide (NO) and other toxic oxygen species which destroy the infected tissue without excluding healthy cells [171]. This may enhance the presence of more potentially harmful antigens able to activate autoreactive CTLs and CD4$^+$ Th1 (self-specific T cells), which in turn may perpetuate the autoimmune destruction. We know that the adrenocortical cells also are able to produce inflammatory cytokines upon stimulation, and that IFN-γ and TNF-α can stimulate the adrenocortical cells along with the tissue resident DCs and macrophages in order to secrete CXCL10. This gives rise to an inflammatory loop were the recruited lymphocytes produce more IFN-γ, leading to secretion of more CXCL10 which again lead to a recruitment of more lymphocytes. Eventually the inflammation

burns out when all adrenocortical cells are destroyed, and there is no longer presence of 21OH antigens.

2. Aims of the study

The overall objective

Our main hypothesis was that adrenocortical cells in AAD are destroyed by cytotoxic T cells, specific for 21OH, responding to a viral infection and interferon production locally in the adrenals. The principal aim was therefore to elucidate pathogenic mechanisms of immune-mediated destruction of the adrenal cortex in AAD.

The specific aims were

I.   To investigate the role of IFNs in AAD disease development including their effect on adrenocortical cells.

II.   To investigate the interferon induced CXCL10 production in patients with AAD.

III.   To investigate humoral and cellular immunity to cytomegalovirus in patients with AAD.

3. Comments to methodology

While a detailed description of the materials and methods is part of each separate paper, this section will elaborate on some of the methods chosen and details not included in the papers.

3.1 Choice of material

Patient material (Paper I-III)

Through the Norwegian registry and biobank for organ-specific autoimmune diseases (ROAS) we have access to serum, plasma, EDTA blood, DNA and peripheral blood mononuclear cells (PBMCs) from patients with confirmed AAD. Samples from this biobank were used in all three papers, and the exact amount is specified in the individual paper. The samples were consecutively chosen and the different patients overlap some in the different studies. All patients signed informed consent approved by the Health Region West Ethics committee (149/96-47.96) and the experiments were conducted in accordance with the declaration of Helsinki.

Healthy controls (Paper II and III)

Heparinized blood samples from gender- and age-matched healthy controls used in these studies were collected from blood donors provided by the blood bank at Haukeland University Hospital. It was either used directly, or PBMC and plasma were isolated using Ficoll-Paque Plus and frozen at -150ºC or -80ºC, respectively. The number of controls used is specified in the individual paper. All controls signed informed consent approved by the Health Region West Ethics committee.

Cell culture experiments (Paper I)

For both practical and ethical reasons we did not have access to primary adrenocortical cells or adrenocortical tissue. Thus the decision was made to use the human adrenocortical carcinoma cell line NCI-H295R. This cell line is well

established and has been shown to exert many of the same properties as primary adrenocortical cells [172]. For this study it was important to investigate whether these cells express the IFN-α/β and IFN-λ receptors and if the same receptors are present in human adrenal tissue. Thus slides mounted with 5 µM sections of human adrenal tissue were purchased from Abcam and used for immunohistochemistry staining.

3.2 Methodological considerations

Enzyme-linked immunosorbent assay (ELISA) for chemokine measurements (Paper I and II)

For paper II two different ELISAs were used when investigating chemokine levels in serum and cell supernatant respectively. The serum concentrations were measured in an ELISA kit from RayBiotech validated for serum/plasma analysis, with a broader standard curve than the ELISA used for cell supernatants. For the cell supernatant measurements the same ELISA kit from R&D systems was employed for both stimulated PBMC of paper II and NCI-H295R cells of paper I.

PBMC stimulation experiments (Paper II)

When investigating the chemokine and interferon production in cells stimulated with cytokines or polyinosine-polycytidylic acid (poly (I:C)), the published results are from experiments performed directly after thawing and washing of the PBMC without resting the cells. AAD patients receive cortisol replacement therapy, and thus the glucocorticoids may interfere with the IFN signaling pathway. By resting the cells for 24h any interference caused by the glucocorticoid could possibly have been avoided. To test this additional experiments were performed on cells rested 24h prior to stimulation, but in general this did not improve the chemokine production.

RNA was isolated and quantitative real-time polymerase chain reaction (qRT-PCR) was performed on cells that had been stimulated with cytokines and poly (I:C) for 24h. Ideally it would have been better to investigate the mRNA expression at an

earlier time point since the expression levels of some ISGs are at its highest after about eight hours [173]. But wanting also to investigate the chemokine and interferon secretion in cell supernatant, 24h was preferred since limited amounts of patient PBMC made it difficult to harvest cells at multiple time points.

Bioassay for type I IFNs (Paper II)

The cell supernatant from the stimulation experiments was used for two different chemokine ELISAs in addition to measuring the type I IFN production. Due to limited material a bioassay based on the reporter cell line, HEK Blue IFN-α/β from Invivogen was employed, where only 20 µl of the cell supernatant was needed. This cell line has a functional type I IFN pathway, and in the presence of IFN-α/β it will turn on the expression of a secreted embryonic alkaline phosphatase (SEAP). Cell supernatant was incubated with the HEK blue cells in a 96 well plate for 24h, then the induced HEK Blue IFN-α/β supernatant were mixed with QUANTI-Blue, a medium that turns from red to blue in the presence of SEAP. The absorbance was measured and IFN-α/β production was calculated in relation to a standard curve with known IFN concentration.

PTPN22 SNP analysis (Paper II)

The SNP analysis was performed as described previously [129]. However, since DNA was not available from the healthy controls, only patients were included in this analysis.

STAT1 and STAT2 activation levels of stimulated PBMC (Paper II)

The STAT1 and STAT2 cell-based ELISA immunoassays were included as a follow-up experiment in paper II, based on the results obtained in the PBMC stimulation experiments. However, PBMC from patients and controls employed in the initial stimulation experiment were no longer available, consequently the STAT experiments were performed on PBMC from new subjects. A cell-based assay was chosen to measure levels of total STAT and STAT activation, allowing use of the same sample on the two different kits simultaneously, thus limiting the need for

cellular material. The cell-based ELISA was also a very quick and efficient method compared to for instance flow cytometry analysis.

CMV peptide specific dextramer analysis (Paper III)

The MHC dextramer technic from Immudex was used to investigate the presence of T cells specific for HLA-B8 and HLA-A2 CMV restricted epitopes. This technic from Immudex allows fast and reliable measurements of T cell specificity considered to be better than the traditional MHC tetramer staining procedures [174, 175].
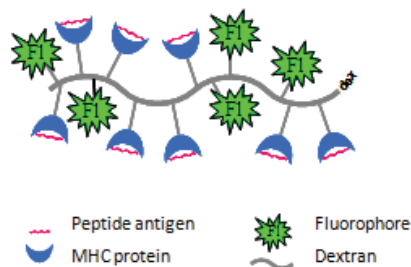
Figure 5: The MHC dextramer technic from Immudex. The MHC Dextramer reagent is a fluorescent labeled MHC multimer. It can be used to detect T cells specific for the antigen the MHC multimer is loaded with. The dextran polymer backbone is loaded with an optimum number of MHC and fluorochrome molecules, allowing the dextramer to interact with several TcR on the individual T cell giving a stable interaction and a high staining intensity. The dextramers can be used to detect antigen-specific T cells in fluids using flow cytometry. Image from http://www.immudex.com/about-products/dextramer-descrip.aspx

MHC dextramers consisting of recombinant HLA-A2 and HLA-B8 loaded with their respective cognate CMV peptides (HLA-A*0201/NLPMVATV/PE and HLA-B*0801/QIKVRVDMV/PE) were purchased from Immudex, along with negative control MHC dextramers loaded with HIV gag-derived peptides (HLA-A*0201/SLYNTVATL/PE and HLA-B*0801/DIYKRWII/PE) and used to test for CMV specific CD8$^+$ T cell responses, both in cells *ex vivo* and after stimulation with CMV peptides.

4. Summary of papers

Paper I

In paper I the aim was to study the possible immunopathological effect that type I and III IFNs could inflict on adrenocortical cells, and if these effects could play a role in the disease development of AAD. All experiments were conducted with the human adrenocortical carcinoma NCI-H295R cell line (H295R). The presence of the IFNAR1 and IFNλR1 chains on the H295R cells, part of the two IFN binding receptors, were confirmed both by flow cytometry and immunofluorescence. The same receptor components were also shown to be present in healthy human adrenal cortex tissue by immunohistochemistry staining.

Stimulation with recombinant IFN-α, IFN-β, IFN-λ and poly (I:C) were shown to exert cytotoxic effect on the H295R cells, by measuring lactate dehydrogenase (LDH) release to evaluate their membrane integrity. The effect was greatest when stimulating with poly (I:C), while using poly (I:C) in combination with the IFNs gave a significantly higher LDH release compared to IFNs alone.

Stimulation with type I and III IFNs significantly increased the expression of MHC class I on the H295R cells measured by flow cytometry, while this upregulation was at its highest after type II IFN stimulation. Poly (I:C) alone did not significantly increase the MHC class I upregulation, although it was elevated compared to unstimulated control. The mRNA expression of *CYP21A2* was measured on RNA isolated from the cell pellet from the stimulation experiment using qRT-PCR. The 21OH expression increased two- to threefold after stimulation with type I/III IFNs and poly (I:C) compared to unstimulated cells.

The H295R cells ability to produce CXCL10 and the related chemokines CXCL9 and CXCL11 was measured by ELISA in cell supernatant from stimulation experiments with type I and III IFNs. Alone the type I/III IFNs did not induce significant levels of chemokine, but in combination with IFN-γ or poly (I:C) the expression of CXCL10 was considerably increased compared to the levels induced by IFN-γ and poly (I:C)

alone. This suggests that there is a synergistic effect of type I/III IFNs on both IFN-γ and poly (I:C). CXCL11 was not produced at the same levels by the H295R cells, and for CXCL9 only IFN-γ appeared to have some effect. In a migration experiment the CXCL10 produced by IFN-γ and poly (I:C) stimulation was sufficient to increase the migration of 21OH specific T cells towards the chemokine in the H295R cell supernatant using a trans well system, compared to the migration induced by unstimulated cells.

Paper II

In paper II the source of the increased serum CXCL10 and CXCL9 concentrations in AAD patients was investigated by studying their propensity to produce chemokines after IFN stimulation. Previous studies have reported elevated serum chemokine levels in AAD patients, which were confirmed in our patient cohort by CXCL10 and CXCL9 ELISA. In an attempt to discover the source of this production PBMC from AAD patients and controls were stimulated with type I and II IFNs and poly (I:C). In all cases the patients were found to produce significantly lower levels of CXCL10 and CXCL9 after a 24h stimulation period, compared to healthy controls as measured by ELISA.

The expression of two ISGs, *USP18* and *IRF7*, was also measured in relation to IFN stimulation and the relative mRNA expression of *USP18* was found to be decreased in the patients for all stimuli. For *IRF7* there were no significant differences after cytokine stimulation, while poly (I:C) induced a significantly lower expression in the patients, indicating a poorer IFN production after TLR3 stimulation. The poly (I:C) induced production of IFN-α/β collectively was also measured using a bioassay, and found to be significantly decreased in the patients.

To investigate the results further the data was correlated against a SNP in the *PTPN22* gene associated with AAD, which have been found to confer an impaired IFN response after TLR stimulation. This was investigated in the patient cohort were DNA was available. The poly (I:C) induced CXCL10 production was stratified between patients carrying the disease associated SNP and those that were

homozygous for the normal variant, and the disease associated carriers produced significantly lower amounts of CXCL10. This effect was not observed for CXCL9.

The total and phosphorylated STAT1 and STAT2 levels was also investigated in relation to cytokine and poly (I:C) stimulation, but no significant differences between patients and controls in that part of the signaling pathway was found.

In a parallel experiment the relative mRNA expression in CD4$^+$ T cells of five ISGs was measured in a new set of patients and controls, to examine if they displayed an activated IFN signature in their peripheral blood. However, although selected patients showed signs of IFN activation, no significant differences between patients and controls were detected.

Paper III

In order to investigate the virus hypothesis further the cellular and humoral response to cytomegalovirus was examined in AAD patients and controls in paper III. A primary or reactivating CMV infection was hypothesized as a possible environmental factor in AAD development. With HLA-B8 being a risk allele in AAD, CD8$^+$ T cell response to HLA-B8 restricted epitopes was investigated in addition to an epitope restricted to the more common class I HLA-A2 subtype.

Initially the IgG and IgM CMV antibody levels were determined in patients and controls. No statistically significant differences were found between the two groups, but the percentage of CMV IgG positive was a bit higher in the patient cohort. Only seropositive patients and controls were included when investigating CMV-specific T cell response.

No *ex vivo* differences could be detected between patients and controls regarding the function or frequency of CMV-specific T cells using dextramers to detect CMV specific T cells by flow cytometry and IFN-γ ELISpot to measure activity. However, a difference was detected in the level of total CD8$^+$ T cells, which were significantly lower in the patient cohort. The cells were also stimulated with CMV epitopes *in vitro* while expanding them over a period of 13 days, but again no significant

differences could be measured. The level of degranulation of CMV stimulated cells was investigated using a CD107a based degranulation assay, without finding any differences.

Although there were no significant differences towards CMV looking at the group as a whole, individual patients showed signs of reactivating CMV infection by fluctuating positive IgM levels over a time period in addition to poor CD8$^+$ T cell responses to the HLA-B8 restricted CMV epitope.

5. Discussion

Autoimmune Addison's disease is an example of a classic organ specific autoimmune disease with an unknown etiology. There's been conducted a lot of research with focus on the genetic aspects of the disease, but with this thesis we wanted to investigate the environmental basis of the disease development and the role played by the adrenal cortex itself. Due to the slow evolvement of the genetic landscape, the increasing incidence of autoimmune diseases could hardly be explained by genetics alone, and thus from 1997 the research focus and publications on the environmental factors of autoimmunity have grown on average 7% each year [176]. In this regard we propose a hypothesis that for individuals genetically predisposed to AAD, this environmental factor could be a viral infection. The virus could serve as a precipitating event in the autoimmune process leading to the disease, while the adrenal cortex itself could be responsible for recruiting lymphocytes and maintaining the inflammatory environment essential for disease development.

5.1 The target tissue

The cells of the adrenal cortex exert certain properties that would allow an immune response to take place, such as expression of different types of TLR [154, 177]. In addition, the cells of the adrenal cortex are able to produce different cytokines and chemokines as response to various stimuli (Introduction section 1.5.8). In paper I we wanted to investigate how the target tissue would respond to the presence of viral induced interferons, and due to lack of primary adrenocortical cells, we used the well-established adrenocortical carcinoma cell line H295R. To test this, we firstly needed to explore the presence of their receptors, which we confirmed with both flow cytometry and immunofluorescence. More specifically we detected the receptor components, IFNAR1 and IFNλR1. We also were able to detect the same components on human adrenal tissue slides using immunohistochemistry. Previous studies have shown that IFNλR1 mRNA expression is detected in the adrenal gland, while IFNAR1 is known to be expressed in different adrenocortical cell lines, including

H295R cells [40, 178, 179]. Confirming that the IFN-α/β and the IFN-λ receptors are expressed both in adrenal tissue and in the cell line, allowed us to use the H295R cells as a model for studying virus induced AAD development.

Interferons cytotoxic to adrenocortical cells

Production of interferons after a viral infection is important for induction of ISGs, but the interferons themselves have also been shown to induce cell death both alone or in combination with viral infections or poly (I:C) [180-183]. Thus the interferons produced in response to an infection could end up being harmful for the affected tissue. In paper I we observed that stimulating the adrenocortical cells with type I and III IFNs alone or in combination with poly (I:C) exerted significant cytotoxicity of the cells, suggesting that the interferons could play an important destructive role on the cells of the adrenal cortex as well, in addition to contributing to the inflamed environment.

Upregulation of HLA class I and 21OH after interferon stimulation

In paper I we found that stimulation of the adrenocortical cells with type I/III IFNs alone or in combination with IFN-γ upregulated expression of HLA class I. Upregulation of HLA class I is important for recruiting CTL to the site of infection, allowing for a CTL mediated destruction of infected cells, and a similar upregulation has been shown to correlate with the disease onset in a mouse model of virus induced T1D [184, 185]. The results in paper I show that adrenocortical cells are prone to IFN induced upregulation of HLA class I, and we envision a similar response in the cells of the adrenal cortex after a viral infection. In T1D hyperexpression of HLA class I is considered a normal trait in insulin containing islets [186]. In addition to HLA class I upregulation, the mRNA expression of 21OH increased significantly after stimulation with type I/III IFNs and poly (I:C). If viral infections and interferons are able to induce the same response in the cells of the adrenal cortex, enhancing the expression of 21OH could lead to higher availability of potentially harmful antigens presented by hyperexpressed HLA class I to CTL recruited to the site of infection. These are both important factors supporting a role for the adrenal cells in the disease development.

The chemokine CXCL10

CXCL10 have been shown to be elevated in sera of patients with different autoimmune diseases, such as autoimmune thyroiditis, SLE and T1D respectively [187-189]. This is also the case for AAD [160, 162], and we found both CXCL10 and CXCL9 to be significantly elevated in patient sera in paper II. The source of the elevated chemokine levels in AAD patients is not known; it could be produced locally due to an inflamed environment of the adrenal cortex or by activated lymphocytes or monocytes in the blood. Both H295R cells and adrenocortical cells have been shown to produce CXCL10 after stimulation with proinflammatory cytokines and poly (I:C) [159, 162], so we know that the cells of the adrenal cortex themselves are capable of this production. In paper II we found that in spite of having elevated chemokine levels in their sera, patient PBMC produced significantly lower levels of these chemokines after IFN stimulation compared to healthy controls. These results supports the notion that the adrenal cells themselves are in fact responsible for the chemokine production detected in patient sera, a view supported by similar studies [159, 162, 190].

Interferons can induce infected or bystander cells to secrete CXCL10, and thus recruit activated T cells to the site of infection [54]. In paper I we demonstrated that adrenocortical cells stimulated with IFN (I/III) in combination with poly (I:C) and IFN-γ did in fact produce CXCL10, and that the level of secreted chemokines was able to attract 21OH specific T cells in a transwell assay in significantly higher amounts than supernatant from unstimulated H295R cells. This neatly demonstrates how a virus infection in the adrenals could lead to recruitment of T cells that are autoreactive against the 21OH it's bound to encounter in the inflamed tissue.

5.2 Interferons in AAD

An interferon signature, or an increased expression of type I IFN regulated genes, have been reported in various systemic and endocrine autoimmune diseases, such as

SLE, SS and GD [191-193]. A similar signature has not yet been reported for AAD, and thus we wanted to investigate this in our patient cohort. Based on a previous study on AAD patients, we chose to look at the expression of five ISGs that was shown to be hypomethylated in $CD4^+$ T cells from AAD patients [194]. However, though there were some patients who showed a clear upregulation of individual genes, there were no significant differences in the IFN score between AAD patients and healthy controls. The patients used in these studies were consecutively recruited and thus disease duration would vary. Two recently published studies showed an IFN signature in pre-diabetic patients [82, 195], suggesting that an IFN signature might have existed in these patients in a pre-AAD time point during the development of the disease.

Even though we found no evidence of an IFN signature we still suspect that type I IFNs has a role to play in the development of AAD, due to the high levels of CXCL10 in sera and how type I IFN treatment have been shown to induce adrenalitis [141, 142]. In paper II we wanted to investigate if there were any differences in how AAD patients and healthy controls responded to interferons, by examining the chemokine release of their PBMC. While the patients have significantly higher amounts of CXCL10 and the related chemokine CXCL9 in their sera, their PBMC did actually produce significantly lower amounts of the same chemokines after stimulation with type I IFNs and IFN-γ. This was also the case when we stimulated the cells using poly (I:C), thus inducing IFN production through TLR3 stimulation. Therefore, the patient PBMC was not only deficient in their chemokine production after direct stimulation through their interferon receptors, but also when stimulated endogenously using a synthetic analog for dsRNA. It seemed evident that the failed chemokine induction was not just a result of poor activation of the IFN receptors, but also due to reduced IFN production, which was demonstrated when we measured type I IFNs after poly (I:C) stimulation and found significantly lower values in the patients.

We did not only measure activation by chemokine secretion we also tested the mRNA expression of two well-known ISGs Ubiquitin-specific peptidase 18 (encoded

by *USP18*) and interferon regulatory factor 7 (encoded by *IRF7*) from the same cells. USP18 is a classic ISG that functions by downregulating the JAK-STAT pathway activated by the interferons [196], and the expression of *USP18* was significantly lower in the patients for all stimulatory conditions which show that the impaired response to interferons is not only affecting CXCL9 and CXCL10 production. IRF7 is a positive feedback regulator of the IFN response, induced to amplify the IFN production after for instance TLR3 stimulation (activated in our case by poly (I:C)) [197]. The *IRF7* expression was significantly lower in the patients after poly (I:C) stimulation, however no differences were detected after IFN stimulation alone. The decreased chemokine production after direct exogenous IFN stimulation is independent of IRF7, while the endogenously IFN stimulated cells not only fail in interferon induced chemokine production but also interferon production itself. To try and elucidate why these patients appear to have a defective response to IFNs we looked into several possible explanations discussed below.

Interferon signaling and activation of STAT1/2

In order to find the reason of the impaired IFN response in the patient cells, we wanted to investigate whether there were any differences in the level of STAT1 and STAT2, and their phosphorylation activity in relation to the stimulation experiments. However, we found no differences either in total STAT1/2 or in regards to phosphorylation after IFN and poly (I:C) stimulation, indicating that the impaired chemokine production is a result of defects in events downstream of STAT signaling or during IFN production.

The effect of glucocorticoid (GC) treatment

Since AAD patients have no endogenous cortisol production, they receive cortisol supplementation therapy. This supplementation is meant to replace the normal cortisol production found in healthy individuals, and though there exists concern whether they receive too much [198] these patients do not appear to have increased serum levels of cortisol compared to healthy controls [199]. Still, GCs are known to exert several immunosuppressive functions [200] and this could in theory explain the

differences we observe in paper II. GC have been shown to inhibit STAT1 phosphorylation after poly (I:C) stimulation [201], but when we investigated the phosphorylation of STAT1 after PBMC stimulation between patients and healthy controls we found no differences, indicating that the GC doses given to the selected patients do not interfere with the results. In addition we performed an experiment where we rested the cells for 24h before stimulation, to avoid GC interference, but no overall improvement of CXCL10 production was observed.

Are AAD patients genetically prone to poor interferon production?

The reason for the decreased chemokine and interferon production could be explained by the fact that the patients are genetically prone to such a phenotype. One example is related to a SNP in the *PTPN22* gene associated with several autoimmune diseases, including AAD [128, 134]. A recent study showed that carriers of the disease allele 1858T, had an impaired interferon production after TLR3 stimulation [135], which we also experienced in paper II in relation to poly (I:C) stimulation. We saw a significant difference in CXCL10 production between carriers of the 1858T allele and the normal C variant. However, there were no correlation between *PTPN22-C1858T* and CXCL9 production which is consistent with CXCL9 being induced primarily by IFN-γ and not by type I IFNs. A similar result was recently published on a cohort of SLE patients, where carriers of the disease associated allele produced significantly lower amounts of IFN-α after stimulation of the TLR7/8 receptor in pDC isolated from PBMC, even though there were no differences in IFN signature or IFN-α serum levels between carriers and non-carriers [202].

If patients carrying this mutation have a poorer response to TLR activation than the patients with the non-mutated gene, it would seem that they could be more prone to infections, and also to prolonged viral persistence. Indeed there exist reports that AAD patients have increased risk of premature death, among others due to infections [203-205]. Patients with AAD have been shown to use higher amounts of antimicrobial agents, and have more infection related hospital admissions than the general public with no obvious correlation to their cortisol treatment [206]. However,

for the deficient immune response we report, only the poly (I:C) induced chemokine production would be affected by this mutation. The impaired CXCL10/9 production induced directly by exogenously added IFNs cannot be explained by the presence of the PTPN22 SNP, since PTPN22 is only involved upstream of endogenous IFN production. PTPN22 could therefore only explain the results partly. When excluding the T carriers there's still a significant difference in IFN induced chemokine production between AAD patients and healthy controls further suggesting that the explanation lies somewhere downstream of the STAT phosphorylation step.

5.3 Cytomegalovirus and Addison's disease

Since cytomegalovirus have a tropism for infecting the adrenals, in particular in immunocompromised individuals, it would seem reasonable to assume that CMV infection of the adrenals is not uncommon, but that the immune system is normally capable to clear or suppress the virus without a prolonged immune activation. However, in an individual that is genetically predisposed to AAD, the primary infection or the latent induction of the immune response it causes could be the precipitating event in the disease development. In paper III we tried to investigate whether AAD patients showed an increased propensity to CMV infections, and their CMV specific CD8$^+$ T cell response. Over 70% of the patients had CMV positive IgG antibodies, which is consistent with reports from the middle-aged healthy population in the western world [207-209], although levels from the healthy controls tested in the same experiments with similar mean age was around 10% lower.

In paper III we found that the *ex vivo* cellular response against CMV did not differ between patients and controls. We investigated the level and activity of CMV specific memory CD8$^+$ T cells, both HLA-B8 and HLA-A2 restricted, without finding any significant differences. Similarly, no differences were detected after *in vitro* stimulation with CMV peptide and expansion of CMV specific CD8$^+$ T cells were similar in patients and controls. However, we found a significant difference in the

level of total circulating CD8^{+} T cells in the patient cohort compared to the healthy controls. The AAD patients had significantly lower CD8^{+} T cell levels among their PBMC, consistent with similar reports in several autoimmune diseases including SLE [210], T1D [211], GD [212] and multiple sclerosis [213]. This CD8^{+} T cell deficiency is believed to be genetically determined, as first degree relatives to autoimmune disease patients also exert this trait [211, 214, 215]. It is possible that the CD8^{+} T cell deficiency, leads to an impaired viral clearance resulting in autoimmunity, which also could explain why infections appear to be more common in AAD patients, as discussed above. This prospect is described thoroughly in a review by M. Pender on Epstein-Barr virus (EBV) infection as the main culprit in genetically compromised individuals, where he suggests that a decreased CD8^{+} T cell level results in an impaired response to EBV, allowing possible autoreactive EBV-infected B cells to accumulate in the target organ [216]. He does not exclude that other viral infections could be responsible for similar responses, however it might seem that EBV is more notoriously associated with autoimmune diseases than for instance CMV [217].

Although we didn't find any significant differences in CMV immunity between the patients and controls in paper III, we did find some interesting observations in individual patients. One female patient in particular had positive CMV IgG levels over a time period of 12 years, while in that same period IgM levels went from negative, to positive, and then negative again suggesting a reactivation of infection. Interestingly this patient, who is HLA-B8 positive, had among the lowest observed levels of CMV specific CD8^{+} T cells. In paper II we found that this exact same patient had next to no chemokine production after type I interferon and poly (I:C) stimulation, but showed clear signs of an activated interferon signature in three of the five ISGs tested. Furthermore, the daughter of this patient also had AAD and, similar to the mother, had almost no detectable CMV-specific CD8^{+} T cells in spite of being anti-CMV IgG positive. We speculate that the co-occurrence of AAD and poor cellular immunity to CMV in this family is part of an inheritable extreme immunological phenotype that makes individuals more prone to develop autoimmunity with suboptimal control of viral infections.

6. Conclusions

In accordance with the specific aims of this thesis, the following conclusions may be drawn:

- Type I and III IFNs alone or in combination with IFN-γ and/or poly (I:C) are cytotoxic to H295R cells and leads to upregulated expression of MHC class I and 21OH. The type I/III IFN receptors are expressed both in human adrenocortical tissue and H295R cells.
- 21OH specific T cells are able to migrate towards CXCL10 secreted by IFN and/or poly (I:C) stimulated H295R cells.
- AAD patients have elevated serum levels of CXCL10 and CXCL9 while their PBMC have impaired CXCL10 and CXCL9 production after type I/II IFN and poly (I:C) stimulation compared to healthy controls.
- AAD patients have normal humoral and cellular immunity towards CMV, but the levels of circulating $CD8^+$ T cells in these patients are decreased.
- Individual patients showed signs of primary or reactivating CMV infection, combined with low levels of CMV specific $CD8^+$ T cells. One of these patients also had decreased CXCL10 production after IFN stimulations and an activated IFN signature, indicating that CMV could have played a role in disease development.

7. Future perspectives

Although autoimmune Addison's disease is a rare disease, the nation-wide Registry for organ specific autoimmune diseases (ROAS), including more than 700 patients, is an invaluable resource for research. Hopefully, with the help of ROAS, we will be able to answer even more of the unanswered questions regarding the pathogenesis of AAD.

For future studies it would have been interesting to correlate more of the genetic data available to what we actually see when studying PBMC from patients for functional studies in the lab, for instance by investigating the effect of the PTPN22*1858T mutation in a larger sample size. The patients used in our experiments were not selected specifically, but were newly recruited patients or patients who had visited their local outpatient ward for their yearly check-up. Since we have DNA and serum from most of our patients at all times, we are able to establish which patients have the mutations in genes associated with AAD and also perform several analyses on sera, for instance check serostatus against different viruses. After having identified patients of interests, we could invite these patients to donate a blood sample for further evaluation using methodology now being implemented in our center.

Next year the laboratory platform will be expanded to include the mass cytometry CyTOF system and the Fluidigm C1 system for single cell analysis, allowing the investigation of multiple parameters in one sample, and thus increasing the information we get from one donation of blood.

In order to test our hypothesis, studying adrenal tissue or biopsies from diseased individuals will be invaluable. Specifically, this could allow us to investigate the presence of viral DNA and proteins, the *in situ* production of IFNs and CXCL10, and also the expression of CXCR3 on infiltrating lymphocytes. However, intact adrenal tissue from deceased patients with AAD is difficult to obtain, and so far only a few studies with limited material have been published. Addison's disease is also known to affect both cats and dogs. A recent report show that adrenal tissue slides from

deceased dogs with confirmed or suspected Addison's disease share similar traits as reported in humans, with lymphocytic infiltrations and atrophy of the adrenocortical cells [218]. Though, for future studies it might be easier to obtain tissue samples from dogs that have died while having the disease or as a result of the disease, to investigate whether there is any trace of a viral infection in the adrenals. Ideally we would have liked to investigate if a virus could induce adrenalitis or accelerate an autoimmune disease development in mice, but as of today there exists no relevant AAD mouse models.

Finally, as our studies suggest that CXCL10 plays a role in the pathogenesis of AAD and is responsible for recruiting autoreactive T cells to the adrenal cortex, the prospect of clinical immunotherapy using monoclonal antibodies against CXCL10 would be quite intriguing. Clinical trials using chemokine inhibitors have been initiated for various autoimmune diseases. In RA a phase II clinical trial, the fully human monoclonal antibody MDX-1100/BMS-936557 (anti-CXCL10) was given to patients already receiving methotrexate, and a significant clinical efficacy was demonstrated. In addition the antibody appeared to be well tolerated by the patients, with very few serious adverse effects [219]. The same antibody gave similar promising results in a phase II study of ulcerative colitis [220].

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