Functional characterization of genetic risk factors in autoimmune Addison's disease

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Scientific environment

The work of this thesis was conducted between 2017 and 2020 at the Department of Clinical Science and KG Jebsen Senter for Autoimmune Sykdommer, Faculty of Medicine, University of Bergen, with Dr. Eirik Bratland as supervisor and Professor Eystein S. Husebye as co-supervisor. Financial support was provided by the Research Council of Norway (grant no. 262677), and travel grants from Dr. Nils Henrichsen og hustru Anna Henrichsens legat, Det alminnelige medisinske forskningsfond and Scandinavian Society of Immunology.

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Summary

Autoimmune diseases occur when the immune system attacks and damages the body's own tissue. *Why* people develop these diseases, and *how* the autoimmune reaction develops are unanswered questions. Autoimmune Addison's disease (AAD) is an organ-specific autoimmune disorder characterized by an immunological attack of the adrenal cortex. The complex genetic architecture underlying AAD has not been entirely established, and the overall aim of this project was therefore to identify and functionally characterize genetic risk factors in AAD.

We discovered several rare and damaging inborn errors of antiviral immunity in AAD patients. Among them, variants in the gene encoding Toll-like receptor 3 (TLR3), which recognizes double-stranded RNAs (dsRNAs) upon viral infection. Functional characterization of the *TLR3* variants revealed a partial loss of function effect on the receptor's signaling activity, leading to impaired interferon (IFN) responses *ex vivo.*

Next, we identified a homozygous stop-gain variant in the gene encoding 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), causing a rare form of congenital adrenal hyperplasia (CAH). The mutation was carried by an AAD patient with circulating antibodies against the major AAD autoantigen 21-hydroxylase (21OH). To our knowledge, this combination represents a novel disease etiology.

Finally, we wanted to identify HLA-specific immunodominant epitopes of 21OH, targeted by autoreactive T cells. We discovered a new immunodominant epitope, ARLELFVVL ($21OH_{434-442}$), presented by HLA-C*0701. This is the first HLA-C*0701 restricted epitope described for a self-antigen in an autoimmune disease. We also confirmed the presence of autoreactive $CD8^+$ T cells responses to the previously proposed epitope LLNATIAEV ($21OH_{342-350}$), restricted to HLA-A2.

Altogether, the work in this doctoral dissertation has provided new insights into *why* certain individuals might be more genetically susceptible to develop AAD, and partly *how* the autoimmune reaction progresses.

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Abbreviations

AAD	Autoimmune Addison's disease
ACTH	Adrenocorticotropic hormone
AH	Ancestral haplotype
AIH	Autoimmune hepatitis
AIRE	Autoimmune regulator
AITD	Autoimmune thyroid disease
ALRs	AIM2-like receptors
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APC	Antigen-presenting cell
APS	Autoimmune polyendocrine syndrome
BACH2	BTB domain and CNC homolog 2
BCR	B cell receptor
CADD	Combined Annotation Dependent Depletion
CAH	Congenital adrenal hyperplasia
CD	Cluster of differentiation
CD	Celiac disease
CLEC16A	C-Type Lectin Domain Containing 16A
CLRs	C-type lectin receptors
CMV	Cytomegalovirus
CNS	Central nervous system
CT	Computer tomography
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CVID	Common variable immunodeficiency
DC	Dendritic cell

DHEA	Dehydroepiandrosterone
DHEA-S	DHEA-sulfate
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EBV	Epstein-Barr virus
EV	Enteroviruses
GWAS	Genome wide association study
gnomAD	Genome aggregation database
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSE	Herpes simplex virus encephalitis
HPA	Hypothalamic-pituitary-adrenal
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IKKε	Inhibitor of nuclear factor kappa B kinase subunit epsilon
IL	Interleukin
ILC	Innate lymphoid cells
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
IRAK4	IL-1R-associated kinase 4
IRF3	IFN regulatory factor 3
ISGs	IFN-stimulated genes
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KIRs	Killer cell Ig-like receptors
LC-MS/MS	Liquid chromatography-tandem mass spectroscopy

LKM-1	Liver/kidney microsomal antibody type 1
LPS	Lipopolysaccharides
MAPK	Mitogen-activated kinase
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
MR	Magnetic resonance
MS	Multiple sclerosis
mTECs	Medullary thymic epithelial cells
MyD88	Myeloid differentiation primary response 88
NALP1	NACHT leucine-rich-repeat protein 1
NF-kB	Nuclear factor-kappa B
NK	Natural killer
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PD1	Programmed cell death protein 1
PGN	Peptidoglycan
PIDs	Primary immunodeficiency diseases
POI	Premature ovarian insufficiency
Poly(I:C)	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I-like receptors

RNA	Ribonucleic acid
ROAS	Norwegian National Registry of organ-specific autoimmune diseases
SEAP	Secreted embryonic alkaline phosphatase
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
ssRNA	Single-stranded RNA
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
T1D	Type 1 diabetes
WES	Whole-exome sequencing
21OH	21-hydroxylase
3βHSD2	3β-hydroxysteroid dehydrogenase type 2

List of publications

Paper I

Sigrid Aslaksen, Anette B. Wolff, Magnus D. Vigeland, Lars Breivik, Ying Sheng, Bergithe E. Oftedal, Haydee Artaza, Beate Skinningsrud, Dag E. Undlien, Kaja K. Selmer, Eystein S. Husebye, Eirik Bratland (2019). **Identification and characterization of rare Toll-like receptor 3 variants in patients with autoimmune Addison's disease**
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Paper II

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Paper III

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Circulating 21-hydroxylase-specific CD8 + T cells in autoimmune Addison's disease are predominantly restricted by HLA-A2 and HLA-C7 molecules.
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Introduction

Protection against invasive pathogens is fully dependent on the immune system, a highly evolved network of specialized cells and molecules. This host defense system is remarkably effective as serious persistent infections are quite rare. Nonetheless, there are some limitations in that severe infections occasionally may occur, and also, in that the immune response sometimes fails to discriminate self from non-self, causing autoimmunity.

The development of autoimmunity recapitulates the same immune responses used to fight off infections and results in destruction of the host's own cells and tissues. Autoimmune disorders may affect almost all organs, whereby the clinical phenotype and severity are dependent on which tissue is being attacked. In autoimmune Addison's disease (AAD), the immune system is directed against the hormone-producing cells of the adrenal cortex, leading to insufficient production of vital steroids. Patients with Addison's disease therefore depend upon lifelong replacement therapy [1].

In the following, a brief introduction of the main components of the immune system is given, followed by a description of immunological tolerance and autoimmunity with emphasis on AAD.

The innate and adaptive immune system

To establish an infection, the pathogen must break through the first line of defense consisting of the skin and mucous barriers that secretes chemical compounds restricting the adherence and growth of microbes. Pathogens that manage to overcome these barriers will meet the two further lines of defense, the innate and adaptive immune systems [2].

The components constituting the innate immune response may be found in all advanced living organisms and has been refined for a longer period throughout evolution than the adaptive immune system, which is believed to have arisen with the vertebrate lineage [3, 4]. The innate immune system mounts the same immediate response each

time it gets exposed to a certain pathogen, whereas the adaptive immune response will improve on every exposure to the same pathogen, making it antigen-specific.

The cellular components of innate immunity

The innate immune response is largely dependent on phagocytosis by mononuclear and polymorphonuclear phagocytes. The mononuclear phagocytes include monocytes, dendritic cells (DCs), and macrophages. DCs are professional antigen-presenting cells (APCs) that can activate immune cells of the adaptive immunity and are found in most tissues including lymphoid organs, skin, lung, and kidneys [3, 5]. Macrophages are specialized cells that engulf and destroy any foreign invader, as well as clearing up debris from dead cells, and are located within the parenchyma of major organs throughout the body. They also serve as APCs to activate adaptive immune responses. Upon recognition of a pathogen, macrophages release chemotactic cytokines to attract other phagocytes, in particular polymorphonuclear phagocytes, to the site of infection. The polymorphonuclear myeloid cells (also known as granulocytes) include neutrophils and eosinophils, releasing reactive oxygen species to kill bacteria and parasites, in addition to basophils and mast cells, the main mediators of inflammation and allergic responses [2]. Another important cell population of innate immunity is the innate lymphoid cells (ILCs). ILCs respond quickly to pathogens by producing various cytokines and are especially abundant at mucosal surfaces [6]. ILCs include the natural killer (NK) cells that can kill infected and malignant cells, in addition to produce diverse cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) [7]. To further control the spreading of microorganisms, the complement system is activated, involving acute phase proteins in the blood that mediate opsonization and lysis of the pathogen, in addition to enhancing the inflammatory response [8].

To recognize pathogens, the innate immune system uses pattern recognition receptors (PRRs) that are specialized to detect small molecules deriving from viruses, fungi, bacteria, and protozoa, known as pathogen-associated molecular patterns (PAMPs) [9].

Binding of PAMPs to PRRs will activate numerous different signaling pathways that produce cytokines promoting the host response to infection [10, 11].

Innate immune receptors

The existence of PAMPs has been known for decades, but the PRRs recognizing them were introduced more recently by Charles Janeway in 1989 [12]. Since then, several classes of PRRs have been discovered including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and AIM2-like receptors (ALRs). These receptors are categorized by their structure and specificity, in addition to their tissue-specific expression and cellular localization [11]. The best characterized PRRs are the TLRs, and at present ten functional human TLRs have been defined. TLR1, TLR2, TLR4, TLR5, TLR6, and probably TLR10 are all expressed on the cell surface, detecting extracellular components of bacterial and fungal cell walls, such as lipopolysaccharides (LPS), di– and triacyl lipopeptides, peptidoglycan (PGN) and flagellin. The remaining TLR3, TLR7, TLR8, and TLR9 are localized within endosomes, where they detect foreign nucleic acids such as double-stranded ribonucleic acid (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG deoxyribonucleic acid (DNA), respectively. These intracellular TLRs are believed to function primarily as viral sensors (Figure 1) [9, 11].

Activation of intracellular TLR signaling is induced by binding of the ligand to the leucine-rich repeats of the receptors' ectodomain. For all TLRs except TLR3, this leads to activation of the adaptor protein myeloid differentiation primary response 88 (MyD88). MyD88 then interacts with interleukin (IL)-1R-associated kinase-4 (IRAK4), which phosphorylates IRAK1 and IRAK2 that, in turn, activate TNF receptor-associated factor 6 (TRAF6) [9, 13]. TRAF6 is further involved in activation of the mitogen-activated kinase (MAPK) pathway, and the two transcription factors nuclear factor-kappa B (NF-kB) and IFN regulatory factor 7 (IRF7), leading to the production of antiviral and proinflammatory cytokines like TNF, IL-12 and IL-6, and type I and III IFNs [14-16].

Upon TLR3 signaling, MyD88 is substituted with the adaptor protein TIR-domain-containing adapter-inducing IFN-β (TRIF), which interacts with both TRAF3 and TRAF6. TRAF3 links TANK-binding kinase 1 (TBK1) with inhibitor of NF-kB kinase subunit epsilon (IKKε), which together phosphorylate IRF3, leading to the production of type I and III IFNs. TRAF6, as mentioned earlier, induces transcription of proinflammatory cytokines and IFNs [14].

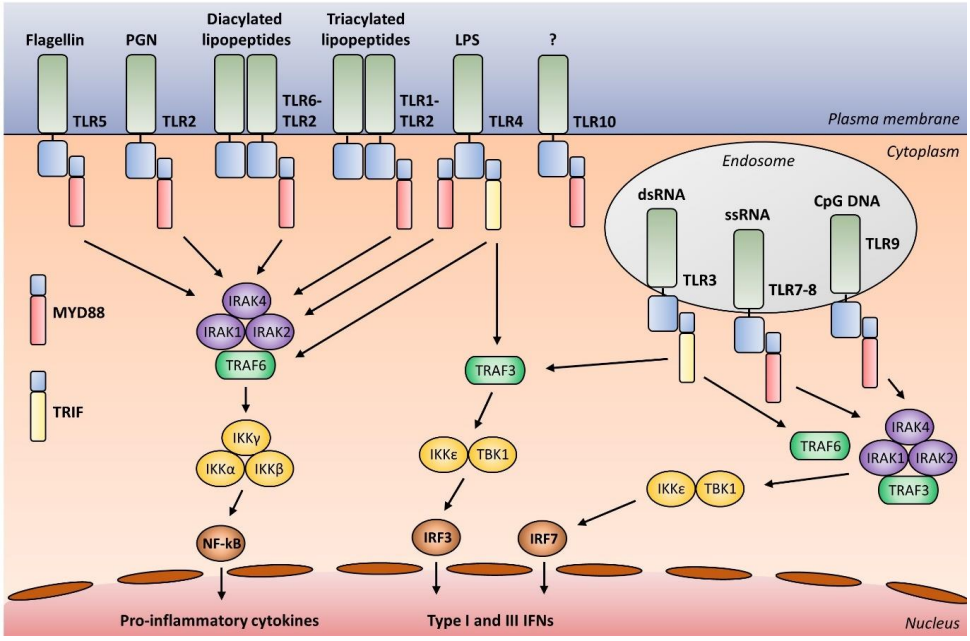

Figure 1. TLR signaling pathways. The extracellular TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and probably TLR10) are activated by molecules deriving from bacterial and fungal cell walls including lipopolysaccharides (LPS), di- and triacyl lipopeptides, peptidoglycan (PGN) and flagellin. The intracellular TLR3, TLR7, TLR8, and TLR9 are activated by nucleic acids within endosomes. Binding of the ligand to the receptors' ectodomain induces activation of the adaptor proteins MyD88 or TRIF. MyD88 recruits IRAK4 to phosphorylate IRAK1 and IRAK2, which further phosphorylate TRAF6, leading to activation of the IKK complex (IKKγ, IKKα, and IKKβ). This complex activates NF-kB, allowing it to translocate into the nucleus and initiate the production of pro-inflammatory cytokines. The other adaptor protein TRIF interacts with both TRAF3 and TRAF6. TRAF3 links TBK1 with IKKε, which promotes phosphorylation of IRF3 and IRF7, leading to the production of type I and III IFNs. The TLRs generally form homodimers upon activation, whereas TLR2 also functions as a heterodimer with TLR1 and TLR6. Figure modified from Wang et al [17].

Apart from the TLRs, RLRs also sense viral RNA products, not within endosomes, but in the cytosol of infected cells. RIG-I and melanoma differentiation-associated gene 5 (MDA5) are two well-defined RLRs, and activate NF-kB and IFN production through the same signaling pathways utilized by the TLRs [18].

Newly synthesized type I IFNs (α and β) and type III IFN (λ) will engage their cognate receptors to activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. The result is the expression of IFN-stimulated genes (ISGs), which induce an antiviral state involving RNA degradation, inhibition of protein synthesis, upregulation of major histocompatibility complex (MHC) class I (described later), chemokine secretion, and apoptosis [19-22].

The adaptive immune system

While the innate immunity mediates a rapid response to infectious agents, the range of innate sensors recognizing them is limited. Consequently, an adaptive and more specific recognition system has evolved, providing a broader repertoire for detecting constantly mutating microbes. Also, after the initial exposure, the adaptive immune response develops memory cells with the capacity of mediating a rapid and enhanced response upon re-exposure to the same pathogen [23].

Cells of the adaptive immune system include thymus-derived T lymphocytes and bone marrow-derived B lymphocytes. During their development, they acquire T cell receptors (TCRs) and B cell receptors (BCRs), respectively, which enable them to recognize a wide range of antigens. The receptors are generated by random combinations of gene segments that are both variable and constant, giving rise to a wide T and B cell repertoire [2]. Following their development in the thymus and bone marrow, T and B cells migrate to secondary lymphoid organs for activation and proliferation. Activation is initiated upon binding of antigens by TCRs and BCRs, presented by professional APCs, in particular DCs, that have migrated to secondary lymphoid organs. The lymphocytes then migrate to the sites of infection and work together with the innate immunity to eliminate the pathogen [23]. There are two types of adaptive immune responses: the humoral response involving antigen-specific

antibodies produced by plasma cells (terminally differentiated B cells), and the cell-mediated response mediated by T cells, which destroys infected cells [24].

T cells

T cells can be divided into two main subsets based on their expression of either cluster of differentiation (CD) 8 or CD4 [25]. $CD8^+$ T cells interact with antigen peptides presented by MHC class I molecules expressed by all nucleated cells, whereas $CD4^+$ T cells interact with antigens presented by MHC class II molecules mainly restricted to APCs. The MHC proteins are encoded by the human leukocyte antigen (HLA) system including class I genes (HLA-A, HLA-B, and HLA-C) and class II genes (HLA-DR, HLA-DQ, and HLA-DP) [23]. In addition to antigen stimuli (signal 1), co-stimulatory signaling is required for T cell activation, involving the interaction of CD80/CD86 on APCs with CD28 on T cells (signal 2), as well as inflammatory cytokines (such as IL-12 or IFNα/β) (signal 3) (Figure 2). The co-stimulatory signal is finely tuned by inhibitory receptors including cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD1) [26].

Following these stimulating events, the production of IL-2 is induced and $CD8^+$ T and $CD4^+$ T cells differentiate into functionally distinct effector populations. Naïve $CD4^+$ T cells, also known as T helper (Th) cells, develop into either Th1, Th2, or Th17 depending on the surrounding cytokine milieu mediated by innate immune cells. IL-12 results in differentiation toward Th1 cells, which support cell-mediated responses in which they secrete IFNγ and IL-2 to activate macrophages and $CD8^+$ T cell development (Figure 2). IL-4 induces differentiation towards Th2 cells that stimulate humoral and allergic responses by releasing IL-4, IL-5, IL-10, and IL-13 [23, 24, 27, 28]. Transforming growth factor-beta (TGF-β) and IL-6 promote the development of Th17 cells, which secrete IL-17 important for protection against extracellular fungi and bacteria. Notably, Th17 cells are known to be implicated in the disease progression of several autoimmune disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriasis [29, 30]. In addition to Th17 cell development, TGF-β, accompanied by IL-2, induces differentiation towards regulatory T cells

(Tregs), critical for regulating T-cell responses through anti-inflammatory cytokines, such as IL-10 [31].

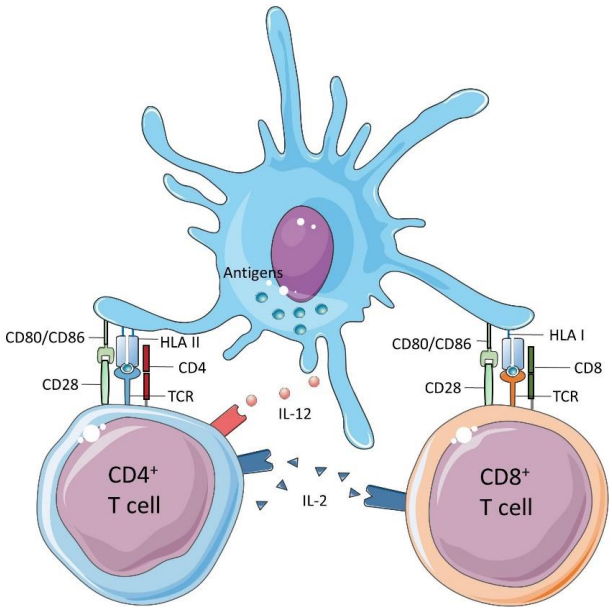

Figure 2. A simplified schematic of T cell activation. Extracellular antigens are taken up by an APC, in this figure a DC, and presented to $CD4^+$ T cells via class II MHC molecules and $CD8^+$ T cells via class I MHC molecules (Signal 1). For proper T cell activation, co-stimulatory signals are required, involving the interaction of CD80/CD86 on the APC with CD28 on the T cells (Signal 2), as well as inflammatory cytokines produced by the APC (Signal 3). Release of cytokine IL-12 will push the $CD4^+$ T cell into the Th1 type, which, in turn, will secrete cytokines, such as IL-2, to support T cell proliferation and differentiation. Figure produced using Medical Servier Art.

Activation of naïve $CD8^+$ T cells results in the conversion to cytotoxic T cells that are essential for defeating pathogens (mostly viruses) in the cytosol of infected cells. Once they are activated, they produce IFNγ to stimulate their cytotoxicity and motility, enabling them to efficiently colonize sites of inflammation [32]. Importantly, IFNγ production is associated with several autoimmune diseases including SLE and AAD [33-35]. Following migration to the site of inflammation, cytotoxic T cells recognize antigens presented by MHC class I molecules on the surface of target cells and kill them by mediating the coordinated action of perforin and granzymes. Perforin

generates pores in the membrane of the target cell, allowing granzymes to enter and activate a caspase cascade. Ligation of Fas-ligand on the cytotoxic T cell to Fas receptors on the target cell can also induce apoptosis, although this mechanism functions mainly to delete activated Fas-expressing lymphocytes after an infection has been cleared, to maintain lymphocyte homeostasis [23, 36].

B cells and antibodies

The adaptive humoral response is mediated by antibody-producing plasma cells developed from B cells. In the bone marrow, B cells pass through several differentiation stages involving rearrangements of the genes encoding antibody, or immunoglobulin (Ig), heavy and light chains to become IgD and IgM-expressing cells. This stage does not require contact with exogenous antigens and is therefore called antigen-independent B cell development [23]. In the next phase, B cells migrate to secondary lymphoid organs to finalize their Ig repertoire. This development stage is mainly dependent on the ability of B cells to function as APCs. The IgM and IgG receptors capture antigens and internalize them for further presentation by MHC class II molecules on the B cell surface. When the B cell then interacts with a T cell, in particular a Th cell specific for the MHC class II/antigen, the T cell starts producing cytokines to promote B cell proliferation. Subsequent interaction between CD40L on T cells and CD40 on B cells promotes differentiation of B cells into short-lived - or memory plasma cells, as well as Ig class switching from IgM and IgD to IgG, IgA or IgE, depending on the surrounding cytokine milieu [23, 27].

Immunological tolerance

Cells of the adaptive immunity are shaped to recognize and neutralize an infinite number of pathogens. Inevitably, some of their receptors may also react to host components. T and B cells must therefore undergo immunological tolerance enabling them to discriminate between self and non-self. There are two mechanisms by which immunological tolerance occurs: i) during lymphocyte maturation in the primary lymphoid organs (central tolerance); ii) during lymphocyte-antigen interactions in the

secondary lymphoid organs (peripheral tolerance). If these mechanisms fail, lymphocytes may start to attack cells of our own body resulting in autoimmunity [37, 38].

Central tolerance

The process of central B cell tolerance is thought to be less complex than the one for T cells. Immature B cells that display self-reactive receptors in the bone marrow either undergo apoptosis or get the chance to rearrange their receptors' specificity to become less self-reactive, resulting in survival [38]. On the other hand, T cells are required to pass through two tolerance mechanisms: positive- and negative selection. Positive selection represents the first step where only double-positive T cells ($CD4^+CD8^+$) showing a defined affinity to self MHC molecules survive. Those without any affinity, constituting 90% of all double-positive cells, undergo a death pathway called "death by neglect" [39]. Following positive selection, double-positive T cells differentiate into single-positive cells (either $CD4^+$ or $CD8^+$) before subjected to negative selection in the thymic medulla. Here, they encounter a variety of self-antigens by interacting with medullary stromal cells including medullary thymic epithelial cells (mTECs) [40]. By utilizing promiscuous gene expression, mTECs and other APCs in the thymus express and present numerous peripheral tissue-specific antigens [41, 42]. These antigens represent diverse tissues such as the lung, heart, stomach, and kidney, and their expression is regulated by the key transcription factor "autoimmune regulator" (AIRE). AIRE is therefore a critical regulator of central tolerance, and deficiency causes autoimmune destruction of several, mostly endocrine, organs manifesting as the syndrome autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also known as autoimmune polyendocrine syndrome type-1 (APS-1) [43, 44]. Besides this well-established role for AIRE in negative selection, there is increasing evidence that AIRE also promotes thymic Treg development [45]. A recent study of Aire-deficient mice showed that AIRE promotes the generation of $Foxp3^+CD4^+$ Tregs in the perinatal period [46]. Given this influence, it has been speculated that Treg defects caused by AIRE deficiency might contribute to autoimmunity [45]. However, this hypothesis requires further testing.

After surviving both positive and negative selection, the remaining $CD4^+$ and $CD8^+$ T cells expressing TCRs without high affinity for self-peptides, migrate to the periphery and secondary lymphoid organs where they have the potential to interact with their cognate antigens presented by APCs [37].

Peripheral tolerance

Although central tolerance is thought to be efficient, some self-reactive lymphocytes manage to escape deletion in the primary lymphoid organs. Thus, peripheral tolerance has evolved to serve as a second elimination process in the periphery. This mechanism is based upon the principle that any escaping self-reactive lymphocyte would need to find a cooperating partner for sufficient co-stimulation and activation. In the absence of such cooperation, autoreactive lymphocytes enter an inactive state, known as anergy, which remains even if they re-encounter the same antigen under appropriate stimuli.

For autoreactive B cells, anergy is induced when they interact with self-antigens without finding a cooperating Th cell. In this condition, B cells are outcompeted by other B cell clones and become excluded from entering the lymphoid follicles to receive survival factors [37, 47].

When self-reactive T cells escape negative selection, peripheral tolerance is promoted by at least three distinct mechanisms: i) anergy; ii) deletion by activation-induced cell death; iii) immune suppression by Tregs [37]. Anergy is induced, as previously mentioned, when the TCR encounters antigens in the absence of co-stimulation. It can also be induced by binding of CD80/CD86 on APCs to CTLA-4 on T cells, which transmits an inhibitory signal [48, 49]. The second mechanism, deletion by activation-induced cell death, is induced when Fas (CD95) and FasL (CD95L) on T cells interact after repeated stimulation of the TCR [50]. The last peripheral tolerance process is mediated by Tregs, which suppress T cell responses either in a contact-dependent manner or by producing immunosuppressive cytokines such as TGF-β or IL-10 [51]. Tregs are therefore important for maintaining self-tolerance, which is underlined by previous research showing that decrease in their numbers and function is associated with several autoimmune disorders such as SLE, multiple sclerosis (MS), type 1

diabetes (T1D), APS-1, RA, and immune dysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome [52-54].

Autoimmunity

From the brief overview of immunological tolerance above, it appears that autoimmunity is a result of defective elimination of self-reactive lymphocytes. Once some of these autoreactive lymphocytes are activated, multiple parts of the immune system may get involved in attacking host tissue, leading to either systemic or organ-specific autoimmune disorders. So far, more than 80 autoimmune disorders have been described, and their incidence and prevalence are still rising [55]. Some of them seem to have shared, yet complex, genetic bases and etiology pathways as they often tend to co-occur within individuals and families [56]. However, they may only develop after exposure to certain environmental factors, making them challenging to study and fully understand.

Genetic factors

Several genetic risk factors for autoimmunity have been identified, but their precise contribution is often hard to specify. Nonetheless, a few autoimmune disorders are induced by single gene alterations, making them easier to study and diagnose. One classic example is the unique disorder APS-1 caused by both autosomal recessive mutations and dominant-negative mutations in *AIRE* [44, 57, 58]. Another example is the autoimmune lymphoproliferative syndrome, also known as the Canale-Smith syndrome, caused by impairment of Fas-induced apoptosis, due to mutations in the *FAS* gene or other genes involved in apoptosis [59]. The IPEX syndrome is also a monogenic autoimmune disease, caused by mutations in forkhead box P3 (*FOXP3*) that regulates the development and suppressive activity of Tregs [60, 61].

Apart from these disorders, most autoimmune diseases are polygenic, resulting from multiple susceptibility variants in many genes. The best-characterized genetic component so far is mapped to the HLA locus encoding class I or II MHC molecules.

Several associations between polymorphisms in the HLA complex and autoimmune diseases have been described. For instance, the haplotypes *DRB1*03:01-DQA1*05:01-DQB1*02:01* and *DRB1*04:04-DQA1*03:01-DQB1*03:02* (DR3-DQ2 and DR4-DQ8) are shown to confer risk to both celiac disease (CD), T1D, autoimmune thyroid disease (AITD), and AAD, explaining why these diseases tend to co-occur within individuals [43, 62, 63]. CD is nonetheless considered one of the most evident HLA-associated diseases as it occurs selectively in individuals expressing the DQ2 or DQ8 haplotypes [64, 65]. In conjunction, specific HLA alleles seem also to be involved in drug-induced autoimmunity by abacavir, an anti-retroviral medication used to treat human immunodeficiency virus (HIV). In treated individuals expressing the HLA-B*57:01, abacavir seems to alter the binding cleft of the HLA class I molecule, increasing self-epitope binding. This leads to autoreactive T cell responses and abacavir hypersensitivity reactions [66, 67].

The role of certain HLA alleles in the pathogenesis of autoimmunity is most probably related to their antigen-presenting capacity; either by enhancing peptide-presentation in the periphery, leading to increased activation of T cells or by an insufficient presentation of self-antigens in the thymus, resulting in more self-reactive T cells or fewer Tregs [68, 69]. Apart from the HLA locus, several other autoimmune susceptibility genes have been identified, encoding cytokines, costimulatory molecules, molecules involved in promoting apoptosis, members of cytokine- or antigen-signaling cascades, and molecules that clear antigen or antigen-antibody complexes [70]. None of these genetic risk factors, however, can alone induce autoimmunity as it is the overall genetic background of the host that determines the probability of disease development [70].

Autoimmune susceptibility genes can also increase immunoreactivity, but they may not necessarily lead to an improved immune response against pathogens. In fact, individuals with autoimmune disorders show increased susceptibility to infections, and manifestations of autoimmunity may only occur after exposure to certain pathogens [71, 72]. This is mirrored by the high degree of genetic overlap between autoimmune disorders and monogenic primary immunodeficiency diseases (PIDs). Variations in the

PID genes including *AIRE, STATs, FOXP3, LRBA* (lipopolysaccharide responsive beige-like anchor), and *CTLA4* are reported to be involved in the immune dysregulation of several autoimmune diseases including APS-1, IPEX, T1D, RA, vitiligo and AITD [73, 74]. Patients with PID caused by mutations in these genes are also prone to develop autoimmune diseases. Therefore, a major reason for autoimmunity could be the lack of clearance of microbial antigens, implying the impact of environmental triggers.

A useful tool for studying the relative contribution of genetic and environmental factors in autoimmune diseases is twin studies. In CD and AAD, the concordance rates among monozygotic twins are 75-86% [75, 76] and 71% [77] respectively, whereas RA and systemic sclerosis have less concordance [78], suggesting higher involvement of environmental factors. Moreover, a true twin concordance rate may only develop after several years, meaning that monozygotic twins have different disease onsets, also suggesting the involvement of non-genetic factors [78]. Another compelling evidence for an environmental contribution is the genotype-independent geographic variation of β-cell autoimmunity involved in T1D [79].

Environmental factors

The well-known environmental factors associated with autoimmunity include infections, vaccines, hormones, chemical exposures, drugs, diet, and cigarette smoking [80-82]. Among these, the most compelling and studied one is infection caused by viruses, bacteria, and other pathogens [83]. Several mechanisms of how infections induce autoimmune responses have been proposed. The pathogen may carry epitopes that are similar to the host's antigens, a phenomenon termed "molecular mimicry". Lymphocytes that react to such epitopes may then also react to self-antigens, resulting in tissue damage and activation of other parts of the immune system. Another mechanism is "epitope spreading", whereby the host tissue is damaged by the immune response to a persistent infection, and/or by necrosis caused by the pathogen itself. Self-antigens are then released, leading to local activation of APCs and presentation of self-peptides that further activate autoreactive lymphocytes. Activation and stimulation of these lymphocytes may also be indirect or non-specific, caused by inflammation

present during an infection, a mechanism called “bystander activation” involving “cryptic antigens” [84]. In contrast to dominant antigens, cryptic antigens are usually invisible to the immune system as they appear in low concentrations. Upon inflammation, however, their concentration can increase due to elevated production of proteases and differential processing of released self-peptides by APCs [85, 86]. This creates the opportunity for autoreactive lymphocytes to develop.

The role of infection underlying autoimmune responses has especially been assigned to T1D, MS, and autoimmune liver disease or autoimmune hepatitis (AIH) [87-89]. Concerning T1D, previous research has demonstrated the presence of enteroviruses (EV) in pancreatic islets from patients [89]. Epidemiological studies have also reported more EV infections in T1D patients than in controls [89, 90]. Regarding MS, several epidemiological and immunological studies indicate an association with Epstein-Barr virus (EBV) [91-96]. However, the results remain controversial; some researchers provide evidence for the expression of EBV in B cells in inflamed post-mortem brain tissue [91], while others do not detect EBV-positive cells, or at least in very few patients only [97-100]. In patients with AIH, previous research demonstrates the presence of antibodies against hepatitis C virus (HCV), in addition to a type of autoantibodies called liver/kidney microsomal antibody type 1 (LKM-1) in patients with chronic HCV infection [87]. Notably, many of the infections associated with autoimmune disorders are opportunistic, primarily affecting individuals who cannot efficiently eradicate the virus, leading to persistent proinflammatory conditions.

Impaired immunity and increased susceptibility to infections have also been demonstrated in patients with AAD in terms of reduced NK cell cytotoxicity and poor responses to IFNs in-vitro [101, 102]. These findings are mirrored by studies reporting that AAD patients have a higher intake of antibiotics and antivirals, both before and after the diagnosis, compared to the general population [103, 104].

Autoimmune Addison's disease

While Addison's disease during the last century was predominantly caused by tuberculous infiltration of the adrenal glands, the autoimmune form currently dominates in developed countries where it is responsible for approximately 80% of the cases in adult patients [83, 105, 106]. AAD is a classic organ-specific autoimmune disease characterized by an immune-mediated attack on the hormone-producing cells in the adrenal cortex, while the adrenal medulla remains intact. Destruction of the adrenal cortex leads to deficiency of the vital steroids cortisol and aldosterone, and patients with AAD therefore depend upon lifelong supplementation therapy [1, 107].

The adrenal cortex

The adrenal gland is a hormone-producing organ, composed of two cell layers with distinct embryological origins. The inner adrenal layer (medulla) arises from the ectoderm lineage and produces catecholamines (such as epinephrine and norepinephrine), whereas the outer adrenal cortex derives from intermediate mesoderm and converts cholesterol into various bioactive steroid hormones (Figure 3 and 4). The adrenal cortex consists of three separate zones, whereby each produces different steroids mediating stress response and regulation of blood pressure. The zona glomerulosa forms the outermost layer and produces the mineralocorticoid aldosterone, important for the maintenance of blood pressure. Zona glomerulosa is under the control of the renin-angiotensin system, which is regulated by blood pressure and changes in sodium and potassium levels. The next cell layer constitutes zona fasciculata that synthesizes glucocorticoids like cortisol under stimulation by the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol induces gluconeogenesis and anti-inflammatory processes during stress. The innermost zona reticularis secrete androstenedione and dehydroepiandrosterone (DHEA), which can further be converted to sex hormones in peripheral tissues [108, 109].

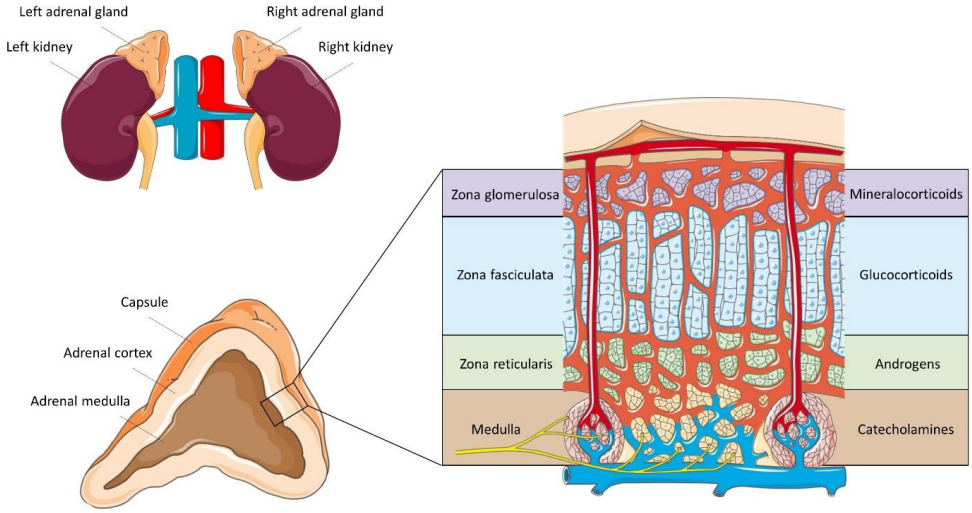

Figure 3. Adrenal anatomy. The adrenal glands are located on top of the kidneys and consist of the outer adrenal cortex and the inner adrenal medulla. The adrenal cortex is subdivided into three distinct layers: Zona glomerulosa, producing mineralocorticoids, zona fasciculata, secreting glucocorticoids, and zona reticularis, producing androgens. The inner medulla synthesizes catecholamines including epinephrine and norepinephrine. Figure produced using Medical Servier Art.

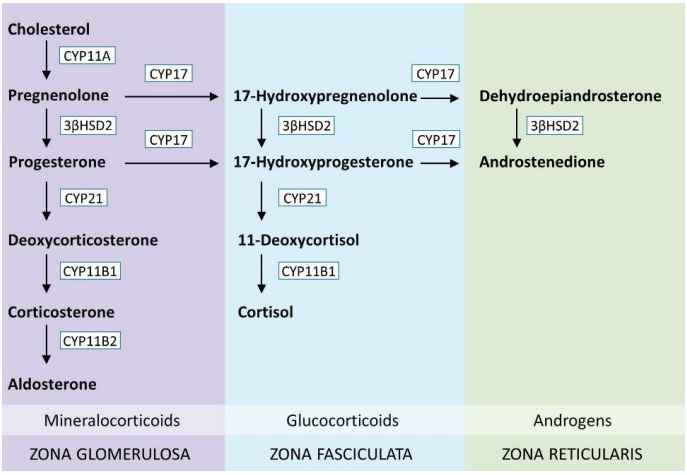

Figure 4. Steroidogenesis in the adrenal cortex. Cholesterol is converted to aldosterone, cortisol, and androgens through different pathways that require the specific enzymes cholesterol side-chain cleavage enzyme (CYP11A), 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), 21-hydroxylase (CYP21), 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2).

Clinical characteristics

AAD is a relatively rare disorder with a prevalence in European populations ranging from 93-220 cases per million [105, 110-114]. Like other autoimmune diseases, AAD affects more women (female: male ratio 1.5-3.5:1) and can occur at any age, although it generally affects individuals between 30 to 50 years of age [105]. Patients have insufficient production of the adrenal steroids due to the autoimmune attack of the adrenal cortex. The pathological changes can develop slowly over many years before the clinical manifestations appear, which include fatigue, nausea, dizziness, weight loss, skin hyperpigmentation, and salt-craving [105, 107, 115]. Hyperpigmentation is due to the absence of the cortisol-mediated negative feedback mechanism of the HPA axis response, leading to increased production of adrenocorticotropic hormone (ACTH) and pro-opiomelanocortin (precursor of ACTH), which enhances stimulation of the melanocortin 1 receptor of skin melanocytes [116, 117]. Salt-craving is caused by aldosterone deficiency, which impairs the kidney's ability to reabsorb salt, causing low blood pressure. This increases the activity of renin to activate the protein hormone angiotensin that, in turn, tries to stimulate the release of aldosterone by the adrenals [115, 118]. Elevated ACTH levels and plasma renin activity are therefore important clinical characteristics of AAD. To correct the insufficient levels of cortisol and aldosterone, patients are treated with hydrocortisone and fludrocortisone, respectively [115].

The dominant target of the adrenal autoantibodies seems to be the steroid cytochrome P450 21-hydroxylase (21OH) and has ever since its discovery in 1992 been the important diagnostic marker of AAD [119]. 21OH is located in the smooth endoplasmic reticulum (ER) of adrenocortical cells and are involved in the synthesis of cortisol and aldosterone (Figure 4) [120]. Autoantibodies against 21OH are reported to be present in >95% of patients at diagnosis in developed countries [121] and 86% of all Norwegian patients [122]. This high frequency, however, tends to decline with a disease duration greater than 20 years. Furthermore, a previous study reported that 21OH autoantibody-positive patients with other autoimmune endocrinopathies or first-degree relatives of patients with organ-specific autoimmune

diseases have a cumulative risk for AAD of around 50% [123]. Thus, presence of these antibodies may predict AAD development. Among the general population, the frequency of 21OH autoantibodies is approximately 0.5% [124].

AAD may not only occur in isolation. Around 50% of AAD patients also suffer from other autoimmune entities such as T1D, AITD, premature ovarian insufficiency (POI), CD, and autoimmune gastritis [124]. The co-existence of at least two of the three endocrine diseases T1D, AITD, and AAD is typically referred to as APS-2 [43]. AAD is also frequently present in the unique disorder APS-1 (prevalence 1:90 000 in Norway) defined clinically by the presence of two out the three manifestations: AAD, hypoparathyroidism, and mucocutaneous candidiasis [125]. Whereas APS-1 is a monogenic disease, AAD (and APS-2) seem to have a more complex etiology involving multiple genes and unknown environmental factors [126].

The genetic basis of AAD

Investigating the genetic basis of complex diseases, such as AAD, is challenging due to several factors. The genotype of a patient does not necessarily correlate with the phenotype; phenocopies may occur, meaning that environmental factors could induce an AAD phenotype in a patient without known disease susceptible variants that matches the phenotype of a patient that does carry such variants. Additionally, AAD is a genetically heterogeneous disease in which variants in any one of several susceptibility genes may cause the same phenotype, and multiple of these may be necessary to produce it (polygenic inheritance). Lastly, AAD show incomplete penetrance, which means that certain individuals who inherit susceptibility alleles might not develop the disease [105].

Despite all these complicating factors, research on monozygotic twins and familial clustering of AAD have revealed the existence of highly penetrant susceptible alleles [105, 127, 128]. The best characterized seem to be located within the HLA class II haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8. Polymorphisms in these haplotypes enable the presentation of a different repertoire of self-antigens in the peptide-binding pocket. Notably, individuals carrying the heterozygous combination DR3-DQ2/DR4-

DQ8 are at higher risk than homozygotes carrying either one of the haplotypes (Figure 5) [113, 129]. This may be due to the formation of heterodimers by the *trans*-encoded DQ molecules, increasing the functional diversity of the MHC complex [130]. HLA class I genes have also been associated with AAD, more specifically those that are part of the conserved allele combination DR3-B8 (Figure 5) [62, 63]. These alleles are further part of the ancestral haplotype defined as 8.1 (AH 8.1) containing the core HLA alleles *HLA-A*01:01, -C*07:01, -B*08:01, -DRB1*03:01, -DQA1*05:01* and -*DQB1*02:01* [131]. Apart from AAD, AH 8.1 has been associated with a wide range of immune-mediated diseases including T1D, CD, SLE, AIH, common variable immunodeficiency (CVID), and IgA deficiency [132, 133].

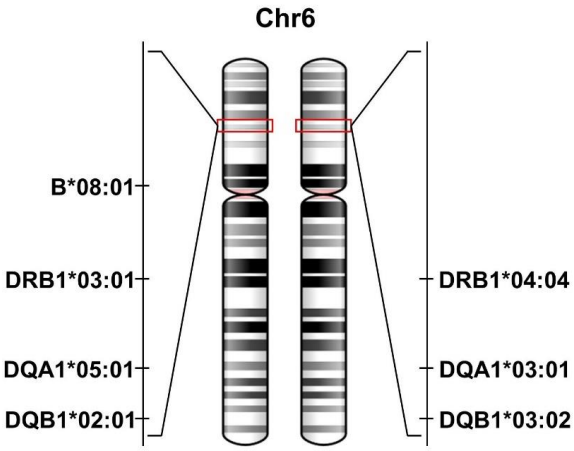

Figure 5. Combination of HLA alleles conferring high risk of AAD development. Figure produced using Genome Decoration Page (NCBI).

Single nucleotide polymorphisms (SNPs) in genes regulating T and B cell activation and differentiation are also associated with increased risk of AAD development (summarized in Table 1). Among these, SNPs in *CTLA4* and *PTPN22* (protein tyrosine phosphatase non-receptor type 22) appear frequently as genetic risk variants (also in other autoimmune disorders), underlining the importance of fine-tuning T cell signaling to avoid development of autoimmunity [134-137]. CTLA-4 downregulates T cell signaling by binding to CD80/86 on APCs, and gene polymorphisms impairing this mechanism may lead to reduced inhibition of T cell responses [138, 139]. PTPN22

inhibits T cell activation by dephosphorylating key signaling molecules downstream of the TCR. This process is dependent on the binding of PTPN22 to C-terminal Src kinase tyrosine kinase, and SNPs (in particular c.1858C>T, p.Arg620Trp) interrupting this physical interaction cause lack of T cell inhibition [138, 140, 141]. Other susceptibility variants are located in the genes encoding BACH2 (BTB domain and CNC homolog 2), important for B cell differentiation and generation of Tregs [129, 142]; NALP1 (NACHT leucine-rich-repeat protein 1), promoting inflammasome assembly and inflammatory cytokine release [143, 144]; and CLEC16A (C-Type Lectin Domain Containing 16A), presumably regulating autophagy and NK cell function [145]. Recently, variants in *AIRE* were also found to be associated with AAD, at least in the Swedish patient cohort [146]. This finding has yet to be replicated in other populations, although many AAD risk loci may differ between studies of different cohorts.

Table 1. Susceptible genes contributing to AAD, outside the MHC region.

Gene	Function	References
CTLA-4	Inhibits T cell responses	[134], [137]
PTPN22	Dephosphorylates signaling molecules downstream of the TCR, important for T cell inhibition	[135], [136]
BACH2	Promotes B cell differentiation and generation of Tregs	[129], [142]
NALP1	Promotes inflammasome assembly and inflammatory cytokine release	[143], [144]
CLEC16A	Regulates autophagy and NK cell function	[145]
AIRE	Master regulator of negative selection in the thymus	[146]

None of the risk genes described so far, except for HLA, seem to have a major impact on an individual's genetic susceptibility to develop AAD; they only explain a small fraction of familial clustering. An explanation for this missing heritability problem is that many of the available genotyping assays focus on SNPs present in more than 1-5% of the patient population [147]. However, the genetic architecture of AAD seems to be complex, involving multiple variants. An important step towards solving this missing heritability problem is therefore to search for rare and low-frequency variants with high impact. This strategy could illuminate the genetic variation in AAD, which is important for better prevention, diagnosis, and treatment of the disease.

Immunopathogenesis

The specific triggers underlying the progression of AAD from one phase to another are still not clear, but there have been considerable advances in our understanding of the pathogenesis over the past few decades.

Histologic studies of adrenal glands from patients with AAD show a significant mononuclear cell infiltration and atrophy of the adrenal cortex, in addition to some fibrosis, while the medulla is spared [148, 149]. These data suggest that during the active phase of the disease there is an extensive destruction and loss of all three cell layers of the adrenal cortex mediated by lymphocytes, plasma cells, and macrophages [150]. Since AAD is primarily associated with class II HLA alleles, it seems likely that initiation of adrenocortical destruction depends upon activation of $CD4^+$ T cells that, in turn, license autoreactive immune cell responses. As there is a potentially unlimited supply of self-antigens, this autoreactive immune attack may continue until the target organ is destroyed and replaced by fibrous tissue.

Irreversible damage of both zona glomerulosa and zona fasciculata is what causes the first clinical signs of adrenal insufficiency [105, 150]. Notably, this might not be until 90% of all adrenocortical cells are destroyed [151]. Before overt, symptomatic adrenal failure, however, autoantibodies against 21OH are typically present. Whether these autoantibodies are involved in disease progression is yet not clear, although it appears that they have no inhibiting effect on the 21OH enzyme *in vivo* [107, 150, 152]. This

is not that surprising since 21OH is an intracellular enzyme. Also, a previous study demonstrated that transplacental passage of 21OH autoantibodies from a mother with AAD was not sufficient to cause autoimmune adrenocortical destruction in the child [153]. Thus, the main mediators of the autoimmune destruction seem to be cytotoxic T cells, although the specific mechanisms remain unclear.

The first indication of T cell proliferation in response to adrenal proteins was provided by Freeman and Weetman, using peripheral blood mononuclear cells (PBMCs) from AAD patients [154]. However, no individual adrenal antigens were identified. Husebye et al. in contrast, used BALB/c and SJL mice, immunized with recombinant 21OH, to demonstrate proliferation of lymph node cells to a specific 21OH-derived epitope, $21OH_{342-361}$ [155]. A few years later, Bratland et al. showed T cell proliferation and IFN-γ production in response to the same epitope ($21OH_{342-361}$), using PBMCs from AAD patients [33]. Since then, epitope mapping has suggested two specific 21OH epitopes targeted by circulating autoreactive $CD8^+$ T cells. The first epitope, published by Rottembourg et al. [34], was mapped to the HLA-B8 restricted sequence EPLARLEL ($21OH_{431-438}$) after performing an ELISPOT assay to detect IFN-γ producing T cells in response to 21OH peptides of 20 amino acids length. Subsequently, using MHC-peptide multimer technology, two responder patients were shown to have higher frequencies of EPLARLEL-specific $CD8^+$ T cells than a HLA-B8-positive control. A few years later, a second epitope was revealed by Dawoodji et al. [156]. This one was mapped to the HLA-A2 restricted sequence LLNATIAEV ($21OH_{342-350}$). T cells specific for this sequence were reported to have the ability to lyse cells pulsed with the LLNATIAEV-peptide, by granzyme B secretion. Together, these studies suggest a central role for 21OH-specific T cells in the pathogenesis of AAD and open up new avenues for research into the features of these cells.

The major and unanswered question of the immunopathogenesis is what causes the lymphocytic infiltration of the adrenal cortex. This issue is challenging to address because all histochemical analyses of adrenal glands from AAD patients are post-mortem due to practical and ethical reasons. Also, the experimental animal models developed so far, have shown limited relevance to the human AAD [105]. Most studies

on the pathogenesis of AAD have therefore been restricted to peripheral blood and serum. Interestingly, evidence of lymphocytic infiltration in the adrenal gland has not only been limited to AAD patients. A previous study on autopsy cases of young and old individuals revealed that approximately 7% of the subjects less than 50 years had mononuclear cell infiltration within the adrenal cortex, and this frequency increased with age [157]. These infiltrations are most likely not significantly pathogenic, as the prevalence of AAD is far more rare, but it might indicate that focal infiltration of the adrenals does occur. In such circumstances, a healthy adrenal gland is producing a sufficient amount of cortisol that would ensure an anti-inflammatory milieu, protecting the cortex from an autoimmune attack [158]. This protective and immunosuppressive effect of cortisol could then help explain why AAD is a rare condition compared to other autoimmune endocrine disorders [105]. In that case, a key step in the pathogenesis of AAD could therefore be a reduction of cortisol at sites of infiltration (e.g. due to adrenocortical damage) which may reduce tolerance to adrenal proteins and lead to activation of lymphocytes. As a result, steroid production decreases along with increased infiltration, resulting in a vicious cycle of adrenocortical cell damage and steroidogenesis impairment. Together, these events may trigger an autoreactivity to adrenal antigens, eventually leading to a complete adrenal failure [105]. The exact mechanisms underlying this selective autoimmune attack and reduced cortisol production in AAD patients remain elusive, but it has been postulated that virus infection of the adrenal gland is a potent trigger [83].

Viruses as triggers of the autoreactive immunity

The coordinated attack of the adrenocortical cells, involving $CD4^+$ T cells, $CD8^+$ T cells and antibody-producing B cells, driven mainly by the self-antigen 21OH, is highly reminiscent of the specific immune response against viruses. In this context, we could speculate that viral infection of the adrenal gland leads to necrosis of adrenocortical cells, resulting in the release of self-antigens and augmentation of lymphocytic infiltration, causing inflammation of the adrenal cortex. This may further lead to uptake of self-antigens and/or viral peptides by APCs. Since 21OH and other steroid enzymes are among the most abundant proteins in the adrenal cortex, they are likely to be

presented as peptides on MHC molecules. Release, uptake, and presentation of self-antigens (epitope spreading) can further activate autoreactive T and B cells, and thereby eventually lead to autoimmune adrenalitis (Figure 6) [83].

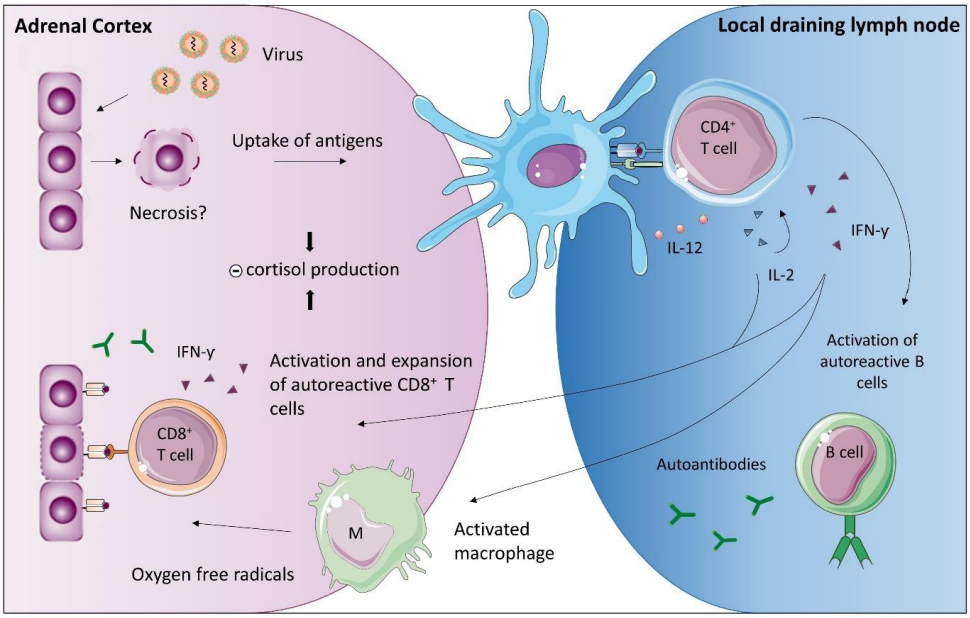

Figure 6. A potential viral pathogenic mechanism in AAD. A simplified model of how virus infection of the adrenal cortex may lead to activation of autoreactive immune cells and adrenal insufficiency. First, persistent viral infection causes necrosis of adrenocortical cells and subsequently release, uptake, and presentation of self-antigens by APCs. Secondly, autoreactive T cells get activated by self-antigen presentation within local draining lymph nodes, which, in turn, license the activation of macrophages (M) and autoreactive B cells. Further necrosis and apoptosis of adrenocortical cells cause decreased cortisol production, increasing the lymphocyte infiltration in the adrenal cortex, eventually leading to AAD. Figure produced using Medical Servier Art.

This hypothetical model for AAD development is supported by previous studies showing that the adrenals are permissive to a variety of viral agents including cytomegalovirus (CMV), EBV, herpes simplex virus types 1 and 2 (HSV-1/HSV-2), HCV and polyomaviruses [83]. Most of these viruses cause opportunistic infections and usually do not lead to autoimmunity, suggesting the requirement of additional host genetic errors and immunodeficiencies. Indeed, this seems to be the case for AAD

patients, as previously mentioned [101-103, 159]. Viral pathogenic mechanisms are also suggested for other complex immune-mediated diseases like asthma, T1D, and Crohn's disease [160]. Unlike these diseases, however, no specific pathogens have been identified for AAD, highlighting the need for research on viral exposure in affected patients. In this way, we may gain a better understanding of the pathogenesis, and thereby design therapies to protect susceptible individuals with inappropriate antiviral responses.

Aims

The overall objective of this project was to identify and functionally characterize genetic risk factors involved in AAD pathogenesis. To fulfill this aim, we used a two-sided strategy: First, we validated and characterized specific mutations revealed by whole-exome sequencing (WES) of 142 AAD patients. Since AAD is a rare condition with a complex genetic basis, we set out to search for and characterize rare genetic variants (allele frequency <1% in the general population) predicted to- or previously demonstrated to be damaging, which could make up for the missing heritability problem in AAD. Secondly, we aimed to elucidate genetic and immunological factors that influence disease progression from one phase to the other. For this, we focused on HLA-specific immunological determinants for autoreactive T cells, as these cells are thought to be the main driver of adrenal gland destruction in AAD.

The specific aims were:

I. Validate and characterize rare *TLR3* variants identified in several unrelated patients.

II. Investigate clinical aspects of a patient carrying a rare homozygous nonsense mutation in the *HSD3B2* gene.

III. Characterize autoreactive $CD8^+$ T cells from AAD patients to identify HLA-class I restricted immunodominant peptides of 21OH.

Methodology

In this section, the most central parts of the methodology used in the thesis will be discussed. A detailed description of the materials and methods can be found in the separate papers.

Patient and control material

Through the Norwegian Registry of organ-specific autoimmune diseases (ROAS), we have access to serum, plasma, EDTA blood, DNA and, PBMCs from almost 1000 AAD patients. All have signed a written informed consent approved by the Health Region West Ethics committee (2018/1417, 146/96-47·96). Samples from healthy controls were collected from blood donors provided by the blood bank at Haukeland University Hospital. All experiments were conducted following the declaration of Helsinki.

Reporter gene assay (paper I)

To determine the functional impact of the *TLR3* variants revealed by WES, we studied the response to polyinosinic:polycytidylic acid (poly(I:C)) stimulation in cells transfected with constructs encoding the wild type or variant TLR3 proteins. This was achieved using Hek Dual Null cells, which are stably transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene induced by NF-kB. As the endogenous *TLR3* gene is specifically knocked out, all expression and activity of the receptor are therefore dependent on transfected TLR3. Following transfection, we assessed TLR3 activity by monitoring NF-kB-induced SEAP production after poly(I:C) stimulation, which was measured by spectrophotometry in the cell supernatants after adding QUANTI-Blue solution.

MHC class I/peptide multimers (paper III)

The MHC multimer technology is a highly useful tool to detect and quantify antigen-specific T cells from PBMCs or other sources/tissues. Recombinant MHC molecules are loaded with peptides of antigen proteins, which can be recognized by cytotoxic T cells. Several of these MHC-peptide complexes are attached to a fluorescent backbone that can be detected and quantified by flow cytometry [161].

In *paper III,* we used MHC class I/peptide multimers (dextramers and streptamers) containing either HLA-A2-, HLA-B8- or HLA-C7-bound peptides. For details about the HLA-A2/B8-dextramers, see paper III. The MHC I-streptamers consisted of an HLA-C7-peptide complex attached to strep-tags, which are short peptides with binding affinity to a streptavidin molecule, named Strep-Tactin. Strep-Tactin molecules are fluorescently labeled, enabling quantification by flow cytometry.

HLA-C typing (paper III)

Since the HLA types of all the patients included in *paper III* were already estimated by allelic imputation by a genome-wide association study (GWAS) [162], we needed to find a quick and reliable way to HLA-type healthy controls. We decided to perform a PCR using sequence-specific primers for HLA-C7, published by Bunce et al. [163]. PBMCs and DNA from 51 healthy controls were then collected for HLA-typing, whereby 30% turned out to be positive for the HLA-C*0701 allele and thereby included in the study.

Results

Paper I:

Identification and characterization of rare Toll-like receptor 3 variants

From the results revealed by the WES analysis including 142 AAD patients, a total number of 135 variants with CADD (Combined Annotation Dependent Depletion) scores >20 in 76 different candidate genes were detected. CADD is an *in silico* variant pathogenicity predictor, whereby scores above 20 indicate that a variant is amongst the top 1% of deleterious variants in the human genome [164].

Searching for rare and potentially damaging variants, we identified several interesting hits in the *TLR3* gene: p.Thr59Asn (T59N), p.Gly221Arg (G221R), p.Phe351Ile (F351I), p.Leu742Phe (L742F) and p.Arg867Gln (R867Q). These were carried by five unrelated AAD patients. According to our in-house database, the variants were absent in Norwegian controls, whereas their allele frequencies in a cohort of Norwegian cancer patients were ranging from 0 to 0.0019. Similar frequencies were found in the genome aggregation database (gnomAD), ranging from 0 to 0.0017. Intriguingly, the F351I variant, detected in two unrelated AAD patients, is only reported once before (in Finland) according to gnomAD. R867Q has been described in a homozygous state in a Finnish patient with HSV encephalitis (HSE) [165]. Functional analysis indicated that this mutant is hypomorphic, causing a partial loss of TLR3's function. T59N has also been described in a patient with HSE but did not show any effect on TLR3's function when tested *in vitro* [166]. Using Sanger sequencing to validate the variants, we subsequently discovered that three of the five patients also carried a more common *TLR3* variant (p.Leu412Phe [L412F]), previously associated with a variety of immune-mediated diseases including cutaneous candidiasis, tick-borne encephalitis and increased susceptibility to CMV infection [167, 168]. L412F has also been associated with autoimmunity including T1D and AAD, although the data are conflicting [167, 169, 170].

With one of the patients carrying both T59N and F351I, these findings revealed that four of the five patients had two variants each. Genotyping of the patients showed that all were compound heterozygotes, including one patient that was homozygous for L412F and heterozygous for R867Q. We further genotyped family members when available, revealing that three of the patients had at least one sibling that was heterozygous for either one of the two *TLR3* variants, and some also carried both. All these siblings were healthy, except for one who also had AAD (heterozygous for T59N).

In silico analysis of the variants demonstrated that F351, L412, L742, and R867 were located at highly conserved residues in the 18 species studied, suggesting that these four variants would be the most damaging. We further tested the functional impact of the *TLR3* variants by studying the response to poly(I:C) in Hek Dual Null cells transfected with constructs encoding the wild type or variant TLR3 proteins. Consistent with the *in silico* analysis, F351I, L743F, and R867Q proved to be the most deleterious variants, reducing TLR3 activity by ~ 50-70%.

For three of the patients carrying the damaging variants, PBMCs were available for experiments. By performing ELISA, we demonstrated an impaired immune response to the dsRNA analog polyadenylic:polyuridylic acid (poly(A:U)) in all three patients compared to healthy controls, as revealed by a significant loss in the production of IL28A/IFNλ2. IFNλs are among the first IFNs produced to suppress viral infection and are induced in a wide range of cell types by PRRs including the TLRs [171, 172].

In addition to *TLR3* variants, the WES analysis identified seven possibly damaging variants (both novel and previously described) among eight additional AAD patients in three different innate immune genes (*IRF3, TICAM1* [TRIF] and *IKBKE* [IKKε]). All are involved in the TLR3-IFN signaling pathway. Interestingly, one of the detected *IRF3* variants was the loss-of-function mutation p.Arg285Gln (R285Q) previously reported and extensively functionally characterized in a patient with HSE [173]. Likewise, one of the *TICAM1* variants (p.Ser160Phe [S160F]), carried by two patients, has also been associated with HSE [166].

In summary, we discovered 13 AAD patients with inborn errors in genes involved in the innate immune response against viruses.

Paper II:

Coexistence of congenital adrenal hyperplasia and autoimmune Addison's disease

Among the many variants revealed by the WES analysis, we also identified a patient homozygous for an extremely rare variant in exon 2 of the *HSD3B2* gene (frequency ~0.00003 in gnomAD), resulting in the exchange of the cysteine codon to a premature stop codon (p.Cys5Ter [C5X]). This leads to 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) deficiency, which is a rare autosomal recessive form of congenital adrenal hyperplasia (CAH) [174]. Subsequent screening of ROAS identified the patient carrying the C5X variant; an adult female with AAD, POI, vitamin B12 deficiency, elevated ACTH levels on multiple occasions, and circulating 21OH- and parietal cell autoantibodies. She did not carry any of the risk HLA alleles associated with AAD. Scrutiny of old medical records revealed that she was initially diagnosed with CAH with hyperandrogenism and severe salt-wasting shortly after birth.

To study her steroid profile, liquid chromatography-tandem mass spectroscopy (LC-MS/MS) was performed of a serum sample taken after an overnight medication fast. Levels of all mineralocorticoids, glucocorticoids, and androgens were below the detection limit, except for tetrahydrocortisol (0.308 nmol/L), 5α-tetrahydrocortisol (0.187 nmol/L), and testosterone (0.066 nmol/L).

Isolated 3βHSD2 deficiency causes accumulation of the Δ5-steroids pregnenolone, 17-hydroxypregnenolone, DHEA, and DHEA-sulfate (DHEA-S) [174]. We therefore measured total pregnenolone, DHEA, and DHEA-S levels by ELISA from previously collected serum samples spanning the years 1996-2013. Nearly normal levels of pregnenolone and DHEA were found in samples from the first two years, but then levels decreased towards the subnormal levels generally seen in AAD patients. The level of DHEA-S was below the normal range, typical of AAD patients, at all time

points. Computer tomography (CT) and magnetic resonance (MR) scans, taken from age 43 to age 51, were re-evaluated and the adrenals were found to be in the lower range of normal thickness, 2-3 mm., indicating adrenocortical atrophy rather than hyperplasia as would be expected in case of isolated CAH.

To the best of our knowledge, these findings constitute the first description of AAD in a patient with 3βHSD2 deficiency.

Paper III:

Circulating 21-hydroxylase-specific CD8$^+$ T cells in autoimmune Addison's disease are predominantly restricted by HLA-A2 and HLA-C7 molecules.

While autoantibodies against 21OH have long been established for AAD patients, experimental data point to autoreactive T cells as the main effector cells in the adrenocortical destruction. So far, two immunodominant T cell epitopes of 21OH have been proposed. The first epitope, provided by Rottembourg et al. [34], was mapped to the HLA-B8-restricted sequence EPLARLEL, and the second epitope, published by Dawoodji et al. [156], was mapped to the HLA-A2-restricted sequence LLNATIAEV. However, data on frequencies of EPLARLEL- and LLNATIAEV-specific CD8$^+$ T cells in AAD patients, and their cytokine responses to the exact epitopes, are limited. Our aim was therefore to address these issues using MHC class I multimers and cytokine analyses.

PBMCs were isolated from patients and controls and used for experiments both *ex vivo* (day 0) and *in vitro* after 13 days of antigen stimulation and expansion. Initial staining (day 0) of cells with HLA dextramers revealed significantly higher frequencies of LLNATIAEV-specific CD8$^+$ T cells in patients compared to controls. These cells also showed strong peptide-induced expansion *in vitro* after 13 days. In contrast, EPLARLEL-specific CD8$^+$ T cells were almost completely absent in both patients and controls and did not show any ability to expand upon stimulation with cognate peptide.

Consistent with these results, IFNγ ELISPOT assays on both *ex vivo* and expanded cells revealed clear production of IFNγ in response to LLNATIAEV, whereas no such response was found for EPLARLEL-stimulated cells. Multiplex cytokine analyses further demonstrated production of IL-6, TNFα, MIP-1α, and MIP-1β in response to LLNATIAEV, but in contrast to IFNγ, these responses faded after 13 days of expansion. Therefore, IFNγ seemed to be the most appropriate and reliable biomarker for peptide-specific $CD8^+$ T cells, especially for expanded cells.

Having demonstrated the lack of response to EPLARLEL, we set out to identify alternative epitopes within the longer peptides $21OH_{431-450}$ (Rottembourg [34]) and $21OH_{430-447}$ (Dawoodji [156]) reported to be immunodominant regions. PBMCs from patients and controls were stimulated with a panel of adjacent 9 amino acids-overlapping peptides covering the longer peptide $21OH_{430-447}$ (G**EPLARLEL**FVVLTRLLQ). Of the peptides tested, ARLELFVVL ($21OH_{434-442}$) induced the strongest IFNγ response among the patients. Furthermore, utilizing an epitope discovery system and complementary biochemical analyses, we discovered that ARLELFVVL did not show any affinity for HLA-B*0801, like EPLARLEL, but instead seemed restricted to HLA-C*0701.

To confirm the immunodominance of ARLELFVVL, we generated an HLA-C*0701/ARLELFVVL streptamer to investigate the frequency of ARLELFVVL-specific $CD8^+$ T cells in AAD patients versus controls. Intriguingly, significantly higher frequencies of peptide-specific cells were found in AAD patients. ARLELFVVL did also induce strong expansion of ARLELFVVL-specific $CD8^+$ T cells in patients by *in vitro* stimulation for 13 days, but not in controls. PBMCs were also tested in ELISPOT assays *ex vivo*, revealing significantly stronger IFNγ responses to ARLELFVVL in patients than in controls. Importantly, these responses were comparable to the responses against the longer peptide $21OH_{430-447}$.

Collectively, these data indicate the presence of two immunodominant $CD8^+$ T cell-targeted epitopes of 21OH in AAD: LLNATIAEV, presented by HLA-A2, and ARLELFVVL, presented by HLA-C7.

Discussion

Since the discovery of Addison's disease in 1855 by Thomas Addison [175], there have been considerable advances in our understanding of the pathogenesis and etiology. Whereas tuberculosis was the predominant cause of adrenal destruction earlier, autoimmune adrenalitis is now the dominating form and accounts for nearly 90% of cases in developed countries. It is well-established that the autoimmune form is caused by a complex interplay between genes and the environment, although environmental factors are less studied. The role for a genetic basis has been particularly evident by the high disease concordance in monozygotic twins and the familial clustering, suggesting the existence of highly penetrant susceptibility alleles [105, 127, 128]. In this project, we have investigated the involvement of *rare* susceptible alleles, with presumably *large effects*. Furthermore, based on previous genetic studies on AAD pointing out the HLA loci as being the most prominent genetic risk factor, we also examined HLA-specific immunodominant epitopes of the major autoantigen 21OH.

Involvement of inborn errors of innate immunity

In *paper I,* we identified several rare missense variants in *TLR3* and associated members of the TLR3-IFN pathway in a subgroup of patients with AAD. The functional impact of the *TLR3* variants on the receptor's response to viral dsRNA was studied utilizing a cell-based reporter assay involving poly(I:C) as the dsRNA analog. Of the five rare variants discovered, three were found to have significantly impaired function; F351I (carried by two unrelated patients) and L742F, both of which have never been functionally characterized before, in addition to R867Q, previously described to be causative (when homozygous) of HSE [165]. Patients harboring F35I or R867Q had healthy siblings heterozygous for the same variants, demonstrating incomplete penetrance. This is in line with previous studies on genetic etiologies of HSE also showing incomplete penetrance of TLR3 pathway deficiencies [165, 173]. Several factors may cause this inheritance pattern including environmental factors such

as infections by viruses or other pathogens, or the genetic architecture of the host, as well as epigenetic factors [165].

Although inborn errors of the TLR3-IFN pathway have been shown to impair antiviral immune responses, previous studies are conflicting on the role of this pathway in protective immunity. TLR3 deficiency in fibroblast seem to impair IFN responses and lead to high levels of viral replication, whereas in leukocytes, TLR3-mediated immunity seems to be surprisingly redundant for the antiviral host defense [165, 176, 177]. This may be due to an extensive activation of cytosolic RNA sensors, including MDA5 and RIG-I [178]. Nonetheless, TLR3 seems to have a clear protective role in the central nervous system (CNS) against HSV- and influenza A virus-induced encephalitis in humans [165, 179, 180]. Studies of TLR3- and TRIF-deficient mice, also suggest an important role for TLR3 outside the CNS, in which these mice have abolished IFN production and considerably higher viral load in non-neural tissues compared to the wild-type mice [181]. This is supported by other studies showing that a wide range of immunopathologies and immunological traits are associated with SNPs in *TLR3* [167, 169, 170]. Recent findings demonstrated that TLR3-pathway deficiency increases the risk of autoimmune encephalitis in the weeks or months after HSE infection [182, 183]. Furthermore, inborn TLR3 deficiency has also been reported in patients with severe influenza pneumonitis [184] and herpes zoster ophthalmicus [185]. In conjunction, infection of TLR3-deficient mice is shown to cause T1D due to abrogated IFN-I responses and necrosis of virus-infected β-cells [186]. Taken together, the role of the TLR3 pathway seems relevant for inducing immunological phenotypes that eventually lead to immune-mediated diseases. Still, more knowledge about the role of the TLR3 pathway in antiviral immunity is needed, especially in the context of autoimmunity that may only occur ensuing certain infections.

Identifying rare genetic variants that influence the development of complex diseases is challenging and the central question, in this case, would be if the identified variants play any etiological roles in AAD. Exome sequencing is a widely-used approach to identify low-frequency variants, but it is difficult to claim any association when only a few individuals are carriers. The effect of the variants may therefore seem low when

standing alone but could confer increased risk in combination with disease susceptible HLA alleles. Moreover, it is important to note that AAD has a heterogeneous genetic etiology, meaning that several different variants could induce the same phenotype, and multiple of these might be necessary to produce it. Therefore, rare variants carried by only some individuals might play a key role in disease development. Moreover, phenocopies may occur, meaning that environmental factors could induce an AAD phenotype in an individual without known susceptibility variants that matches the phenotype to an individual that does carry high-risk variants [105]. This may explain why the AAD patients in the current study do not exhibit any unique phenotype. At the same time, we might speculate that the genotype could predict the etiology of the disease, even though it does not correlate with the phenotype. Thus, AAD patients with inappropriate antiviral responses, due to inborn errors of innate immunity, may share the same environmental trigger such as viral infections (Figure 7).

The suggested role of viruses in AAD is based upon previous studies showing that the adrenals are permissive to a variety of viral infections and that AAD patients show reduced NK cell cytotoxicity and poor response to IFNs in-vitro, both of which may cause inappropriate antiviral responses [101, 102]. Furthermore, certain genetic risk factors associated with AAD are variants in genes that participate in the immune response against infectious diseases, such as *PTPN22* (also participating in TLR-driven type I IFN production) [135, 187-189] and *BACH2* [142, 190], which together can affect the course of viral infections [191]. Epidemiological and cohort studies have also reported that AAD patients have more infections and increased use of anti-microbial agents (such as antivirals) than the general population, which does not correlate with glucocorticoid dosage [103, 192]. Moreover, a previous study of AAD patients in the Polish and United Kingdom cohorts showed that there is a month-of-birth effect on the risk of AAD development, in which the incidence of AAD peaks in the winter months. This may be linked to the increase of viral infections during this time of year that could trigger the development of AAD later on [193].

Identification of an extremely rare homozygous mutation in the HSD3B2 gene

The steroidogenesis is a fine-tuned mechanism of the adrenal cortex that converts cholesterol into a variety of bioactive signaling molecules. Disturbed steroid biosynthesis is often associated with adrenal disorders, whereby the severity is depending on which steroid enzyme is being deficient. Apart from AAD, underlying causes of steroid enzyme deficiencies include inborn autosomal recessive mutations resulting in CAH [116]. In *paper II* we report an adult female patient with primary adrenal insufficiency due to both CAH, resulting from 3βHSD2 deficiency, *and* AAD. 3βHSD2 deficiency is a rare autosomal recessive condition, constituting <0.5% of all CAH cases, and impair the first steps of steroid hormone production and sexual development [194].

At inclusion in ROAS, the patient was classified as having AAD and POI with vitamin B12 deficiency, and 21OH and parietal cell autoantibodies. However, genetic analysis revealed that she also had CAH due to an extremely rare homozygous stop-gain mutation in *HSD3B2,* representing, to our knowledge, a novel disease etiology. Scrutiny of old medical records further revealed the presence of small-sized adrenal glands instead of hyperplastic glands, as normally seen in CAH. The patient had also no overproduction of Δ5-steroids following elevated ACTH levels, suggesting complete adrenal insufficiency.

Steroid profiling by LC-MS/MS revealed the presence of tetrahydrocortisol and 5α-tetrahydrocortisol, which is consistent with her ongoing replacement therapy including hydrocortisone. Additionally, the very low, but detectable, level of testosterone may be due to the conversion of DHEA by 3βHSD1 in the periphery. Previously measurable pregnenolone levels suggest that the autoimmune destruction of the adrenal cortex commenced sometimes in the 1990-ties. However, her initial steroid levels were not *above* the normal range as would be expected in case of isolated 3βHSD2 deficiency, and may indicate an even earlier initiated adrenocortical destruction. The treatment of CAH, starting only 1 week after birth, could have masked the classical manifestations of complete adrenal insufficiency resulting in delayed diagnosis of AAD. Hence, the

diagnosis of AAD could not be confirmed from symptoms, but only by detecting 21OH autoantibodies.

According to literature, we could only find one reported case of CAH occurring together with complete adrenal insufficiency suspected to be AAD. This patient, however, did not have 21OH autoantibodies or HLA risk alleles but was weakly positive for autoantibodies against 17α-hydroxylase [195]. Therefore, it is conceivable that there might be other rare unreported cases of AAD due to early diagnosis of CAH, masking the clinical symptoms of adrenal insufficiency. Notably, several other AAD patients included in our WES analysis were heterozygous carriers of rare missense variants in *HSD3B2*. Although family members of CAH patients, carrying heterozygous *HSD3B2* mutations, seem to have maintained normal 3βHSD2 activity in vivo [196], genetic variations in *HSD3B2* are associated with other conditions such as idiopathic hypospadias and prostate cancer [197, 198].

Apart from *HSD3B2* variants, WES also revealed heterozygous missense variants in the steroidogenesis-related genes *CYP11A* and *STAR* (unpublished data). Additionally, our group has previously detected several exonic and intronic variants in *CYP21A2* (encoding 21OH) in AAD patients, but as all were in linkage disequilibrium (LD) with high-risk HLA-DR alleles they were not considered as independent risk factors [199]. However, it is possible that such subtle molecular abnormalities and inborn errors of steroidogenesis could lead to decreased negative feedback inhibition of the HPA axis and hence elevated levels of ACTH (seen in AAD and CAH patients). Since ACTH regulates expression of steroidogenesis-related genes [200], this in turn may upregulate transcription of steroidogenic enzymes including 21OH, potentially leading to ER stress and accumulation of misfolded proteins, and/or generation of novel adrenal antigens presented by stressed adrenocortical cells. In combination with viral infections, this could have biological consequences and contribute to inflammation and immunological destruction of the adrenal cortex (Figure 7).

Immunodominant HLA-restricted epitopes of 21OH in AAD

In *paper III*, we were able to detect significantly higher frequencies of LLNATIAEV-specific $CD8^+$ T cells in AAD patients than in controls, utilizing isolated PBMCs and MHC class I dextramers. These cells also displayed a stronger ability of IFNγ production and clonal expansion compared to controls upon stimulation with cognate peptide. This could reflect a previous antigen experience *in vivo* in an inflammatory setting, and thus the $CD8^+$ T cells in AAD patients would have a more memory-like phenotype. Likewise, autoreactive T cells in T1D patients are previously demonstrated to be skewed towards a memory phenotype, whereas those detected in healthy controls seem to be predominantly naïve [201, 202].

Surprisingly, we did not detect any significant T cell response to EPLARLEL in terms of low frequencies of EPLARLEL-specific $CD8^+$ T cells and weak peptide stimulated IFNγ production. Neither did they show any ability to expand upon peptide stimulation. This lack of response spurred us on to investigate alternative immunodominant epitopes. Having found that AAD patients showed positive IFNγ responses against the longer peptide G**EPLARLEL**FVVLTRLLQ ($21OH_{430-447}$), we tried to map alternative epitopes using short, overlapping peptides. Strong T cell responses were detected to peptide 5 ($21OH_{434-442}$, ARLELFVVL), but this epitope did not show affinity for HLA-B*08:01 (like EPLARLEL) according to an epitope discovery system. We therefore started to search for alternative HLA class I molecules able to present this peptide, with a particular emphasis on those encoded by AH 8.1, containing the predisposing HLA-B8, -DR3, and -DQ2 alleles.

The extended form of AH 8.1 is usually defined by the haplotype *HLA-A*01:01, -C*07:01, -B*08:01, -DRB1*03:01, -DQA1*05:01* and *-DQB1*02:01* [131]. This combination has been highly conserved throughout evolution, hence HLA-B*0801 is in strong LD with the class I alleles HLA-C*0701 and -A*0101. Using the NetMHC 4.0 server for HLA peptide binding prediction, ARLELFVVL was predicted to bind HLA-C*0701. Following generation of C7*ARLELFVVL streptamers, we managed to detect high frequencies of ARLELFVVL-specific $CD8^+$ T cells in several HLA-C7

positive patients, but not in HLA-C7 positive controls. These cells also displayed the ability of clonal expansion and IFNγ production upon stimulation with ARLELFVVL, suggesting antigen experience *in vivo*. Intriguingly, these ARLELFVVL-specific responses were comparable to the responses to GEPLARLELFVVLTRLLQ, suggesting that the reactivity against the longer peptide are primarily directed against ARLELFVVL, and not EPLARLEL as previously suggested. Detection of both LLNATIAEV- and ARLELFVVL-specific $CD8^+$ T cells supports the hypothesis that the autoimmune reaction in AAD is triggered by various epitopes of 21OH (Figure 7) [105]. This would require a broad MHC peptide-binding repertoire, which is in line with why the heterozygous combination of the DR3-DQ2 and DR4-DQ8 haplotypes, increasing the variety of MHC molecules, confers higher risk of disease development than a homozygous combination of either of the haplotype [63, 203]. This seems also to be the case for T1D, in which the risk of disease development is greater in individuals expressing the heterozygous genotype (DR3-DQ2/ DR4-DQ8), presumably because DQ heterodimers facilitate presentation of several different beta cell antigens [204].

DR3, DQ2, and HLA-C7 are all part of AH 8.1, as previously mentioned, which confers risk of developing a wide range of immune-mediated diseases. Nonetheless, AH 8.1 has been highly conserved through generations and is today one of the most common haplotypes in northern Europe [132, 133]. Carriers of AH 8.1 might develop aberrant immunity including increased antibody production and decreased NK cell activity [132, 205, 206]. Increased antibody production is in line with AAD, and other AH 8.1-associated autoimmune disorders, being characterized by the presence of autoantibodies, even though they often are characterized by Th1-mediated immune responses [132]. Also, decreased NK activity fits well with the previous finding that AAD patients seem to have reduced NK cell cytotoxicity, potentially making them more vulnerable to viral infection [102]. The various immune dysfunctions arising from AH 8.1 suggest involvement of several genes beyond the classical high-risk HLA alleles, such as *C4* (involved in the complement cascade), *IKBL* (involved in the regulation of NF-kB) and *TNF* (involved in inflammatory conditions) [132, 133]. However, the independent impact of these genes has been difficult to determine due to

the strong LD within AH 8.1. Many studies have therefore focused on HLA to understand key events of autoimmune pathologies.

Of the AH 8.1-associated HLA class I alleles, HLA-B8 stands out as an important autoimmune-prone factor, whereas HLA-C7 has received little attention [207]. Our study seems therefore to represent the first demonstration of an HLA-C7 restricted epitope in the context of autoimmune diseases. The limited focus on HLA-C, in general, may be due to its low expression, but maybe more importantly because of the strong LD with the predisposing HLA-B, -DR and DQ that makes it difficult to detect any independent associations for individual HLA variant loci [63, 133, 208]. However, since AH 8.1 is so well conserved and seems unique in its association with several autoimmune diseases [132, 133, 209], it is conceivable that HLA-C7 to some extent does contribute to disease susceptibility. Besides, a growing number of publications have demonstrated that HLA-C alleles, including C7, play a role in T1D, SLE, and RA [204, 208, 210-215]. For instance, the HLA-C*0602 allele is a major risk factor in psoriasis by which $CD8^+$ T cells react to epitopes derived from streptococcal M proteins and type 1 keratin, presented by HLA-C*0602 [216]. However, the majority of the HLA-C/autoimmunity associations are not linked to antigen-presentation, but rather the capacity of HLA-C to act as a ligand for killer cell Ig-like receptors (KIRs), expressed by NK cells [207].

KIRs and HLA-C interact to regulate the cytotoxic activity of NK cells, ensuring a balanced immune response and self-tolerance [207, 217]. Polymorphisms of KIRs and HLA can therefore disrupt this balance, potentially leading to inflammation and autoimmune diseases later on [218]. Concerning HLA-C7, its role in autoimmune diseases has been particularly evident in patients with both T1D and CD, where it contributes to elevated levels of the weak inhibitory KIR ligand C1 [215]. Predominantly weak regulation and inhibition of NK cells could lead to higher cytotoxic activity and inflammation, accelerating an initiated autoimmune process. With regards to AAD, excessive NK activity does not seem to play a major role in the autoimmune attack of the adrenal cortex, especially since NK cells seem rather impaired in some AAD patients [102]. On the other hand, it has been demonstrated that

HLA-C7 zygosity affects NK cells' cytotoxicity, whereby homozygotes for HLA-C7 have a lower frequency of NK cells with lytic activity than heterozygotes [219]. We may therefore envisage that it is the HLA-C7 zygosity in AAD patients (carrying AH 8.1) that impairs their NK cell cytotoxicity, and further make them vulnerable to viral infection, which may trigger AAD later on. Therefore, as well as being an immunodominant antigen-presenting molecule shown in this study, HLA-C7 might also contribute to AAD development by affecting innate immune responses.

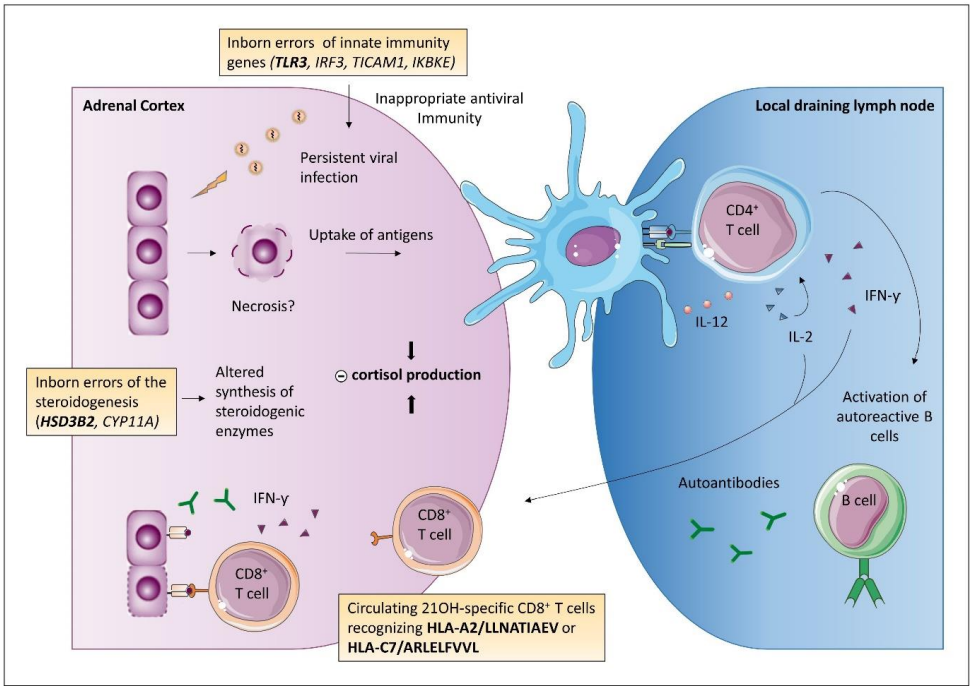

Figure 7. A model for the pathogenesis of AAD. Inborn errors of innate immunity could cause persistent viral infection and lead to necrosis of adrenocortical cells and subsequent release of 21OH and other autoantigens engulfed by APCs. Next, autoreactive T cells get activated by self-antigen-presentation by APCs within local draining lymph nodes, which, in turn, license the activation of autoreactive T and B cells with specificities for 21OH and/or other adrenocortical antigens. Circulating 21OH-specific $CD8^+$ T cells, recognizing the immunodominant epitopes LLNATIAEV presented by HLA-A2 or ARLELFVVL presented by HLA-C7, produce IFNγ to stimulate their cytotoxicity and motility and cause further necrosis and apoptosis of adrenocortical cells, leading to decreased cortisol production. Besides, inborn errors of steroidogenesis-related genes may influence the pathogenesis through altered ACTH stimulation of steroidogenic enzyme synthesis. This may cause ER stress and/or generation of novel antigens presented by stressed adrenocortical cells. Altogether, these events may result in inflammation and increased lymphocyte infiltration of the adrenal cortex, eventually leading to AAD. Figure produced using Medical Servier Art.

Conclusions

The conclusions to the work in this doctoral dissertation are as follows:

I. Several AAD patients carry rare and damaging variants in the TLR3-IFN signaling pathway, some of which impair the TLR3 signaling function in response to dsRNA (poly(I:C)).

II. TLR3 pathway deficiencies affect the patients' IFN production, potentially making them more susceptible to viral infections.

III. The identified *TLR3* variants show incomplete penetrance as several siblings of the patients were healthy carriers, which is consistent with previous studies demonstrating that the penetrance of TLR3 pathway deficiencies are incomplete for HSE.

IV. We report the first description of a patient with complete adrenal insufficiency due to both AAD and 3βHSD2 deficiency caused by an extremely rare variant in the *HSD3B2* gene. To our knowledge, this combination represents a novel disease etiology.

V. Unreported cases of AAD may exist due to early diagnosis of CAH, masking the clinical symptoms of autoimmune adrenalitis.

VI. AAD patients have significantly high frequencies of $CD8^+$ T cells reacting to the previously reported HLA-A2-restricted 21OH epitope LLNATIAEV ($21OH_{342-350}$).

VII. LLNATIAEV-specific $CD8^+$ T cells display a strong ability of clonal expansion and cytokine production (including IFNγ) in response to its cognate peptide, compared to healthy controls.

VIII. $CD8^+$ T cells specific for the proposed HLA-B8-restricted 21OH epitope ($21OH_{431-438}$) EPLARLEL, were almost absent in all the AAD patients tested. Neither did the epitope induce significant IFNγ response or $CD8^+$ T cell clonal expansion.

IX. We have identified a new immunodominant region of 21OH, ARLELFVVL ($21OH_{434-442}$), detected in a large proportion of HLA-C7-positive patients. Following stimulation with ARLELFVVL, all patients exhibited clear $CD8^+$ T cell clonal expansions and IFNγ production.

Future perspectives

This thesis demonstrates the presence of several inborn errors in innate immunity among patients with AAD, which might indicate that they have impaired immune response against viruses in terms of deficient IFN production. Given these findings and previous experimental data, one of the most important future tasks would be to confirm if there is a true association between viral infection and autoimmune adrenalitis. Currently, we are analyzing the viral exposure among AAD patients utilizing a Virscan analysis. Virscan is a high-throughput method to analyze antiviral antibodies from sera using immunoprecipitation and DNA sequencing of a bacteriophage library displaying peptides covering the whole human virome. This analysis might enable us to sort out specific viral candidates potentially initiating adrenocortical autoimmunity. Furthermore, it is desirable to perform adrenal cortex small-needle biopsies via endoscopic ultrasound in AAD patients to evaluate presence of virus particles and viral nucleic acids in adrenocortical tissue. However, as this procedure is rather invasive, it may face ethical challenges.

Another interesting follow-up study would be to investigate the response of PBMCs and fibroblasts from AAD patients, in particular from those with variants in the TLR3-IFN signaling pathway, to *in vitro* exposure to live viruses. Such studies could provide answers to whether AAD patients, in general, have aberrant immune responses against live viruses, as well as how genetic deficiencies of innate immunity influence the pathogenesis of AAD. Moreover, prospective studies should be carried out, as they would be invaluable to discover key events between infections and signs of adrenalitis or adrenal insufficiency. Individuals with a strong family history of AAD could then be followed from birth to disease onset to monitor their viral exposure in relation to signs of adrenal impairment. If a role for viruses in AAD can be proved and specific viral candidates can be identified, vaccines or antiviral drugs would be obvious choices to protect susceptible individuals and to prevent disease development.

In *paper III* of the present study, we report the identification of a new immunodominant epitope, ARLELFVVL, restricted by HLA-C7, as well as confirming the presence of

HLA-A2*LLNATIAEV-specific $CD8^+$ T cells in AAD patients. Future research should investigate whether LLANTIAEV and ARLELFVVL promote expression of HLA-A2 and HLA-C7, respectively, in the adrenal cortex. Since hyperexpression of HLA class I is observed in pancreas samples from T1D patients [220], it is conceivable that the same mechanism can occur in AAD, influencing the $CD8^+$ T cell-driven destruction of adrenocortical cells. As we are planning to perform immunohistochemical staining of adrenocortical tissue from deceased AAD patients, we could stain for HLA-C7 and HLA-A2 molecules to see if they are hyper-expressed. Furthermore, the cytotoxic potential of ARLELFVVL-specific $CD8^+$ T cells should be explored to assess wether they lyse HLA-C7/ARLELFVVL-expressing cells, as shown for HLA-A2-restricted $CD8^+$ T cells recognizing LLNATIAEV [156].

It will also be important for future studies to perform a comprehensive investigation of the phenotypic characteristics of ARLELFVVL- and LLNATIAEV- specific T cells, using the established HLA-multimer reagents. Recent advances in next-generation sequencing technologies have made it possible to examine the clonality and diversity of such T cells on a single-cell level. Utilizing MHC class I multimer technology to sort these antigen-specific T cells, followed by single cell immune profiling to identify the cells' TCR sequences, we can assess whether they have an identical antigen specificity and gene rearrangement, as well as analyzing their transcriptomes.

Elucidating the diversity of 21OH autoreactive T cells would aid our understanding of disease mechanisms, as well as facilitate the use of these cells as disease biomarkers and therapeutic targets for intervention at a stage when the majority of adrenocortical cells are still viable.

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Appendix

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Identification and characterization of rare toll-like receptor 3 variants in patients with autoimmune Addison's disease

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ABSTRACT

Autoimmune Addison's disease (AAD) is a classic organ-specific autoimmune disease characterized by an immune-mediated attack on the adrenal cortex. As most autoimmune diseases, AAD is believed to be caused by a combination of genetic and environmental factors, and probably interactions between the two. Persistent viral infections have been suggested to play a triggering role, by invoking inflammation and autoimmune destruction. The inability of clearing infections can be due to aberrations in innate immunity, including mutations in genes involved in the recognition of conserved microbial patterns. In a whole exome sequencing study of anonymized AAD patients, we discovered several rare variants predicted to be damaging in the gene encoding Toll-like receptor 3 (TLR3). TLR3 recognizes double stranded RNAs, and is therefore a major factor in antiviral defense. We here report the occurrence and functional characterization of five rare missense variants in *TLR3* of patients with AAD. Most of these variants occurred together with a common *TLR3* variant that has been associated with a wide range of immunopathologies. The biological implications of these variants on TLR3 function were evaluated in a cell-based assay, revealing a partial loss-of-function effect of three of the rare variants. In addition, rare mutations in other members of the TLR3-interferon (IFN) signaling pathway were detected in the AAD patients. Together, these findings indicate a potential role for TLR3 and downstream signaling proteins in the pathogenesis in a subset of AAD patients.

1. Introduction

Autoimmune Addison's disease (AAD), is a chronic endocrine disorder characterized by an immunological attack on the hormone-producing cells in the adrenal cortex [1]. The self-antigen 21-hydroxylase (21-OH) seems to be the dominant target of adrenal autoantibodies and autoreactive T cells [1–3]. The underlying causes of AAD are complex, involving both genetic and environmental factors. So far, the best characterized genetic factors are related to antigen presentation and T cell regulation, like certain HLA combinations and common variants in the genes *CTLA4* and *PTPN22* [4–6]. Most of these genetic risk factors are shared with other organ-specific autoimmune diseases, including type 1 diabetes (T1D), celiac disease and thyroiditis, which often co-occur with AAD in patients with autoimmune polyendocrine syndrome type 2 (APS-2). On very rare occasions AAD can also be monogenic, as part of autoimmune polyendocrine syndrome type 1 (APS-1), and caused by mutations in the *AIRE* gene encoding the autoimmune regulator protein [7]. However, apart from HLA genes and *AIRE*, the genetic factors discovered so far are only moderately increasing the susceptibility to develop AAD. In particular, the clustering of AAD in some families suggest the existence of highly penetrant AAD susceptibility alleles [8,9], but the specific alleles remain elusive. The involvement of rare variants in the pathogenesis of AAD is in line with AAD being a rare disorder,

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and may provide an explanation to the missing heritability problem of AAD. A possible strategy would therefore be to search for rare genetic variants (allele frequency <1% in the general population) affecting the phenotype [10].

Previous studies have demonstrated that errors in innate immunity are associated with autoimmunity [11–14]. Recent findings by others and us have suggested that alterations in the innate immune response might be present in AAD as well [15–17]. Persistent viral infections have further been proposed to act as triggers for immunopathological tissue destruction, resulting in autoimmune disease [11,13]. To detect and eliminate infectious agents, the innate immune system uses several recognition systems, including pattern-recognition receptors (PRRs) [18,19]. Some of the best characterized PRRs are the toll-like receptors (TLRs), which are not only expressed in innate immune cells, but also in the parenchyma of different organs such as the endocrine glands [19–21]. In particular, amongst the ten human TLR paralogs, single nucleotide polymorphisms (SNPs) and rare variants in the *TLR3* gene have been associated with several immune mediated diseases and different outcomes of infections [11,22–27]. TLR3 is localized intracellularly, within endosomes, and recognizes double-stranded RNA (dsRNA), which is the viral replication intermediate in most viruses and the actual genome in others [28,29]. Binding of dsRNA by TLR3's ectodomain initiates a signaling cascade resulting in activation of interferon (IFN) regulatory factor (IRF)-3/IRF-7 and nuclear factor kB (NF-kB) that stimulates cytokine production [22,30]. Mutations in *TLR3* or associated key-members of the TLR3-IFN pathway, may cause sporadic cases of herpes simplex virus type-1 (HSV-1) encephalitis (HSE) [31]. Both dominant and recessive mutations have been described, but incomplete clinical penetrance has been observed for most genetic etiologies of HSE.

In the present work we have identified several rare *TLR3* variants in AAD patients, and characterized these with a novel NF-kB-based reporter assay. Some of these variants have not previously been functionally characterized. In addition, peripheral blood mononuclear cells (PBMCs) from patients with *TLR3* variants were stimulated with dsRNA to examine any impairment of IFN production.

2. Materials and methods

2.1. Patients and controls

The initial patient cohort consisted of 142 AAD patients that were subjected to whole-exome sequencing (WES). All patients were diagnosed with AAD based on clinical criteria as recommended by the Endocrine Society [32], and were recruited from the National Registry of Organ-specific Autoimmune Diseases (ROAS) at Haukeland University Hospital, Norway. The control group consisted of an in house database containing 308 exomes sequenced in research projects with no link to AAD or related phenotypes. We also compared our results with those from 795 exomes available from the Norwegian Cancer Genomics Consortium (NCGC) website (http://www.cancergenomics.no). This dataset contains more than 1.5 million individual variants coming from 1590 normal chromosomes of various cancer patients [33].

2.2. Ethics

The study was approved by the Regional Ethics Committee, project numbers 2013/1504, 2015/2053 and 2018/1417, and informed consent was obtained from each patient and family member included in the study.

2.3. Blood samples and DNA extraction

EDTA blood samples from patients and controls were used for purification of DNA and for the isolation of PBMCs. Genomic DNA was extracted from whole blood in house, using a QIAamp DNA Blood Mini Kit (Qiagen), or performed by the Nord Trøndelag Health study (HUNT) biobank (Levanger, Norway). Blood samples were kept at −80 °C until DNA extraction using the MasterPure™ DNA purification kit version II B1 (Epicentre). Concentration of extracted DNA were normalized to 50 ng/μl. PBMCs were isolated from whole blood using Ficoll density gradient centrifugation, and were cryopreserved at −150 °C.

2.4. Whole-exome sequencing

WES was done on an Illumina HiSeq (Illumina), after exome capture with the Roche Nimblegen SeqCap EZ Exome Library V2 kit (Roche Diagnostics Corporation). The sequences were aligned to the reference human genome (hg19) by using bwa mem (v0.7.12, [34]). The alignments were then refined by the Genome Analysis Toolkit (GATK, Version 2.8, [35,36]) and PCR duplicates were marked by Picard (v1.124, http://broadinstitute.github.io/picard). The variants were called by UnifiedGenotyper by GATK (Version 2.8, [35,36]) and annotated by Annovar (v2017Jul16, [37]). Filtering of the exome variants was done in FILTUS [38]. To identify rare variants, we applied filters removing variants with allele frequencies greater than 1% in any of the databases gnomAD, 1000 Genomes Project, or our in house database.

2.5. Validation of WES results and follow-up of patients

Confirmation of the detected *TLR3* variants were obtained by Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Family members of selected patients were sequenced using the same sequencing kit. PCR was performed using AmpliTaq Gold™ DNA Polymerase with Gold Buffer and $MgCl_2$ (Applied Biosystems). For one of the patients, no DNA from family members was available. To determine the haplotype of this patient, exon 4 of *TLR3* was amplified from genomic DNA and cloned into pCRTM2.1-TOPO vector, using TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. One Shot TOP10 chemically competent *E. coli* cells (Invitrogen) were transformed with cloned vector according to the manufacturer's recommendations, and plated onto ampicillin containing plates and grown overnight at 37°C. Plasmid DNA from single colonies were then amplified, purified using ExoProStar 1-Step enzymatic clean up system (GE Healthcare Life Sciences), and sequenced as described above. All primer sequences used for PCR and Sanger sequencing were designed using Primer3web version 4.0.0 and are available upon request.

2.6. In silico predictions

To estimate the degree of evolutionary conservation of the identified *TLR3* variants, 18 protein sequence homologs to the human TLR3 protein were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) method [39] and ran by Jalview multiple sequence analysis workbench version 2.10.4b1. The specific areas containing the variants of interest were aligned separately to avoid using gaps in the alignment.

Furthermore, the variants of TLR3 and the TLR3/IFN signaling pathway detected in patients were classified by the *in silico* variant pathogenicity predictors SIFT [40], PolyPhen2 [41], MutationTaster2 [42], PROVEAN [43] and CADD [44]. In particular, CADD score > 20 was used as a threshold for classifying variants as potentially damaging.

The structural implications of the F351I mutant were also analyzed using The PyMOL Molecular Graphics System v. 1.5 [45]. The x-ray crystallography structures of the ectodomains of human and mouse TLR3 [46,47] were used as templates.

2.7. Cell culture

HEK-Dual Null (NF/IL8) cells (InvivoGen) were grown in DMEM (1X) + GlutaMax (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma Aldrich) and 100 μg/ml Normocin (InvivoGen) at 37 °C with 5% CO_2 in a humidified incubator. These cells are stably transfected

with two different reporter genes (Secreted embryonic alkaline phosphatase (SEAP) and the *Lucia* luciferase) inducible by NF-kB and IL-8, respectively, and are triply knocked out for TLR3, TLR5 and the TNF receptor genes.

2.8. Plasmids and site-directed mutagenesis

The following SNPs in Human TLR3 cDNA in pCMV6-Entry/TLR3 (OriGene) were produced using the QuickChange site-directed mutagenesis kit (Strategene): T59N, G221R F351I, L412F, L742F and R867Q. The mutagenic primers were designed with PrimerX and are available upon request. All SNPs were confirmed by Sanger sequencing.

2.9. Reporter gene assay

HEK-Dual Null (NF/IL8) cells were harvested and plated in 6-well plates (6×10^5 cells/well) for 24 h. The cells were then transiently transfected with a mixture of the Lipofectamine 2000 reagent (Thermo Fisher Scientific) and plasmids pCMV6-Entry/TLR3 (OriGene) encoding the full-length wild type (WT) gene, and/or TLR3 mutants. The cells were then incubated for 48 h, before being harvested and plated in a MicroAmp Optical 96-well Reaction Plate. 100 μg/ml of polyinosinic-polycytidylic acid (poly(I:C)) (Invivogen) were then added to each well. The cells were allowed to incubate for 22–24 h to reach 70–80% confluency. 20 μl of cell supernatant of each well were then mixed with 180 μl of SEAP-inducer compound (InvivoGen) according to the manufacturer's protocol. The SEAP activity was assessed by reading the optical density (OD) at 620–655 nm after 2 h. The units of SEAP activity were normalized to untreated controls within the same transfection, and the activity of the mutant TLR3 proteins was measured as a percentage of activity relative to the WT protein.

2.10. Western blots

Hek-Dual Null (NF/IL8) cells were transiently transfected with WT or mutant TLR3 as described above. The cells were then harvested and lysed using cOmplete Lysis-M buffer 0 (Sigma Aldrich), the lysates were centrifuged, and the supernatants obtained. The protein concentration of the supernatants was estimated using a DC protein assay kit (Bio-Rad) and equal amounts of protein were then mixed with LDS sample buffer (4X) (Life Technologies/Invitrogen) and Sample Reducing Agent (10X) (Life Technologies/Invitrogen). After 10 min at 70 °C, the samples were loaded on a NuPAGE 10% Bis-Tris Gel (Novex) and blotted onto polyvinylidine difluoride membrane (Invitrogen). The membrane was incubated with a mouse *anti*-DDK (FLAG) monoclonal antibody (clone OTI4C5, Origene). Mouse *anti*-GAPDH (clone 6C5, Millipore) was used as loading control. Bands were visualized using secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Invitrogen) and the ECL system (Thermo Scientific).

2.11. PBMC stimulations

Cryopreserved PBMCs from patients, family members and controls, were thawed and plated onto 24-well plates (2×10^6 cells/well) and stimulated with either poly(I:C) (100 μg/ml) or polyadenylic-polyuridylic acid (poly(A:U)) (10, 50 and 100 μg/ml) for 24 h. The production of cytokines in the supernatants was determined by ELISA kits according to the manufacturer's instructions. These included IL28A/IFNλ2, IFNγ (both from BioLegend) and IFNβ (R&D systems).

2.12. Statistical analysis

In order to estimate whether rare and possibly damaging variants in *TLR3* were enriched among AAD patients, we compared the proportion of individuals carrying variants with CADD scores above 20 in our AAD cohort with the appropriate ethnic subpopulations of the Genome Aggregation Database (gnomAD), using a two-sided Fisher's exact test. Only exomes from controls were used for the analysis. All data from experiments using HEK-Dual Null (NF/IL8) cells are expressed as means of at least three independent experiments. All collected data were then tested by an ordinary one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. To test for IL28A/IFNλ2 differences between controls and patients, multiple t-tests were used followed by the Holm-Sidak method to correct for multiple comparisons. For all tests, $P < 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism v.8.0.1 software.

3. Results

3.1. General findings of WES

Initially, we searched for variants in a candidate list of 194 genes (available upon request) related to 1) APS-1 disease manifestations apart from AAD (e.g. chronic mucocutaneous candidiasis), 2) *AIRE,* molecular partners of *AIRE,* or related genes, 3) other autoimmune diseases or syndromes consisting of autoimmune manifestations, and 4) steroidogenesis, vitamin D metabolism, or adrenal disorders. A total number of 135 variants with CADD scores > 20 in 76 different genes was detected in the patients. 94 patients carried at least one variant (ranging from 1 to 6), while 38 patients did not carry any rare variants. The results from the last 10 patients were excluded from further analysis as the sequencing data failed to pass quality control. No significant findings were noted with regard to total number of coding variants, or enrichment of rare variants in any of the list of 194 candidate genes. However, when searching for rare and potentially damaging variants, we identified several interesting hits in *TLR3*. A complete list of all detected rare variants with CADD

Table 1
Overview of patients with *TLR3* variants. Abbreviations: AAD = autoimmune Addison's disease; APS-2 = autoimmune polyendocrine syndrome type 2; F = female; Het = heterozygous; Hom = homozygous; M = male; T1D = type 1 diabetes.

Patient	Sex	Autoimmune disease	Age of onset	HLA status	Affected relatives	*TLR3* variants (NM_003265.2)	Zygosity
P1	M	Isolated AAD	22	High risk		c.176C > A (p.Thr59Asn)	Het
						c.1051T > A (p.Phe351Ile)	Het
					Brother: AAD	c.176C > A (p.Thr59Ans)	Het
					Brother: T1D		
P2	M	Isolated AAD	24	Neutral		c.1051T > A (p.Phe351Ile)	Het
						c.1234C > T (p.Leu412Phe)	Het
					Cousin: T1D		
P3	F	Isolated AAD	33	High risk		c.1234C > T (p.Leu412Phe)	Hom
						c.2600G > A (p.Arg867Gln)	Het
					Mother: hypothyroidism		
P4	M	Isolated AAD	47	Neutral		c.1234C > T (p.Leu412Phe)	Het
						c.2224C > T (p.Leu742Phe)	Het
P5	F	APS-2 + celiac disease	19	High risk		c.661G > A (p.Gly221Arg)	Het
					Father: T1D		

scores > 20.0 can be found in Supplementary Table 1.

3.2. Five TLR3 missense variants in five unrelated patients with AAD

WES revealed five rare *TLR3* (transcript NM_003265.2) missense variants in five AAD patients: c.176C > A, c.661G > A, c.1051T > A (in two patients), c.2224C > T and c.2600G > A, leading to the predicted amino acid changes p.Thr59Asn, p.Gly221Arg, p.Phe351Ile, p.Leu742Phe and p.Arg867Gln, respectively. In the following these variants will be referred to as T59N, G221R, F351I, L742F and R867Q. None of these variants were detected among the 308 exomes in our in house database, but some were present at low frequencies in the NCGC exome database (summarized in Table 2). Using Sanger sequencing to validate the variants, we discovered that three patients carrying the F351I, L742F and R867Q single nucleotide variants (SNVs), respectively, also carried the more common *TLR3* variant c.1234C > T (p.Leu412Phe), from here on referred to as L412F. One patient carried both the T59N and the F351I variant. Therefore, four of the five patients had two variants each. All variants, except G221R, were present at low frequencies in gnomAD, which includes data from ~140,000 unrelated individuals [48]. G221R was absent in gnomAD, but was detected in a single allele in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort as part of the UK10K consortium [49]. The F351I variant detected in two AAD patients, is extremely rare, with only one allele (detected in Finland) deposited in gnomAD. T59N and R867Q have previously been implicated in HSE. R867Q has been described in homozygous combination in a Finnish patient with HSE, and was shown to be a hypomorphic allele resulting in a partial form of autosomal recessive TLR3 deficiency [31], and T59N was detected in a HSE patient along with a rare variant in the TANK-binding kinase 1 *(TBK1)* gene, but did not confer any impairment on TLR3 function *in vitro* [50].

3.3. Clinical features of patients with rare TLR3 variants

The five patients carrying *TLR3* variants, three males and two females, were all typical AAD patients (Fig. 1 and Table 1): All had autoantibodies against 21-OH and 3/5 had the HLA class II genotype (DR3-DQ2/DR4.4-DQ8) conferring the highest risk of developing AAD [5]. Patient 1, 2, 3 and 4 (P1–P4) had isolated AAD, while Patient 5 (P5) had APS-2 consisting of AAD, T1D and hypothyroidism, as well as celiac disease. P1–P3 and P5 reported a family history of organ-specific autoimmunity, including AAD in a brother of P1. P1–P3 did not report any serious viral infections, but P1 and P2 had records of recurrent upper respiratory tract infections requiring antibiotics. P1 had also been suffering from chronic sinusitis that required surgery. P4 died in 2016 at the age of 87, probably due to cardiac arrest, and no follow-up study could be done for this patient. The patient had been admitted to hospital care with possible L2-L3 fracture and lower respiratory tract and urinary infections shortly prior to his death.

For P1–P3, DNA was available from family members (Fig. 1B). The brother of P1 was diagnosed with AAD and carried T59N, whereas the brother diagnosed with T1D harbored two WT alleles. The unaffected father carried F351I. Four additional healthy siblings were also sequenced; three were heterozygous for T59N and one of them carried F351I. The latter, a seemingly healthy brother of P1, tested negative for 21-OH autoantibodies. For P2, the mother and one sibling were genotyped. Both of them were heterozygous for L412F, and both were healthy. P3 was homozygous for L412F and heterozygous for the hypomorphic R867Q variant. Unfortunately, the patient's mother who was diagnosed with hypothyroidism was deceased and therefore no DNA was available for genotyping. Two healthy siblings were genotyped, whereby one was heterozygous for L412F variant, and the other homozygous, in addition to being heterozygous for R867Q. This sibling was negative for 21-OH autoantibodies. In order to genotype P4, we used a TOPO cloning kit, revealing that also L412F and L742F were positioned on opposite alleles. Collectively, the results indicated that the variants in P1–P4 were compound heterozygous (except P3 that was homozygous for L412F and

Table 2
Overview of rare variants of *TLR3* (NM_003265.2) found in AAD patients.

Variant annotation				Frequencies			*In silico* prediction tools				
TLR3 variant	Protein change	Chromosomal location NC_000004.11 GRCh37 (hg19)	Exon number	AAD frequency	In-house database frequency	NCGC[a] frequency	GnomAD frequency	SIFT	PolyPhen2	Mutation taster	PROVEAN	CADD[b]
c.176C > A	p.Thr59Asn	g.186997949C > A	2	0.0038 (1/264)	0	0.00062	0.0002	Deleterious	Benign	Disease causing	Neutral	25.7
c.661G > A	p.Gly221Arg	g.187003501G > A	4	0.0038 (1/264)	0	0	0	Tolerated	Damaging	Disease causing	Neutral	23.4
c.1051T > A	p.Phe351Ile	g.187003891T > A	4	0.0076 (2/264)	0	0	0.00003	Deleterious	Possibly damaging	Disease causing	Deleterious	24.6
c.2224C > T	p.Leu742Phe	g.187005064C > T	4	0.0038 (1/264)	0	0.0013	0.0017	Deleterious	Probably damaging	Disease causing	Neutral	26.1
c.2600G > A	p.Arg867Gln	g.187005912G > A	5	0.0038 (1/264)	0	0.0019	0.0007	Deleterious	Probably damaging	Disease causing	Neutral	31.0

[a] NCGC = Norwegian Cancer Genomics Consortium exome database, http://invitro.hpc.uio.no:8082/vcf-miner/.
[b] CADD (Combined Annotation Dependent Depletion) scores above 20 indicate that a variant is amongst the top 1% of deleterious variants in the human genome.

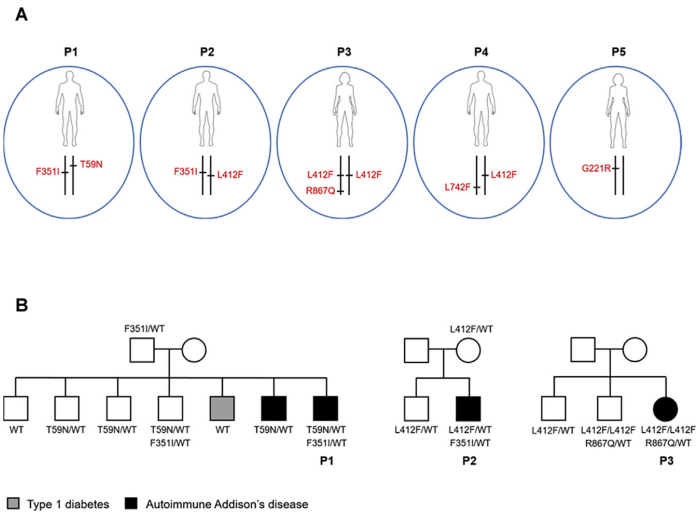

Fig. 1. *TLR3* genotypes and pedigrees for AAD patients with rare *TLR3* variants. (A) Overview of the five unrelated AAD patients carrying *TLR3* variants (red). P1–P4 were compound heterozygous for two *TLR3* variants (except for the homozygous state of L412F in P3), whereas P5 was heterozygous for one variant, G221R. (B) Family pedigrees of P1, P2 and P3, with allele segregation. Individuals affected by AAD or T1D are shown in black or grey, respectively. Healthy relatives are shown in white. Since P3 was homozygous for L412F, genotyping of parents was omitted. Mother of P1 and father of P2 were not genotyped.

heterozygous for R867Q), hence no WT variant was present (Fig. 1A).

3.4. In silico studies of the variants

Sequence alignment analysis of the six *TLR3* variants indicated that F351, L412, L742 and R867 were highly conserved in the 18 species studied, whereas T59 and G221 were less conserved, especially among the fish species (Fig. 2). Thus, based on evolutionary conservation, F351I, L412F, L742F and R867Q would be expected to be more damaging than T59N and G221R.

T59N, G221I, F351I, L742F and R867Q were all classified by the *in silico* pathogenicity prediction tools SIFT, PolyPhen2, MutationTaster, PROVEAN and CADD, through the respective web services (Table 2). A schematic diagram of the TLR3 protein is presented in Fig. 3. T59N, G221R, F351I and L412F are all located in different parts of the ectodomain of TLR3, which is essential for the recognition and direct binding of dsRNA at acidic pH [51,52]. L742F is located in the linker region, which bridges the transmembrane domain and the TIR domain [31]. Furthermore, R867Q is located in the TIR domain essential for the interaction with TRIF and thus the intracellular signal transduction upon binding of dsRNA [31,51]. All these variants, especially F351I, L412F, L742F and R867Q could potentially have a negative impact on the function of the TLR3 protein.

When taking into account all the alleles containing *TLR3* variants predicted to be damaging (CADD score > 20), we found 6/264 (2.27%) AAD patients and 213/26926 (0.79%) controls of Swedish and North Western European origin. This difference was significantly different (Odds ratio, 2.92 (95% CI 1.38–6.37), two-sided P value $= 0.02$). The difference was also significant when comparing rare variants in *TLR3* in AAD patients (6/264, 2.27%) and Norwegian exomes of the NCGC database and our in house database (19/2206, 0.86%): Odds ratio, 2.68 (95% CI 1.12–6.77, two-sided P value $= 0.043$).

3.5. Effects of variants on TLR3 activity

To determine the functional impact of the six variants, we studied the response to poly(I:C) stimulation in cells transfected with constructs encoding the WT or variant TLR3 proteins. This was achieved using Hek Dual Null cells, stably transfected with a SEAP reporter gene induced by NF-kB. As the endogenous *TLR3* gene is specifically knocked out, all expression and activity of the receptor is therefore dependent on transfected TLR3. Stimulation of TLR3, and subsequent activation of NF-kB, is achieved by treating the cells with a TLR3 ligand such as poly(I:C). The units of SEAP activity were normalized to untreated controls within the same transfection, and the activity of the mutant TLR3 proteins was measured as a percentage of activity relative to the WT protein.

T59N, G221R and L412F demonstrated approximately the same level of activity as WT TLR3 (Fig. 4A). F351I had reduced activity by ~70% ($P < 0.001$), L742F had reduced activity by ~ 50% ($P < 0.01$), and R867Q had reduced reporter activity by ~ 55% ($P < 0.01$) which increased to ~ 60% together with L412F ($P < 0.001$) compared to the WT. Thus, consistent with the *in silico* studies, the F351I, L742F and R867Q TLR3 variants were dysfunctional.

Although TLR3 exists as a monomer in solution, dimerization is required in order for TLR3 to bind dsRNA [53]. To examine whether the WT allele could rescue the activity of dimers in presence of the mutants, we performed co-transfections of equal amounts of both variants and WT (Fig. 4B). The WT protein fully restored the function of TLR3 when expressed together with the variants, except from T59N and L742F, which still showed reduced activity, but without being statistically significant.

To mimic the patients' genotypes, in which all variants were located on opposite alleles, four different co-transfections were performed. These included (1) constructs encoding T59N together with constructs encoding F351I (P1), (2) constructs encoding F351I together with constructs encoding L412F (P2), (3) constructs encoding L412F together with constructs encoding both L412F and R867Q (P3), and (4) constructs encoding L412F together with constructs encoding L742F (P4) (Fig. 4C). The F351I, L742F and R867Q TLR3 proteins combined with L412F showed a significant reduction in receptor activity by 40–45% ($P < 0.001$ for all combinations).

3.6. Expression of the TLR3 variants

The reduction or complete loss of TLR3 activity caused by the mutations could be an effect of low protein expression/stability, or of an impairment of a specific function. To examine the protein expression of

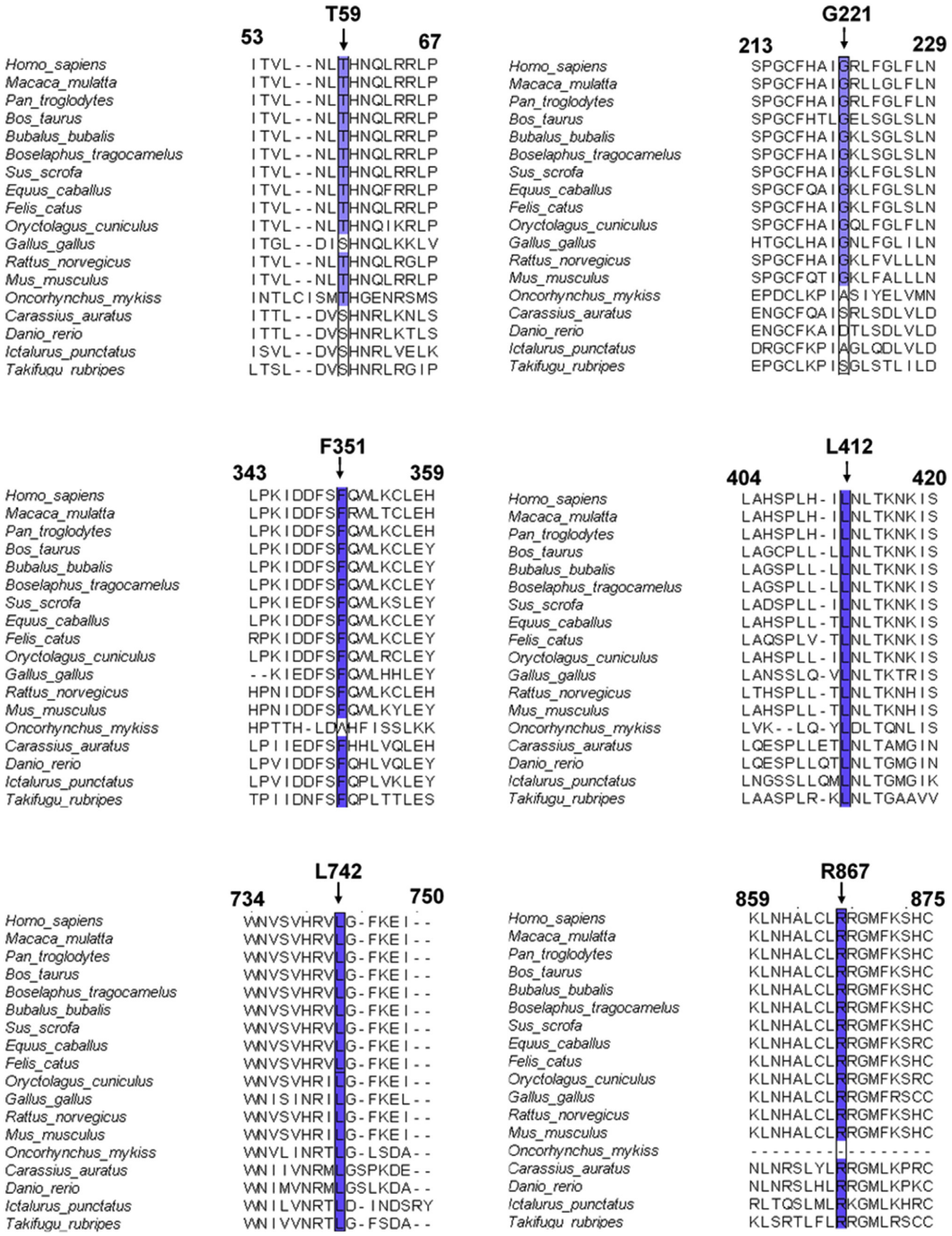

Fig. 2. Sequence alignment of TLR3 from multiple species and conservation of the identified variants. Alignment of the TLR3 region in humans and the corresponding regions in the other 17 species studied, containing the six identified variants. The conserved amino acids where the variants are located are shaded in purple.

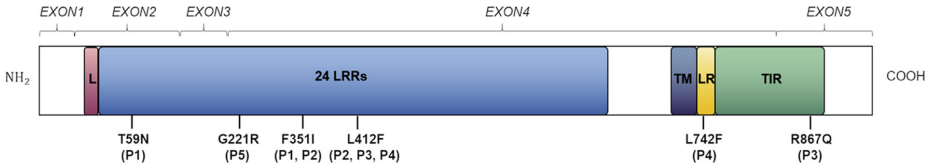

Fig. 3. Schematic presentation of the TLR3 protein with functional domains. Model of the TLR3 protein with the location of each identified variant, and the different domains (leader sequence (L), 24 leucine-rich repeats (LRRs), the transmembrane domain (TM), linker region (LR) and Toll/interleukine-1 receptor (TIR) domain).

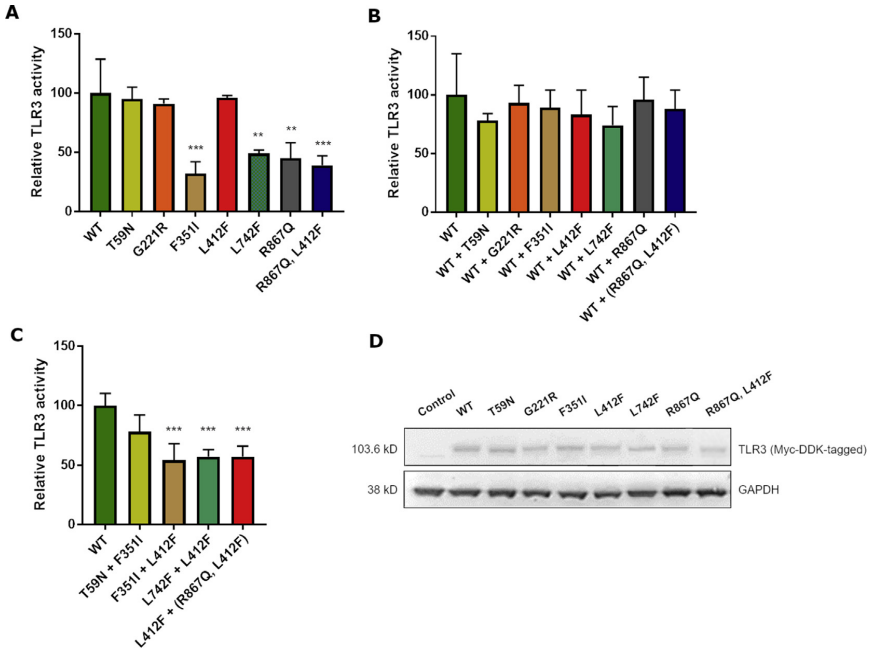

Fig. 4. Activity and expression of the WT and mutated TLR3 alleles. (A) Activity levels were determined by TLR3-dependent reporter expression induced by NF-kB in TLR3-deficient Hek-Dual Null cells transfected with various TLR3 proteins (WT TLR3, T59N, G221R, F351I, L412F, L742F, and R867Q mutant TLR3), followed by stimulation with 100 μg/ml poly(I:C). Activity levels are presented as percentages relative to the activity of the WT protein. An ordinary one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test was used to test for statistical differences between WT and mutated TLR3 proteins (**$P < 0.01$). (B) Activities of the WT alone and combined with the TLR3 mutated proteins, transfected in equal amounts. (C) Activity of the WT and co-transfected TLR3 mutants mimicking the genotype of the four patients carrying two different variants (***$P < 0.001$). (D) A representative Western blot showing the expression of WT and TLR3 mutants in Hek-Dual Null cells. The cells were transfected with plasmids encoding the WT or the mutated TLR3 proteins listed above the gel image.

the *TLR3* variants, we performed a Western blot using *Anti*-DDK (FLAG) as the primary antibody (Fig. 4D). The results from three independent experiments demonstrated that the expression levels of all variants (T59N, G221R, F351I, L412F, L742F and R867Q) were comparable with the WT. Thus, the decrease in activity was most likely not an effect of altered expression levels.

3.7. Impaired responses to dsRNA in PBMCs from patients with TLR3 variants

For P1–P3, we were able to collect live PBMCs for functional studies. For stimulation experiments with PBMCs, we chose to use the alternative TLR3 ligand poly(A:U) over poly(I:C). There are several additional receptors for dsRNA, in particular in PBMCs, and poly(A:U) has been shown to be more exclusive to TLR3 than poly(I:C) [54]. PBMCs from P1, P2 and P3, as well as three healthy blood donors with only WT *TLR3* alleles, were stimulated with increasing amounts of poly(A:U). The secretions of different IFNs into the cell culture supernatant were then measured using ELISA. For IFNβ and IFNγ the production was either undetectable or no differences were observed between patients and controls (results not shown). The secreted levels of IL28A/IFNλ2 were also relatively low (1–10 pg/ml), but a dose dependent response was observed for the controls but not the patients (Fig. 5). For the highest dose of poly(A:U), 100 μg/ml, the difference between patients and controls was significant ($P < 0.01$ for fold change and $P < 0.05$ for absolute levels). Thus, the PBMCs from AAD patients with certain combinations of deleterious *TLR3* variants are functionally impaired in response to dsRNA.

3.8. Structural analysis of the F351I TLR3 variant

Since the structure of the ligand-binding ectodomain of TLR3 has been solved by x-ray crystallography, we were able to analyze the structural implications of the rare and predicted deleterious variant,

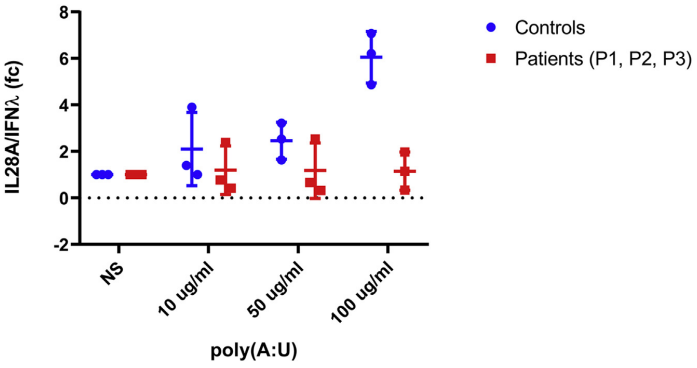

Fig. 5. PBMC responses to stimulation with dsRNA. IL28/IFNλ levels (fold change) were determined by ELISA in cell culture supernatants of PBMCs obtained from P1–P3 (red) and three healthy controls (blue). The cells were either unstimulated (NS) or stimulated with 10, 50 or 100 μg/ml poly(A:U) for 24 h. Broad and narrow horisontal bars show the mean and standard deviation, respectively, for each group per condition. Multiple t-tests were used to test for statistical differences between patients and controls, followed by the Holm-Sidak method to correct for multiple comparisons (**$P < 0.01$).

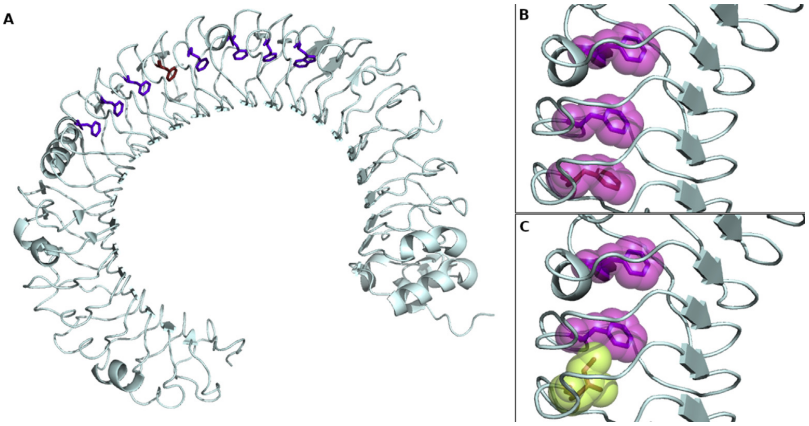

Fig. 6. Consequences of the F351I variant on TLR3 ectodomain protein structure. (A) Overview of the TLR3 ectodomain with the phenylalanine spine in purple (F351 in red). The importance of consecutive aromatic amino acids, in particular phenylalanine, in this regard is probably due to the favorable action of π-electrons on top and below the aromatic rings. This excess of electrons gives the face of the ring a small net negative charge, while the hydrogen atoms on the edge of the ring gets a correspondingly small positive charge. The aromatic rings of the consecutive phenylalanines therefore interact with each other with their rings in parallel but slightly offset so that the edge of each ring is interacting with the face of the next. (B) Close-up view of F351 (in red) and two of its neighbouring phenylalanines. (C) Modelling of the F351I variant (in yellow), indicating both a disruption of the phenylalanine spine, but probably also steric hindrance in the interior of the TLR3 ectodomain.

F351I. The F351 residue is part of a phenylalanine spine that helps stabilize the interior of the convex face of the horseshoe-shaped tertiary structure of the TLR3 ectodomain (Fig. 6A, B). This particular structural feature are conserved in many of the TLR proteins, but also in other proteins containing leucine rich repeat (LRR) domains [55, 56]. Changing the phenylalanine at residue 351 to an isoleucine will not only disrupt the spine of consecutive phenylalanines, but perhaps also destabilize the convex shape of the TLR3 ectodomain by steric effects (Fig. 6C).

3.9. Additional rare variants in the TLR3-IFN signaling pathway in AAD patients

As mutations in a number of genes in the TLR3-IFN pathway have been described to result in impaired TLR3 signaling [57], we reexamined our WES data to search for rare variants in these genes in AAD patients. Specifically, we searched for variants in the genes *IKBKE, IKBKG, IRF3, TBK1, TICAM1, TRAF3* and *UNC93B*, as none of these were among our original candidate list of 194 genes. As with *TLR3*, we focused on variants with frequencies less than 1% in gnomAD, 1000GP and our in house exome database, that received a CADD score >20. Both novel and previously described variants were discovered in *IRF3*, *IKBKE* and *TICAM1* (Table 3). Intriguingly, one AAD patient carried the previously described c.854G > A (p.Arg285Gln) mutation in *IRF3*, from here on referred to as R285Q. This autosomal dominant mutation, leading to haploinsufficiency, has previously been reported in an adolescent with HSE [58]. Furthermore, two AAD patients had a variant in *TICAM1*, whereby one of the variants (c.479C > T (p.Ser160Phe)) also has been described in an adult patient with HSE [50, 59].

4. Discussion

In the present study, we have identified and characterized several rare variants in *TLR3* and associated members of the TLR3-IFN signaling pathway in patients with AAD. The discovery of mutations in these genes indicates that inborn errors of immunity could influence the genetic susceptibility to develop AAD.

Our exome sequencing approach revealed five rare missense variants in *TLR3* in five different AAD patients. All variants were absent in healthy Norwegian controls, whereas their allele frequencies in a cohort of Norwegian cancer patients were ranging from 0 to 0.0019. Similar frequencies were found for the *TLR3* variants in the gnomAD database, ranging from 0 to 0.0017. To study if the identified variants displayed an impaired response against viral dsRNA in general, we designed and established a cell-based reporter assay using poly(I:C) as the stimulating factor. Of the five rare missense variants discovered, three were found to have significantly impaired function; F351I, an extremely rare variant carried by two of our patients, L742F, which has, together with F351I,

Table 3
Overview of rare variants of the TLR3-IFN signaling cascade (other than *TLR3*) found in AAD patients.

Variant annotation					Frequencies				*In silico* prediction tools				
Gene	Variant	Protein change	Chromosomal location GRCh37 (hg19)	Exon number	AAD frequency	In-house database frequency	NCGC[a] frequency	GnomAD frequency	SIFT	PolyPhen2	Mutation taster	PROVEAN	CADD[b]
IRF3	c.613G > A	p.Glu205Lys	NC_000019.9 g.501655574C < T	6	0.0038 (1/264)	0	0	0.000018	Tolerated	Possibly damaging	Disease causing	Neutral	24.6
NM_001571.5													
IRF3	c.854G > A	p.Arg285Gln	NC_000019.9 g.501653333C > T	6	0.0038 (1/264)	0	0.000063	0.000039	Deleterious	Probably damaging	Polymorphism	Deleterious	26.8
NM_001571.5													
IKBKE	c.275G > A	p.Gly92Arg	NC_000001.10 g.206648254G > A	5	0.0038 (1/264)	0	0	0	Deleterious	Probably damaging	Disease causing	Deleterious	28.9
NM_014002.3													
IKBKE	c.560G > A	p.Arg187Gln	NC_000001.10 g.206650040G > A	7	0.0038 (1/264)	0	0.0019	0.0013	Deleterious	Benign	Disease causing	Deleterious	24.4
NM_014002.3													
IKBKE	c.1393C > T	p.Leu465Phe	NC_000001.10 g.206653842C > T	13	0.0038 (1/264)	0.0033	0.00063	0.00050	Tolerated	Possibly damaging	Disease causing	Neutral	22.0
NM_014002.3													
TICAM1	c.479C > T	p.Ser160Phe	NC_000019.9 g.4817911G > A	2	0.0076 (2/264)	0.0016	0.0038	0.0022	Deleterious	Probably damaging	Polymorphism	Deleterious	23.1
NM_182919.3													
TICAM1	c.1537C > A	p.Leu513Met	NC_000019.9 g.4816853G > T	2	0.0038 (1/264)	0	0	0	Deleterious	Probably damaging	Disease causing	Neutral	23.3

[a] NCGC = Norwegian Cancer Genomics Consortium exome database, http://invitro.hpc.uio.no:8082/vcf-miner/.
[b] CADD (Combined Annotation Dependent Depletion) scores above 20 indicate that a variant is amongst the top 1% of deleterious variants in the human genome.

never been functionally characterized before, and R867Q, previously described to be causative (when homozygous) of HSE [31]. The reduced activity was probably not due to low levels of translated protein, as Western blot analyses revealed approximately the same levels of variant proteins as the WT. Instead, we believe that the variants displaying impaired function are disrupting key functional domains of the TLR3 protein. Using the available crystal structures of the TLR3 ectodomain, we were able to model the structural consequence of the F351I variant. It appears that F351 is a crucial part of the phenylalanine spine of the ectodomain, which serves to stabilize the interior of the convex side of the domain. Switching F351 into a non-aromatic amino residue, such as isoleucine, could therefore be detrimental to the stability of the ecto-domain. Unfortunately, no structural analysis could be made of L742F and R867Q, as these residues are located in domains of TLR3 that have not yet been structurally solved. It is possible that the exchange of the leucine at position 742 with a phenylalanine may affect the protein's stability, solubility, oligomeric state and proteolytic resistance [60]. Variants in this region have been reported as possibly pathogenic before, in particular the G743D variant in cis with the R811I variant was shown to result in autosomal dominant TLR3 deficiency by haploinsufficiency in a HSE patient [31]. The exchange of an arginine to an asparagine at position 867 makes the mutant protein partially dysfunctional [31]. The previous reporting of R867Q as a hypomorphic allele is consistent with our functional characterization of the variant.

Interestingly, four of the five patients carrying rare *TLR3* variants were compound heterozygous for an additional *TLR3* variant, except for the homozygous state of L412F in P3. For patients P2–P4, these genotypes resulted in an impaired signaling activity in our *in vitro* reporter assay. For three of the patients the additional allele was the common L412F variant which has been associated with a wide range of aberrant immune responses, including an APS-1-like phenotype characterized by cutaneous candidiasis, increased susceptibility to cytomegalovirus infections and autoimmunity, including AAD and hypothyroidism [61]. However, the L412F variant was not found to be associated with AAD in a Polish cohort of 168 AAD patients [62]. In our functional characterization of the TLR3 variants, we could not detect any functional defect of L412F compared to the WT, which is in agreement with some previous reports [63], but in contrast to others [64,65]. On the other hand, L412F did not rescue the TLR3 activity when co-expressed with the hypomorphic alleles F351I, L742F and L412F + R867Q. Finally, we demonstrated that PBMCs from P1–P3 had impaired responses to dsRNA, as revealed by a significant loss in production of IL28A/IFNλ2.

Although TLR3 deficiency and other inborn errors of the TLR3-IFN signaling pathway have been shown to confer defects in innate immunity, their ascribed clinical phenotypes appear surprisingly narrow. Apart from infections of the CNS by HSV-1, HSV-2 and probably varicella zoster virus (VZV), potentially leading to encephalitis, TLR3 and associated partners appear functionally redundant for immunity against most other viruses [63,66]. This may be due to the extensive activation of cytosolic RNA sensors, including MDA5 (encoded by the gene IFIH1) and RIG-I [67], when TLR3 is deficient. At the same time, a wide range of immunopathologies and immunological traits have been associated with common polymorphisms in *TLR3* [68], strongly suggesting that genetic variations in TLR3 induce immunological phenotypes that eventually lead to immune-mediated diseases.

Interestingly, in addition to variants in *TLR3*, we also identified AAD patients carrying previously described variants in genes encoding other proteins in the TLR3-IFN signaling pathway: The autosomal dominant variant R285Q in IRF3 [58], and several other, both rare and novel, missense variants in *IRF3*, *TICAM1* and *IKBKE*.

Identifying rare genetic variants that drive the onset of a disease is challenging, and a central question here would be if the identified variants in the TLR3-IFN signaling pathway play any etiological roles in AAD. Importantly, approaches typically used for testing associations of genetic variants with phenotype based on simple regression models are

underpowered for rare variants [69]. Although numerous studies have demonstrated the ability of WES to identify low frequency variants associated with autoimmune disease, it is difficult to conclude any association when only a few individuals carry the variants. In addition, studying the genetic basis of complex diseases such as AAD is challenging because the genotype of a patient does not necessarily clearly correlate with the phenotype; phenocopies may occur, meaning that environmental factors could induce an AAD phenotype in a patient without known disease susceptible variants that matches the phenotype of a patient that does carry such variants. This may explain why none of our patients carrying *TLR3* variants, exhibit a unique phenotype compared to the other 127 AAD patients. Hence, several different variants could induce the same phenotype, and multiple of these may be necessary to produce it [8]. On the other hand, even though the genotype does not correlate with the phenotype, we could speculate that it may predict the etiology of the disease. In that case, patients with damaging variants in innate immune genes may share the same triggers, such as viral infections, initiating or perpetuating the immunological attack of adrenal tissue [70].

Another caveat in our study is the presence of the *TLR3* variants in unaffected carriers and family members, indicating incomplete penetrance, which is a common phenomenon for mutations associated with AAD [8]. The impact of rare *TLR3* variants may therefore seem relative subtle when standing alone, but could confer an increased risk of developing AAD when present together with HLA risk alleles. In this regard, it should be noted that the patients (P1 and P3) sharing *TLR3* genotypes with healthy siblings, both carried the HLA class II haplotypes giving the highest risk to develop AAD. However, incomplete penetrance seems to characterize the majority of mutations in the TLR3 pathway causing HSE, especially for the autosomal dominant mutations involving haploinsufficiency [57,58].

Taken together, our findings support that decreased or aberrant antiviral defense is associated with risk for AAD. This observation is consistent with previous research showing that patients with primary adrenal insufficiency have higher intake of antimicrobial agents, especially antifungal and antiviral drugs, and increased hospital admission as a result of infection compared to controls [17]. Moreover, the adrenal cortex has previously been shown to be permissive to a variety of viral, fungal and bacterial agents [71–75]. Decreased resistance or inappropriate responses to viruses can therefore lead to a persistent viral infection in the adrenal glands, resulting in destruction of adrenocortical cells, which may initiate an autoimmune process [1]. AAD may therefore only develop if there is an adrenal infection that effectively triggers the condition. The specific pathogens involved in this process, however, are yet to be discovered. Interestingly, experimental infections with HSV-1 in mice have shown that the adrenal cortex is among the first locations colonized by the virus [74]. Given the previous shown association between *TLR3* mutations and HSE, we tested for an increased susceptibility to HSV-1 infections in AAD affected individuals. However, no significant difference in IgM and IgG antibodies against HSV-1 between patients and healthy controls was detected (data not shown).

In conclusion, this study provides the first identification of several rare and damaging variants in the TLR3-IFN pathway in patients with AAD and highlights the significance of studying the interplay between genes and viral infections in immune mediated diseases.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2019.100005.

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CASE REPORT
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Coexistence of Congenital Adrenal Hyperplasia and Autoimmune Addison's Disease

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Background: Underlying causes of adrenal insufficiency include congenital adrenal hyperplasia (CAH) and autoimmune adrenocortical destruction leading to autoimmune Addison's disease (AAD). Here, we report a patient with a homozygous stop-gain mutation in 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), in addition to impaired steroidogenesis due to AAD.

Case Report: Whole exome sequencing revealed an extremely rare homozygous nonsense mutation in exon 2 of the *HSD3B2* gene, leading to a premature stop codon (NM_000198.3: c.15C>A, p.Cys5Ter) in a patient with AAD and premature ovarian insufficiency. Scrutiny of old medical records revealed that the patient was initially diagnosed with CAH with hyperandrogenism and severe salt-wasting shortly after birth. However, the current steroid profile show complete adrenal insufficiency including low production of pregnenolone, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEA-S), without signs of overtreatment with steroids.

Conclusion: To the best of our knowledge, this is the first description of autoimmune adrenalitis in a patient with 3βHSD2 deficiency and suggests a possible association between AAD and inborn errors of the steroidogenesis.

Keywords: adrenal insufficiency, congenital adrenal hyperplasia, 3β-hydroxysteroid dehydrogenase type 2 deficiency, autoimmune adrenalitis, autoimmune Addison's disease

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INTRODUCTION

3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) catalyzes the conversion of Δ5-steroids into Δ4-steroids (**Figure 1**). Deficiency of 3βHSD2 causes a rare autosomal recessive form of congenital adrenal hyperplasia (CAH) characterized by high levels of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and androstenediol, and lack of cortisol, aldosterone, and androstenedione (1). There are two isozymes of 3βHSD encoded by *HSD3B1* and *HSD3B2*. 3βHSD2 is expressed in the gonads and the adrenal cortex, whereas 3βHSD1 is expressed in peripheral tissues, converting circulating DHEA to testosterone (1). 3βHSD2

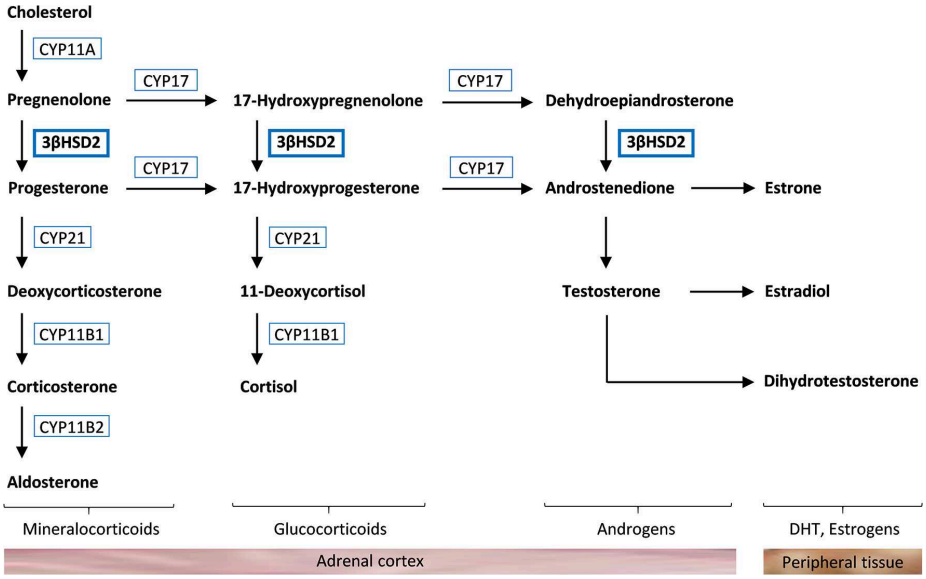

FIGURE 1 | Steroidogenesis in the adrenal cortex. Cholesterol is converted to aldosterone, cortisol, and androgens through different pathways that require specific enzymes [cholesterol side-chain cleavage enzyme (CYP11A), 17α-*hydroxylase* (CYP17), 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2), 21-hydroxylase (CYP21), 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2)]. Androstenedione and testosterone are further converted to dihydrotestosterone (DHT) and estrogens in peripheral tissue.

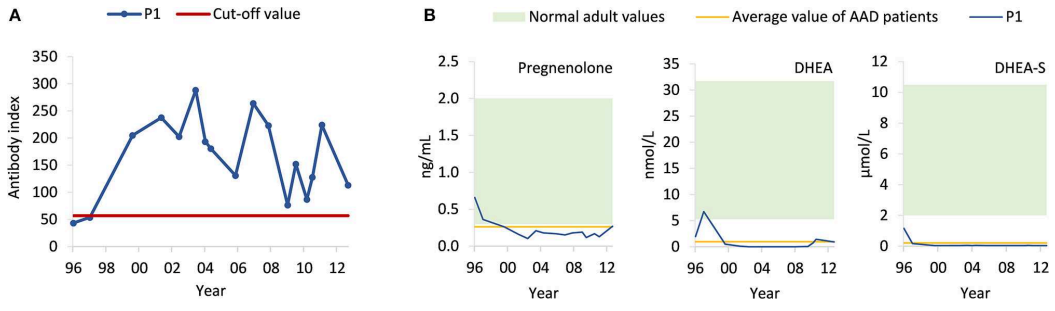

FIGURE 2 | Levels of circulating 21OH autoantibodies in the patient (P1) and steroid profiling. **(A)** Using radioimmunoprecipitation assay, levels of 21OH autoantibodies (blue) were measured in serum samples taken at different time points from 1996 to 2013. The cut-off value (red) was set to obtain the maximal accuracy as calculated by an interlaboratory study (6). **(B)** Using ELISA, levels of pregnenolone, DHEA, and DHEA-S were measured in serum samples from the patient (P1, blue) taken from different time points from 1996 to 2013. Normal adult values (green) are based on established reference values from the textbook "Gynecologic Endocrinology" (pregnenolone) (7), hormone laboratory at Oslo University Hospital (DHEA) (https://ehandboken.ous-hf.no/api/File/GetFile?entityId=105475) and hormone laboratory at Haukeland University Hospital (DHEA-S) (https://analyseoversikten.no/analyse/14). The average value of AAD patients (yellow) is measured from serum samples of patients included in our biobank.

deficiency can therefore cause relatively high levels of testosterone in females, whereas it cannot compensate for the absence of adrenal and gonadal synthesis of testosterone in males. This causes ambiguous genitalia in males, whereas female newborns exhibit mild virilization or normal sexual differentiation, and may remain undiagnosed until a salt-wasting crisis occurs (1–3).

Adrenal insufficiency can also be due to autoimmune adrenalitis, or autoimmune Addison's disease (AAD), characterized by an immunological attack of the adrenal cortex leading to decreased production of cortisol and aldosterone (4). The self-antigen 21-hydroxylase (21OH) is the dominant target of adrenal autoantibodies and autoreactive T cells (4). Therefore, autoantibodies against 21OH and

low serum cortisol levels are important diagnostic markers for AAD.

To the best of our knowledge, we here report the first patient affected by both inborn 3βHSD2 deficiency and acquired AAD.

CASE REPORT

A whole-exome sequencing study involving 142 AAD patients (5) revealed a patient with a rare homozygous mutation in exon 2 of *HSD3B2* [frequency ∼0.00003 in the Genome Aggregation Database (gnomAD)] at nucleotide position 15 (NM_000198.3:c.15C>A), resulting in the exchange of the cysteine codon to a premature stop codon (p.Cys5Ter). Subsequent screening of the Norwegian Addison Registry identified the patient harboring the mutation, a 55-year-old female with AAD, premature ovarian insufficiency and vitamin B12 deficiency, accompanied by 21OH- and parietal cell autoantibodies (**Figure 2A**). Notably, she did not carry any of the major histocompatibility complex (MHC) alleles conferring high risk to develop AAD (4). She tested negative for other autoantibodies such as anti-thyroid peroxidase, and had normal levels of thyroid stimulating hormone (0.40–4.50 mIU/L). Scrutiny of early medical records showed that

TABLE 1 | Steroid profile of the patient.

Steroids	LLoQ (nM)	Patient serum sample
Progesterone	0.114	<LLoQ
11-deoxycorticosterone	0.023	<LLoQ
Corticosterone	0.114	<LLoQ
Tetrahydrocorticosterone	0.114	<LLoQ
18-hydroxycorticosterone	0.069	<LLoQ
Aldosterone	0.0023	<LLoQ
Tetrahydroaldosterone	0.062	<LLoQ
17-hydroksyprogesterone	0.114	<LLoQ
11-deoxycortisol	0.114	<LLoQ
21-deoxycortisol	0.023	<LLoQ
Cortisol	0.914	<LLoQ
Tetrahydrocortisol	0.114	0.308
5α-tetrahydrocortisol	0.114	0.187
18-hydroxycortisol	0.046	<LLoQ
18-oxocortisol	0.046	<LLoQ
Cortisone	0.914	<LLoQ
Tetrahydrocortisone	0.343	<LLoQ
5α-tetrahydrocortisone	0.343	<LLoQ
Dehydroepiandrosterone	0.617	<LLoQ
Dehydroepiandrosterone sulfate	22.862	<LLoQ
Androstenedione	0.023	<LLoQ
Testosterone	0.023	0.066
Dihydrotestosterone	0.023	<LLoQ
Epitestosterone	0.023	<LLoQ

Overview of the levels of 24 analytes in a fasting serum sample from the patient, including the Lower Limit of Quantification Levels (LLoQ) for each analyte. The analysis was performed using LC-MS/MS.

TABLE 2 | Overview of rare variants of *HSD3B2* (NM_000198.3) found in AAD patients.

Mutation annotation				Frequencies				*In silico* prediction tools				
***HSD3B2* variant**	**Protein change**	**Chromosomal location NC_000001.10 GRCh37 (hg19)**	**Exon number**	**AAD cohort**	**In-house exome database**	**NCGC[a]**	**gnomAD[b]**	**SIFT[c]**	**PolyPhen2[c]**	**Mutation taster[c]**	**PROVEAN[c]**	**CADD[c]**
c.15C>A	p.Cys5Ter	g.119958057C>A	2	0.0076	0	0	0.000032[d]	NA	NA	Disease causing	NA	36.0
c.707T>C	p.Leu236Ser	g.119964831T>C	4	0.0038	0	0	0.0038	Tolerated	Benign	Polymorphism	Neutral	13.74
c.931C>T	p.Gln311Ter	g.119965055C>T	4	0.0038	0	0	0.0000040	NA	NA	Disease causing	NA	34.0
c.995A>C	p.Lys332Thr	g.119965119A>C	4	0.0038	0	0	0	Deleterious	Probably damaging	Disease causing	Deleterious	25.0

[a] *NCGC, Norwegian Cancer Genomics Consortium exome database, http://invitro.hpc.uio.no:8082/vcf-miner/.*

[b] *gnomAD, The Genome Aggregation Database, https://gnomad.broadinstitute.org/.*

[c] *In silico variant pathogenicity predictors: SIFT (9), PolyPhen2 (10), MutationTaster2 (11), PROVEAN (12), and CADD (Combined Annotation Dependent Depletion) scores above 20 indicate that a variant is amongst the top 1% of deleterious variants in the human genome (13).*

[d] *The variant has previously been reported in gnomAD, but never in a homozygous state as in this case report.*

she exhibited hyperpigmentation of genitalia and clitoris hypertrophy already at birth. One week of age, she started to vomit and developed hyponatremia (127 mmol/L) and hyperkalemia (6.1 mmol/L). Elevated levels of 17-ketosteroids were detected. She was therefore diagnosed with CAH and supplemented with cortisone acetate and eventually fludrocortisone. Her sister had also been diagnosed with CAH, but unfortunately died in an adrenal crisis at 2 years of age.

Given these conflicting findings, computer tomography (CT) and magnetic resonance (MR) scans, taken from age 43 to 51, were re-evaluated and the adrenals were found to be in the lower range of normal thickness 2–3 mm, indicating adrenocortical atrophy rather than hyperplasia as would be expected in case of isolated CAH. We then obtained a steroid profile by liquid chromatography tandem mass spectroscopy (LC-MS/MS) of a serum sample taken after an overnight medication fast (**Table 1**). Levels of all mineralocorticoids, and most glucocorticoids and androgens were below the detection limit, except for tetrahydrocortisol (0.308 nmol/L), 5α-tetrahydrocortisol (0.187 nmol/L), and testosterone (0.066 nmol/L). Total pregnenolone, DHEA and DHEA-S levels were measured by enzyme-linked immunosorbent assay (ELISA) from previously collected serum samples spanning the years 1996–2013 (**Figure 2B**). Normal levels of pregnenolone and DHEA were found in samples from the first 2 years, but then levels decreased toward the subnormal levels generally seen in AAD patients. DHEA-S was also below the normal range, typical of AAD patients, at all time points. ACTH was measured at several time points, revealing elevated levels on multiple occasions. The highest levels were observed in 2011 at 130 pmol/L (normal range 2.0–11.6 pmol/L). Importantly, we detected no subsequent increase in pregnenolone, DHEA, and DHEA-S in spite of elevated ACTH levels (**Figure 2B**).

She is currently, at age 57, treated with 20 mg hydrocortisone (Plenadren™) and 100 μg fludrocortisone, and does not have suppressed ACTH-values.

DISCUSSION

This is the first report of a patient with primary adrenal insufficiency due to both 3βHSD2 deficiency and AAD. At inclusion in the national Addison registry, she was classified as having AAD with vitamin B12 deficiency, and 21OH- and parietal cell autoantibodies. However, genetic screening and scrutiny of old records revealed a rare form of CAH due to a stop-gain mutation in *HSD3B2*. The presence of small sized adrenal glands instead of hyperplastic glands, normally seen in CAH, and no overproduction of Δ5-steroids following elevated ACTH levels, suggest that the adrenal cortex is not functioning. Presence of tetrahydrocortisol and 5α-tetrahydrocortisol is consistent with her ongoing replacement therapy which includes hydrocortisone. The low, but detectable, level of testosterone may be due to conversion of DHEA by 3βHSD1 in the periphery. Previously measureable pregnenolone levels suggest that the autoimmune destruction of the adrenal cortex commenced sometimes in the 1990-ties.

According to literature, we could only find one reported case of CAH occurring together with complete adrenal cortex insufficiency suspected to be autoimmune adrenalitis. This patient, however, had neither 21OH autoantibodies, nor MHC risk alleles, but was positive for autoantibodies against 17α-hydroxylase (8). Therefore, we speculate there might be other rare unreported cases of autoimmune adrenalitis due to early diagnosis of CAH, masking the clinical symptoms of AAD. Interestingly, several other AAD patients included in our exome sequencing analysis carry rare heterozygous non-synonymous variants in *HSD3B2* (**Table 2**). Although it appears that family members of CAH patients, carrying heterozygous *HSD3B2* mutations, maintain normal 3βHSD2 activity *in vivo* (14), genetic variations in *HSD3B2* are associated with other conditions such as idiopathic hypospadias and prostate cancer (15, 16). Therefore, both subtle molecular abnormalities and deleterious mutations in *HSD3B2* could have biological consequences, and may play a role in the pathogenesis of the immune-mediated adrenocortical destruction in AAD.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The name of the repository and accession number(s) are not available.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committee for Medical and Health Ethics. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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