The role of autoantibodies in cell mediated autoimmune Addison`s disease

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Ragnhild Stenberg Berg



Institute of Medicine and Department of Biomedicine Faculty of Medicine and Dentistry University of Bergen June 1. 2012

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Abstract

Background: Addison's disease (primary adrenal insufficiency) is a prototypic organ-specific autoimmune disease affecting the adrenal cortex. Autoimmune Addison's disease (AAD) is often characterized by autoantibodies against intracellular cytochrome p450 enzymes, in particular 21-hydroxylase (210H) which is central in the steroid hormone synthesis of cortisol and aldosterone. AAD is thought to be T-cell mediated, but these 210H-autoantibodies (210H-aab) may still play a central role. Our hypothesis is that 210H-aab may contribute to an enhanced T-cell response against 210H leading to an autoimmune attack against the adrenocortical cells.

Aim: This study aimed to investigate the role of autoantibodies in antigen processing and presentation in the pathogenesis of AAD.

Methods: Total IgG from patients and healthy controls were purified chromatographically and were detected as 21OH-aab by western blot and ELISA analyzes. The ability of 21OHaab to enhance T-cell responses against 21OH was evaluated in an *ex-vivo* interferon-gamma (IFN- γ) ELISPOT assay, using peripheral blood mononuclear cells (PBMC) pre-stimulated with 21OH protein or 21OH protein in complex with 21OH-aab. LPS-matured monocyte derived dendritic cells (DC) pulsed with 21OH protein alone, or 21OH in complex with 21OH-aab were used to established short-term CD4⁺ and CD8⁺ T-cell lines. These T-cell lines were then tested against a 21OH peptide panel to determine if 21OH-aab could influence the repertoire of 21OH-derived peptides presented by DCs. Again, the production of IFN- γ , assayed by ELISPOT and ELISA, was used as readout for T-cell activation. In addition, the cytotoxic potential of the short-term CD8⁺ T-cell lines were evaluated in a lactate dehydrogenase (LDH) assay employing adrenocortical carcinoma cells, lymphoblastoid B cell lines, or autologous PBMC as targets.

Results and conclusions: High levels of 21OH-aab was detected in Addison's patients compared to healthy controls, and western blot analyzes confirmed that purified IgG-aab bound strongly to 21OH. The *ex-vivo* ELISPOT analyzes revealed an enhanced T-cell response in the presence of 21OH-aab. IFN- γ stimulated monocytes expressed both CD32 and CD64 Fc γ -receptors and blockade of these lead to a decreased T-cell response, suggesting for a more efficient uptake of immune complex through these receptors. However B-cells cannot be excluded due to their strong expression of CD32 receptors. T-cell lines induced by DCs pulsed with 21OH protein in complex with autoantibodies produced more IFN- γ when restimulated with 21OH protein and peptides compared to T-cell lines pulsed with 21OH protein and peptides compared to T-cell lines was also enhanced in the presence of autoantibodies. To conclude 21OH-aab may have an important role in the pathogenesis of AAD.

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Abbreviations

17-OH – 17-hydroxylase 21-OH - 21-hydroxylase AAD – Autoimmune Addison's disease ACTH – Adrenocorticotropic hormone ADCC – Antibody-dependent cell mediated cytotoxicity AP – Alkaline phosphatase APC – Antigen presenting cell APS 1/2 – Autoimmune polyendocrine syndrome type 1/2BCR – B-cell receptor B-cell – Bone marrow derived lymphocyte CRH - Corticotrophin releasing hormone CTLA-4 – Cytotoxic T-Lymphocyte Antigen 4 DC – Dendritic cell DMSO – Dimethyl sulfoxid ER - Endoplasmic reticulum FcyR – Fc-gamma receptor FDC – Follicular dendritic cells FITC – Fluorescein isothiocyanate GC – Germinal center GM-CSF - Granulocyte monocyte colonystimulating factor H295R – Human adrenocortical cancer cell HLA - Human leukocyte antigen

IFN-γ – Interferon gamma Ig – Immunoglobulin IL – Interleukin LPS – Lipopolysaccharide MHC – Major histocompatibility complex NK-cell – Natural killer cell line (NCI-H295R) PBMC - Peripheral blood mononuclear cells PAMP - Pathogen-associated molecular patterns PRR – Pattern recognition receptors RT – Room temperature SCC – Side chain-cleaving SD - Standard deviation T1D – Type 1 diabetes T-cell – Thymus derived lymphocyte TCR – T-cell receptor $T_{\rm H}1/2 - T$ -helper lymphocyte TLR – Toll-like receptors Treg – T-regulatory lymphocyte TSA - Tissue-restricted antigens/selfantigens UoB – University of Bergen

1. Introduction

1.1 The immune system

The immune system is a complex system with many interactions, containing numerous components specialized to defend the body against infections by microorganisms such as bacteria, viruses, fungi and parasites [1]. When a pathogen invades an organism it has to overcome several hindrances represented by the innate and adaptive immune system in order to colonize the host and cause disease [2]. The innate immune system responds fast to invading microorganisms and alerts the antigen specific adaptive immune system to generate immunological memory against microorganisms. Immunological memory is the ability for clones of antigen-specific lymphocytes to respond rapidly and efficiently against a previously encountered pathogen [1, 3]. For an adequate immune response it is therefore necessary with a proper communication between the innate and adaptive immune system.

Lymphocytes have a major role in the immune system and are divided into two groups based on their place of development - thymus derived lymphocytes (T-cells) and bone marrow derived lymphocytes (B-cells) [2, 4]. Activation of lymphocytes by antigens happens in spleen and lymph nodes in distinctive T- and B-cells compartments. T-cells are activated through T-cell receptors (TCR) on their surface and are differentiated into effector cells in order to kill other cells or to activate or regulate a T-cell response depending on the activating signal [1]. B-cells may in turn be activated by T-cells after recognition of antigen by their Bcell receptor (BCR) and are important for both long-lasting and specific protection of antigens. Activated B-cells may differentiate into plasma cells to produce antigen-specific antibodies or into specific memory cells making a protection for a secondary infection [5].

1.1.1 The innate immune system in control of adaptive immunity

The innate immune system consist of both cellular and molecular components with different effector functions, such as phagocytic cells (macrophages and neutrophil granulocytes), natural killer cells, acute phase proteins, cytokines such as interleukins (IL), and components of the complement system [2]. Furthermore, the innate immune system has two overall functions; to respond fast to microbes and to stimulate the adaptive immune system [2, 3]. The innate immune system is activated immediately during an invasion of pathogens by instant recognition without prior encounters with the immune system [1].

Invariant germ-line encoded pattern recognition receptors (PRR) contribute to discrimination between self- and foreign molecules by binding to conserved molecular patterns present on microorganisms called pathogen-associated molecular patterns (PAMP) [3, 6]. Depending on the function, PRR may be divided into endocytic receptors which are expressed on phagocytes to endocytose pathogens into lysosomes for degradation and peptide processing prior to presentation on the major histocompatibility complex (MHC) class II (Figure 1.1).

Another PRR is the toll-like receptor (TLR) which also works as signaling receptors binding to PAMP. Toll-like receptor (TLR)-4 was the first human TLR to be identified and is essential for the recognition of lipopolysaccharide (LPS) [3]. LPS is one of the best known PAMP and is found in the cell wall of all gram-negative bacteria [6]. PAMP-activated TLR stimulate a transduction signal inside the antigen presenting cell (APC) enhancing the expression of immune-response genes. The APC will start expressing costimulatory molecules on the cell surface and producing cytokines and co-stimulatory molecules which in turn will activate T-cells of the adaptive immune system [2, 3]



Figure 1.1: Activation of the adaptive immune system through innate immunity. Pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide (LPS) are recognized by pattern recognition receptors (PRR); endocytic receptors and Toll-like receptors (TLR) on an antigen presenting cell (APC). Pathogen is endocytosed and processed in lysosomes and presented as peptides on major histocompatibility complex (MHC) class II to a T-cell. Simultaneously cytokines are secreted and costimulatory molecules, B7, are expressed on the APC surface binding to CD28 to activate T-cells to proliferate and differentiate into effector cells. Reproduced from [3].

1.1.2 Adaptive immunity

The adaptive immune system is based on clonal selection from a repertoire of lymphocytes bearing highly diverse antigen-specific receptors that enable the immune system to recognize any foreign antigens [3]. The antigen-specific receptors, such as the TCR and the BCR, have a large range of variety as they are somatically generated in a process where multiple gene fragments are set together in several different combinations [2].

APC and T-cells are both involved in the initiation of the adaptive immune response. Processed peptides in APC are presented to T-cells and recognized trough their TCR [2]. Even though the adaptive primary response is slow the immunological memory provides a more rapid secondary response by a subsequent encounter where memory T-cells specific for the pathogen in question have a lower activation threshold [1].

1.1.2.1 How are antigens presented by antigen presenting cells to T-cells?

Professional APC is experts in the uptake of antigens and to present them to lymphocytes to provide their proliferation and differentiation. Dendritic cells (DC), macrophages and B-cells are the main cell types involved in the presentation of antigens to naïve T-cells [2]. These cells vary in their means of antigen uptake, MHC class II expression and their localization (Table 1).

	Dendritic cell	Macrophage/monocyte	B-cell
Location	Organs and epithelial barrier tissues	Lymphoid tissue, connective tissue and body cavities	Lymphoid tissue and peripheral blood
Antigen uptake	Pinocytosis, phagocytosis by toll-like receptors	Phagocytosis	BCR-mediated uptake
MHC class II- expression	Normal, increased by maturation and IFN- γ	Low or none, effected by IFN-γ	Normal, increased by IL-4
Characteristics, when activated	Migration to regional lymphoid tissues for peptide-presentation of internalized proteins to naïve T-cells.	Phagocytosis of microbes and production of cytokines, activating and attracting inflammatory cells activating macrophage lead to killing of microbe	Antigen presentation to CD4 ⁺ helper T-cells leading to activation of effector T- cells and subsequently B-cell activation and antibody production
Immune response	Clonal expansion and differentiation of effector T-cell	Cell mediated immunity	Humoral immunity

Table 1: Antigen presenting cells[7, 8].

Antigens may be recognized by DC in peripheral tissues trough PRR and engulfed trough phagocytosis [6]. The APC then become activated and up-regulates the expression of costimulatory molecules, such as B7 (CD80 or CD86) which together with the antigen receptor is necessary for lymphocyte activation [1, 2]. Activated DC migrates to local lymph

nodes to present processed antigen-peptide to lymphocytes through the MHC class II receptor [1, 2].

The MHC genes consist of a linked set of genetic loci encoding many of the proteins involved in antigen presentation to T-cells, including the MHC class I and II proteins that present peptides to the TCR [9]. MHC class I is expressed on all nucleated cells and peptides presented by these are targeted by CD8⁺ T-cells. Activated CD8⁺ T-cells will kill the target cell presenting the relevant peptide via MHC class I [10]. The APC are presenting intracellular molecules to CD8⁺ T-cells and the MHC class I are divided into isoforms A, B and C [1].

MHC class II molecules are usually only expressed on professional APC and may promote Tcell activation and differentiation. The T-cells in turn may activate B-cells to start producing antibodies as well as produce cytokines to help other parts of the adaptive immune response. DR, DQ and DP are genes coded by MHC class II and present extracellular antigens to T-cells [1].

T-cell activation requires a persistent contact between the TCR on the surface of naïve T-cells and the peptide presented by an MHC molecule on APC. The peptides recognized by the TCR are linear and seated in the groove of the MHC molecule after its antigen processing inside the APC [1, 11].

The TCR is a membrane-anchored heterodimer glycoprotein containing covalently bound α and β -chain, where each has a constant and variable domain (Figure 1.2) [2]. The constant domains mediate effector functions while the variable domains are responsible for the variety in the antigen binding site and antigen specificity together with the CDR hyper variable loops. The cytoplasmic domain of the TCR are too small and lack the signal transduction activity, it`s non-covalently bound to CD3. CD3 consists of four polypeptide chains; γ , δ , ε and ζ , in a TCR complex which are required for transduction of activating signals further into the cell [11, 12].



Figure 1.2: T-cell receptor (TCR)-complex. The TCR consists of a complex with interacting molecules required for both extracellular antigen recognition and intracellular signaling. The TCR consist of a heterodimer α - and β -chain and the CD3 complex containing four polypeptide chains; γ , ζ , ε , and δ which are important for the intracellular signal transduction. Modified from [13]

1.1.2.2 Activation of T-lymphocytes

When a TCR binds to a peptide-MHC complex tyrosine residues on the cytoplasmic domain of CD3 are phosphorylated, leading to a signal transduction into the nucleus (Figure 1.3, signal 1). Simultaneously, the co-receptor molecules CD4 or CD8 expressed on T-cells, binds to the MHC molecule [9].

PAMP activation of PRR induces APC to up-regulate the expression of adhesion molecules CD80/86 also known as B7, leading to co-stimulation which is necessary for a stable binding between the two cells (Figure 1.3, signal 2) [14]. B7 expressed on APC may bind to CD28 or CTLA (Cytotoxic T-Lymphocyte Antigen)-4 on the T-cells. CD28 transmits a stimulatory response while the CTLA-4 transmits an inhibitory stimulus weakening and eventually turning off the T-cell response. The signal transduction explained above may activate genes for cytokines which are released and may in turn activate proliferation or further differentiation of T-cells. Secreted IL-12 from the APC binds to the IL-12 receptor on the T-cell and may promote development of T_{H} -cells or CTL which again will secrete IFN- γ when activated (Figure 1.3, signal 3) [11, 1].



Figure 1.3: Activation of a T-cell in three steps. A T-cell is usually activated in three steps by an APC. When an antigen is presented on the MHC class on APC it attracts naïve T-cells which bind to the peptide-MHCcomplex. To maintain the bond between the cells for a complete activation, the APC up-regulate its expression of stimulating molecules such as CD80/86 (B7) against T-cell surface molecules. Depending on the antigen and the interacting T-cell the APC then secretes cytokines, here IL-12, which will activate the T-cell to secrete IFN- γ and thereby stimulate it further into effector cells. Modified from [1].

T-cells may be divided into two subgroups based on their expression of $CD4^+$ - and $CD8^+$ -coreceptors interacting with MHC class II and I, respectively. $CD4^+$ T-cells may be divided into several types of effector T-helper cells (T-_H1, T-_H2 and T-_H17) with different functions (summarized in Table 2) [1]. Regulatory T-cells (T-reg) that regulate the activation of other T-cells also express $CD4^+$ -receptors and are necessary for maintaining the peripheral tolerance to self-antigens. $CD8^+$ T-cells consist of cytotoxic T-cells (CTL) which kill infected cells that present peptides from intracellular viral protein and intracellular microbes bound to class I MHC molecules [2, 15].

Table 2: An overview of subgroups of CD4⁺ T-lymphocytes. Activated T-cells produces large amounts of cytokines which is used to discriminate between the different T-cell populations. CD4⁺ T-cells are divided into $T_{-H}1$, $T_{-H}2$, $T_{-H}17$ and T-reg based on their different functions and cytokine secretion profile [16-19].

	Effector function	Secretes
T _H -1	Cell mediated immunity, Anti-tumor response and T-cell mediated cytotoxicity, viral and bacterial infections. May be involved in autoimmunity	IL-2, IFN-γ, TNF-α/β
<i>T_H-2</i>	Humoral immunity, extracellular defense, protective against infections with helminthes. May be involved in allergies	IL-4-6, IL-10, IL-13
T _H -17	Protective against certain bacterial and fungal infections. May be involved in autoimmunity	IL-17A, IL- 17F, IL-21-22
Treg	Maintaining peripheral tolerance to self-antigens	IL-10, TGF-β

1.1.2.3 Activation of B-cells and production of antibodies

B-cells are developed in the bone marrow and may work as antigen presenting cells by ingesting antigens and displaying them to T-cells to induce an immune response. In order to perform these tasks B-cells express several membrane molecules such as the BCR-complex which have a critical role in the antigen uptake (Figure 1.4).

The BCR is an immunoglobulin (Ig) made up of two heavy chains and two light chains that are bound together by a disulfide binding [2, 20]. BCR binds extracellular antigens in hypervariable regions and the binding site is only 600-1700Å where only the antigenic epitope, which is the antigen structure recognized by the antigen receptor, will manage to fit [11]. Complex antigens have therefore several epitopes, like a mosaic structure [2].

BCR are rearranged by somatic recombination giving a great variation in antigen recognition as B-cells are programmed to express only one kind of antibody against a single antigen [2]. Circulating BCR is referred to as antigen-specific antibodies secreted by plasma cells. The membrane-bound BCR is associated with the signal transduction proteins Ig α (CD79a) and Ig β (CD79b) with immunoreceptor tyrosine activation motifs (ITAM`s) on their cytoplasmic tails [11, 20]. ITAM enable intracellular signaling and are required for signal transduction of the extracellular antigen into the cell and for the transport of the BCR to the B-cell surface [2].



Figure 1.4: The B-cell receptor (BCR)-complex. The B-cell receptor is an immunoglobulin (IgG) with both variable and constant domains and is associated with Ig α and Ig β (BCR) molecules in a B-cell receptor complex which are important for the intracellular signal transduction. Modified from[13].

Naïve B-cells circulate in peripheral lymphoid tissue and blood to encounter their specific antigen and maturate into effector cells. Activated B-cells migrate into germinal centers (GC) of the secondary follicle, areas in spleen and lymph nodes, for presentation of antigens by a

meshwork of follicular dendritic cells (FDC). FDC is a cell population in GC expressing high levels of adhesion molecules [5]. The antigen engagement lead to a cross-linkage of BCR's and ITAM on the Iga/ β -sheaths are brought close together and phosphorylated by the Src kinase family Lyn [5, 8]. Phosphorylated ITAM's lead to signal transduction that may induce gene expression and internalization of the antigen. The BCR-genes have undergone somatic hypermutation and class switching during B-cell proliferation in response to different stimuli from FDC. Ingested antigen is processed within endosomes and loaded on the MHC molecule before it is presented on the cell surface.

Then a helper T-cell will activate the B-cell in a cell-to-cell interaction by binding to a costimulatory molecule CD40 on the B-cell. Cytokine secretion by T_{H} -cell will provide further activation and induce generation into memory B-cells and plasma cells (Figure 1.5) [5]. Memory B-cells encounter antigens in the GC and will prevent a second infection against an already encountered antigen [5]. Plasma cells are immunoglobulin-secreting cells and produce large numbers of antigen-specific antibodies which are released into the circulation to neutralize the encountered antigen [2].



Figure 1.5: Activation of B-cells in a T-cell dependent manner. B-cells are APC and express BCR and MHC class II which are recognized by an active T-cell receptor. Costimulatory molecules, CD40 and CD40 ligand enhance the activation together with cytokine production, here IL-10. Modified from [21].

1.1.2.4 Antibodies

Antibodies are circulating proteins known for their incredible capacity in recognizing different structures on foreign antigens. Compared to other antigen binding structures such as TCR and MHC, antibodies may discriminate between multiple antigens in a wider range and may also bind to antigens in a greater strength [2].

The antibody is made up of two identical polypeptide heavy chains and light chains bond together by disulfide bonds making a Y-formation with both constant and variable domains (Figure 1.6). The antigen binding site is localized at the N-terminal containing both a light and heavy chain, each with three hypervariable regions where the compositions of different amino acids give rise to the high diversity in antigen recognition [2].

The antigen binding site together with a heavy and light constant domain makes the antigen binding fragment Fab, which if separated from rest of the antibody still may recognize antigens [22]. The effector functions to the antibody are localized to the constant domains of the heavy chain C-terminals. The amino acid sequence of the constant domain determine which immunoglobulin class the antibody belongs to; IgM, IgG, IgA, IgD or IgE. The outcome of an immune response depends on the Ig-class as they have different effector functions in the immune system [2]. The heavy constant domains are also known as the crystallizable fragment (Fc). The occurrence of antibodies may be either as membrane-bond to B-cells (as the BCR) or secreted in the circulation and tissue for preventing infections by pathogens [2].



Figure 1.6: Immunoglobulin, antigen-specific antibody. Antigen-specific antibody is made of two identical heavy chains (C_H and V_H) and two light chains (C_L and V_L) that are bound together by disulfide bonds (S-S). The Fab domain is involved in antigen recognition while the Fc domain determines the effector functions which are depended on the Ig class.

1.2 Tolerance and autoimmunity

1.2.1 Tolerance and development of T-lymphocytes

Tolerance is the mechanism of the immune system to control and prevent immune responses against self-antigens and a failure in tolerance may lead to autoimmunity. Tolerance is maintained during lymphocyte development where lymphocytes undergo different selection mechanisms to prevent maturation into autoreactive lymphocytes. Autoreactive T-cells are also kept in check by additional control mechanisms in the periphery [2, 23]

Central tolerance is implemented in generative lymphoid organs; the thymus and the bone marrow. Initially, a positive selection step is performed to sort out the T-cells that are able to recognize self-MHC molecules. Next, the TCR with high affinity to self-antigens are sorted out by negative selection [2]. The central tolerance mechanisms will delete cells with strong recognition to self-antigens by apoptosis, or redirect them into T-reg lymphocytes which actively control responses against self-antigen in peripheral tissues [24].

Peripheral tolerance is related to mature lymphocytes released into circulation and may be induced by an incomplete T-cell activation or a continuous stimulation to self-antigens in peripheral organs such as spleen and lymph nodes. The outcome may be anergy, an unresponsive state against self-antigens leading to inactivated T-cells, or apoptosis of the autoreactive T-cell due to constant exposure to self-antigen [14].

1.2.2 Autoimmunity

Autoimmunity is a condition where the immune system respond to self-antigens due to an imbalance between the immune response and tolerance [1]. A breakdown in self-tolerance may give raise to autoreactive lymphocytes responsible for an autoimmune response where self-structures are recognized and destroyed by the immune system [24, 25]. If such autoimmune responses persist and lead to tissue damage it's called an autoimmune disease [1].

Autoimmune diseases have an incidence of 3-5 % in western countries [26, 27] and may be divided into systemic and organ-specific disorders. In systemic autoimmune diseases such as lupus erythematous (SLE), rheumatoid arthritis and psoriasis, antigens are not tissue-specific as they are expressed systemically and the disease may affect multiple organs in an individual [27]. Addison's disease and type 1 diabetes (T1D) are defined as local or organ-specific

diseases as autoantibodies are directed against antigens produced exclusively in specific organs or even cells.

Loss of tolerance and the genesis of autoimmunity, may involve both environmental and genetic mechanisms [27], and the autoimmune regulator (AIRE) is one of the genes that may cause autoimmunity when mutated. AIRE work as a transcription factor during central tolerance and multiple studies have determined AIRE as an inducing factor for the expression of TSA (tissue-restricted antigens/self-antigens) in thymus, which are crucial for the negative selection of T-cells [24, 25]. Even a single gene mutation in AIRE may therefore have severe complications as it may induce release of self-reactive T-cells [25] and lead to a reduced central tolerance identified as a cause of the autoimmune polyendocrine syndrome Type 1 (APS 1) [24, 28].

APS 1 is a recessive autoimmune disease with tissue-specific manifestations and autoantibodies against self-antigens in affected organs, such as 210H in the adrenal cortex. In most cases the APS 1 patients have multiple disorders such as Addison's disease, hypoparathyroidism or chronic mucocutaneous candidiasis [24, 29].

1.3 Autoimmune Addison`s disease

1.3.1 Adrenal cortex

The adrenal glands lie right atop of the kidneys and consist of a cortex and a medulla (Figure 1.7). The adrenal medulla produces epinephrine and norepinephrine and has a role in the sympathetic nervous system, while the adrenal cortex produces steroid hormones as mineralocorticoids, glucocorticoids and androgens. The adrenal cortex is divided into three histological zones: zona glomerulosa, zona fasciculata and zona reticularis. The zona glomerulosa produces aldosterone which participates in the organism's salt and water metabolism and is also important for maintaining the blood pressure.

Cortisol is secreted from zona fasciculata and is important for regulation of energy, bone metabolism and immune-cell function [28, 30]. Cortisol is secreted in response to ACTH secretion from the pituitary gland which again is stimulated by corticotrophin releasing hormone (CRH) from hypothalamus [31]. Androgens induce development and growth of reproductive organs in males, but the adrenal androgens play a minor role compared to testosterone which is exclusively produced in the testicles [31].



Figure 1.7: Kidneys and different layers of the adrenal glands. Adrenal glands are localized on top of the kidneys with their cortex and medulla important for normal steroid hormone production in the body. Adrenal cortex is divided into three zones; glomerulosa, fasciculata and reticularis. Aldosterone is produced in zona glomerulosa important for regulation of the salt and water balance, while cortisol is released in response to stress by the zona fasciculata. At last androgens from the zona reticularis induce development of the male reproductive system, while catecholamines are secreted from medulla due to stress. Reproduced from [http://images.yourdictionary.com/adrenal-gland].

1.3.2 Addison's disease

Addison's disease (primary adrenal insufficiency) is a prototypic organ-specific autoimmune disorder caused by an immunological destruction of the hormone producing cells of the adrenal cortex [32-34]. The adrenal medulla is not affected by the immunological attack. The disorder is leading to a lack of glucocorticoids and mineralocorticoids such as cortisol and aldosterone [31, 33, 35].

In 1855 Thomas Addison described the clinical features of Addison's disease and it was often attributed to tuberculosis, however the use of antibiotics after the 19th century changed this pattern [33, 34]. Presently 80-90 % of the causes of Addison's disease in the Western world are caused by an autoimmune attack [31-33].

Autoimmune Addison's disease (AAD) may occur isolated or in association with other endocrine autoimmune diseases [31, 34]. Almost 50 % of patients with AAD have a polyendocrine syndrome termed autoimmune polyendocrine syndrome type 2 (APS 2) with additional co-morbidities such as thyroiditis, T1D, premature ovarian failure or pernicious anaemia [28]. As described above, AAD may also be part of the rare disease APS 1, which has a prevalence of 1:90 000 in Norway [36].

Addison's disease has a low incidence, thus the total prevalence are approximately 700 in Norway (4.9 million population) with 144 cases per million [37]. The highest prevalence is found in women aged between 30-50 years, but AAD may occur at all ages [38]. The disease is chronic and patients need lifelong hormone therapy with glucocorticoids and mineralocorticoids [31]. At present, this therapy is not fully restoring normal physiological hormone production [32].

Diagnosing Addison's patients may be a challenge as most of the symptoms are unspecific; nausea, abdominal pain, weight loss, vomiting and fatigue, however some characteristic symptoms are salt craving and hyperpigmentation in areas with scars and folds due to overstimulation of ACTH (Figure 1.8) [34, 37]. Characteristic symptoms of Addison's disease typically occur after 90 % of the adrenal tissue is degraded and each cell layer of the cortex is eventually replaced by fibrous tissue [32, 34, 39].



Figure 1.8: Hyperpigmentation in folds of hands of an Addison's patient. Modified from [28]

1.3.3 HLA risk alleles

The development of AAD has a clear heritability with a strong risk linkage to the Human leukocyte antigen (HLA) class II haplotypes DR3-DQ2 (DRB1*03-DQA1*0501-DQB1*0201) and DR4.4-DQ8 (DRB1*0404-DQA1*0301-DQB1*0302) [33, 40], especially with the heterozygous combination of the DR3 and DR4.4 proteins which may induce an earlier disease breakout [37].

The DRB1*0404 allele is more frequent in AAD patients compared to other DRB1*04 alleles [37]. DR4.4 differs from DR4.1 (the most common DR4 molecule in the healthy Norwegian population) in only two amino acids [41]. However, both of these are located in the peptide binding pocket and therefore influences which peptides that are presented from a given protein antigen [32, 40, 42].

1.3.4 Pathogenesis

Studies have described hyper expression of MHC class II in remaining tissue of adrenal cortex of Addison's patients compared to healthy adrenal tissue. This may reflect an exposure to pro-inflammatory cytokines like IFN- γ , or maybe also induce enhanced activation of CD4⁺ T-cells against the adrenal cortex cells [32, 33]. Pathogenesis of AAD and the additional destruction of adrenal cortex are thought to be a result of three main incidents, looked into in Figure 1.9.

Due to environmental stress such as infections, adrenocortical cells may die and intracellular 21OH could be released to the extracellular environment and ingested by accumulated APC in the adrenal cortex (Figure 1.9, phase 1) [32]. Presentation of 21OH-peptides through MHC class II and I will activate CD4⁺ and CD8⁺ T-cells in lymph nodes, which may start an activation of autoreactive T- and B-cells with production of 21OH-aab (Figure 1.9, phase 2). This again may result in a highly specific and integrated immune response against the adrenal cortex with cytotoxic CD8⁺ T-cells, together with autoantibodies and the complement system as mediators in the destruction of the adrenal cortex (Figure 1.9, phase 3)[32, 33].



Figure 1.9: Pathogenesis of Autoimmune Addison's disease. Adrenocortical cells may suffer from environmental stress or virus infections which may lead to cell death and release of intracellular 21OH. 21OH will in turn be ingested by APC that travel to local lymph nodes and present peptides to T-cells. Autoreactive T- and B-cells may go through clonal expansion and for instance start production of 21OH-autoantibodies which may attack own adrenal cortex tissue together with $CD8^+$ T-cells, macrophages secreting free radicals, complement system or by cytokines produced by $CD4^+$ T-cells inducing apoptosis. Reproduced from [32].

1.3.5 Autoantibodies

Autoimmune diseases are often characterized by specific autoantibodies which may have a role in the pathogenesis of the disease [23, 26]. In Addison's disease autoantibodies against intracellular cytochrome p450 enzymes of the steroid synthesis in adrenal cortex are found and used diagnostically; 21-hydroxylase (cytochrome p450c21, 210H), 17 α hydroxylase (p450c17, 170H) and cholesterol side chain-cleaving enzyme (p450cSCC, SCC). These enzymes are expressed in all layers of adrenal cortex, and 170H and SCC are in addition produced in all steroid producing cells such as in testes and gonads [29, 43]. 210H, which is exclusively expressed in the adrenal cortex, is the dominant antigen, confirmed recently in a large Norwegian cohort where 86 % of 426 patients had 210H-aab [37].

21OH (54 kDa [35] or 55 kDa [44]) is an intracellular enzyme associated with the endoplasmic reticulum (ER) membrane and important for steroid synthesis of cortisol and aldosterone [28, 40, 45, 46].

Several studies have shown that the antibodies against 21OH and the adrenal cortex predict the clinical onset of AAD [34, 47]. Boe et al. [29] determined that the 21OH specific autoantibody in the adrenal cortex is a IgG₁ subtype. IgG₁ in humans is associated with activation of T_{H} -1 cells which are involved in e.g cell mediated immunity (Table 2). In addition, the Fc-domain of IgG may bind to Fc γ -receptors (Fc γ R`s) expressed on phagocytic cells; Fc γ R I (CD64), II (CD32) and III (CD16), and trigger biological functions such as phagocytosis and endocytosis [11, 48]. Such activation may lead to peptide presentation by MHC class I or II which may in turn activate effector T-cells binding to this peptide-MHC complex [49]. However, CD16 are also expressed on natural killer (NK)-cells and may be involved in antibody-dependent cell mediated cytotoxicity (ADCC) rather than increased phagocytosis of 21OH immune complexes [48].

Taken together, IgG1 autoantibodies against 21OH may be involved in destructive processes against the adrenal cortex in AAD [29, 32]. However, the overall function of these autoantibodies in AAD is still unknown and more research is needed to investigate different mechanisms that 21OH-aab may be involved in. Instead, T-cell mediated autoimmunity is assumed to be the major cause of AAD and 21OH are indeed targeted by antigen-specific T-cells [40].

1.4 Aim of the study

In a previous study [40] a considerable increased T-cell response against 21OH was observed when cells from Addison's patients were stimulated in the presence of autologous plasma containing 21OH-aab. As AAD is believed to be T-cell mediated, the question is which role these 21OH-aab have in the mechanism behind the development of the disease.

The main aim of this study was to investigate if 21OH-aab in Addison's patients may enhance the T-cell response against 21OH and thereby amplify a specific immune response against the adrenal cortex.

Several objectives were set to solve this aim:

- 1. Detect and purify 21OH-autoantibodies in Addison's patients
- 2. Determine which APC that activates a T-cell response against 210H and by which Fcγ-receptors on APC the immune complexes are taken up
- 3. Investigate which peptides of 210H that are presented by APC and activates a T-cell response
- 4. Investigate if target cells express similar HLA molecules as effector CD8⁺ T-cells by flow cytometry and immunofluorescence
- 5. Determine if cytotoxicity is increased due to an enhanced T-cell response by stimulation of DC with CD8⁺ T-cells and 210H peptide derivate

2. Materials

To this project samples from patients with autoimmune Addison's disease (AAD, n=34) were collected from a local bio bank (ROAS) at the Medical department at Haukeland University Hospital. Control samples were collected from blood donors (from here named healthy controls) (n=13) at the Blood Bank at Haukeland University Hospital. A written agreement approved by the Committee of Ethics at the University of Bergen confirmed that patients and healthy controls consented to research. Addison's patients selected for this study were based on the presence of 210H-aab, both high and low levels as measured by a fluid phase radioimmunoassay (RIA) [50].

Recombinant 210H (~54 kDa, 130 μ g/ml in sterile PBS) was purified as described [51]. Briefly, the cDNA encoding 210H was cloned into the genome of baculovirus with a hexahistidin-tag on the N-terminal, where histidine binds to divalent metal ions such as nickel. Insect cells (*Spodoptera frugiperda, Sf.*) infected with baculovirus were lysed to isolate 210H after separation of cytosol and cell membrane, and the recombinant 210H was purified by immobilized metal ion affinity chromatography [51]. The 210H sequence is displayed in appendix 6.1.

HLA genotypes from some of the patients were available from a previous study [52].

Name	Manufacturer	Catalogue nr.
NUPAGE [®] LDS Sample Buffer (4x)	Invitrogen TM	NP0007
NUPAGE® Reducing Agent (10x)	Invitrogen TM	NP0009
NuPAGE®10 % Bis-Tris Gel 1.0 x 15 well	Invitrogen TM	NP0303BOX
SeeBlue®Plus2 Prestained Standard (1x)	Invitrogen TM	LC5925
MagicMark TM xp Western Standard	Invitrogen TM	LC5603
Simply Blue [™] SafeStain	Invitrogen TM	LC6060
iBlot Gel Transfer Stacks PVDF mini	Invitrogen TM	IB4010-02
MOPS SDS Running Buffer (20x)	Invitrogen TM	NP0001
NUPAGE®Antioxidant	Invitrogen TM	NP0005
Immobilon [™] Western Chemiluminescent		
AB substrate	Millipore	WBKDS0100
BCIP (100x)	Biorad	170-6532
NBT (100x)	Biorad	170-6539

2.1 Materials for LDS-PAGE and western blot

Name	Content	
Wash buffer, PBS-T	PBS, 0.05 % (v/v) Tween-20	
Block solution (ELISA) PBS-T 3% BSA	3 % (v/w) BSA in PBS-T	
FC-buffer	PBS 1 % BSA	
Running buffer	I: 40 ml MOPS SDS Running buffer (20X)	
	760 ml Milliq-H ₂ O	
	II: 200 ml Running buffer I	
	500 μl NUPAGE [®] Antioxidant	
Developing buffer	0.1M NaHCO3, 1mM MgCl2 in ddH2O at pH 9,8	
Blocking solution, WB	5 % v/w Non-Fat Dry Milk. PBS-T	
4% (v/v) Paraformaldehyde in PBS	Made prior to the study and stored at -20 °C	

2.2 Buffers for SDS-PAGE, western blot, ELISA and immunofluorescence

2.3 Materials for IgG purification, ELISA, ELISPOT and cytotoxicity analysis

Name	Manufacturer	Catalogue nr.
Protein A HP SpinTrap kit	GE Healtcare	28-9031-32
Ab Buffer kit	GE Healthcare	28-9030-59
DEPC Treated Water	Invitrogen TM	46-2224
p-Nitrophenol phosphate substrate	SIGMAFAST TM	N1891
solution		
ELISA MAX [™] Deluxe Sets Human	BioLegend	430104
IFN-y		
Nunc-Immuno TM 96-well flat-bottomed	Thermo Fischer Scientific	442404
plates with Maxi Sorp TM		
ELISpotPRO for Human IFN-y kit	Mabtech	3420-2AST-2
LDH Cytotoxicity Assay Kit	Cayman Chemical Company	10008882

2.4 Antibodies

Name	Dilution	Manufacturer	Catalogue nr.
AP-conjugated goat Anti-human	1:2000,	Sigma-Aldrich [™]	A3187
IgG (γ-chain specific)	1:5000		
AP-conjugated donkey Anti-	1:5000	Santa Cruz Biotechnology	Sc-2022
Goat IgG			
mouse Anti-human CD32	1:100	eBioscience	16-0329
Functional grade purified			
mouse Anti-Human CD64 (Fc	1:100	eBioscience	16-0649
gamma receptor 1) Functional			
grade purified			
goat Anti-Human 210H	1:200	Santa Cruz Biotechnology	Sc-48466
Unconjugated mouse Anti-	2 µg	BioLegend	343301
Human HLA-A2			
Alexa488 Fluor® donkey Anti-	1:200	Invitrogen TM	A21202
Mouse IgG (H+L)			
FITC-conjugated mouse Anti-	1:5	Biolegend	343303
Human HLA-A2			

Name	Manufacturer	Catalogue nr.
Ficoll-Paque™ Plus	GE Healthcare	17-1440-02
Trypan Blue stain 0.4 % with Countess [®]	Invitrogen™	T10282
X-VIVO 15	Lonza	BE04-418F
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich [®]	D8418
Human Sera Type AB (male) plasma		
derived	Bio Wittaker®	14-191E
MACS [™] BSA Stock Solution	Miltenyi Biotec	130-091-376
autoMACS [™] Rinsing Solution	Miltenyi Biotec	130-091-222
MACS [®] Separation Columns 25 MS		
Columns	Miltenyi Biotec	130-042-201
MACS Separator	Miltenyi Biotec	-
CD8 MicroBeads, human	Miltenyi Biotec	130-045-201
CD4 MicroBeads, human	Miltenyi Biotec	130-045-101
CD14 MicroBeads, human	Miltenyi Biotec	130-050-201
Dynabeads [®] CD19 pan B	Invitrogen TM	111.43D
DETACHaBEAD® CD19	Invitrogen TM	125.06D
GM-CSF (100x)	Biolegend	572904
IL-4 (50x)	Biolegend	574006
LPS (1000x)	Sigma-Aldrich	L1887
IL-7	eBioscience	34-8079-82
IL-2	Peprotech	200-02
Dulbecco's Modified Eagle's		
Medium/Nutrient Mixture F-12 Ham	Sigma-Aldrich [®]	D2906-1L
Na ₂ CO ₃	Sigma®	S-5761
BD Nu-Serum	BD Bioscience®	355100
ITS+	BD Bioscience®	354352
RPMI 1640 without L-Glutamine	Lonza	BE12-167F
L-Glutamine 100x	Lonza	17-605E
FBS 100x	Life Technologies	10108-165
Hepes Buffer 1M	Bio Wittaker®	BE17-737E
PEN-STREP 10.000 U Penicillin/ml	Bio Wittaker®	DE17-602E

2.5 Materials for cell culture

2.6 Apparatus and Equipment's

Manufacturer
Eppendorf
Eppendorf
BD Accuri Cytometers
Sanyo
Hoefer
Alpha Innotech
Invitrogen TM
Tecan
Zeiss
Nano®Drop
Biorad
Lab-Tec
Molecular Devices
Heidolph Instruments

2.7 Software

Name	Purpose
GraphPad Prism 5	Graph production and statistical analysis
Adobe Photoshop CS5	Picture modification
Adobe Illustrator CS5	Figure production
LSM Image Browser	Immunofluorescence images
Zeiss LSM 510 Software	Immunofluorescence analysis
CFlow®Sampler Analysis	Flowcytometry modification
CTL ImmunoSPOT Analyzer	ImmunoSpot Software for ELISPOT Analysis

2.8 Miscellaneous

Name	Manufacturer	Catalogue nr.
PBS Tablets	Invitrogen®	18912-014
Phosphate Buffered Saline, PBS	Bio Whittaker®	BE17-516F
Bovine serum albumin (BSA)	Sigma [®]	A7030-100G
Tween-20	Cayman Chemical company	400035
Triton X-100	Sigma-Aldrich®	T9284
FBS	Life technologies	10108-165
$H_2SO_4 \ 1 \ M$	Merck	
Nuclease Free Water	Ambion®	AM 9932
FcR Blocking Reagent, human	Miltenyi Biotech	130-059-901
ProLong [®] Gold antifade reagent	Invitrogen™	P36935
with DAPI		

3. Methods

3.1 Detection of 210H-autoantibodies

In order to identify patient plasma with high levels of 21OH-autoantibodies (21OH-aab), an in-house ELISA was performed essentially as described [51]. Briefly, Nunc-ImmunoTM 96-well flat-bottomed plates with Maxi SorpTM surface were coated with 100 μ l 21OH protein (5 μ g/ml in PBS). After incubation at 4 °C overnight, excess protein solution were decanted and blocking solution were used to block unspecific binding sites for 30 minutes at 37 °C. Plates were washed four times with wash buffer before 100 μ l plasma samples (1:100) diluted in blocking solution were pipetted in duplicates or triplicates on the 96-well plate. Plasma samples were incubated on shaking platform for 1 hour at room temperature (RT) or overnight at 4 °C.

Subsequently, the plate was washed four times with wash buffer and incubated with alkaline phosphatase (AP)-conjugated goat Anti-Human IgG (1:2000) for 1 hour at RT on a shaking platform. After five final washes in wash buffer, visualization of immune complexes by p-Nitrophenol phosphate substrate solution was performed and incubated in the dark for 10- 30 minutes and the optical density was measured at 405 nm by a SpectraMax Plus spectrophotometer. For each plate, a cut-off value distinguishing between plasma samples positive and negative for 210H-aab were calculated from a series of 12 healthy controls, using the mean OD values from these plasma samples tested in parallel, plus 3 standard deviations (SD).

3.1.1 LDS-polyacrylamide gel electrophoresis (LDS-PAGE) and Western blotting

Western blotting was performed to confirm that 21OH-aab detected by ELISA were present in patient plasma, and that purified IgG still contained 21OH-aab. Different sources of 21OH-containing protein lysates were prepared for electrophoresis with NuPAGE[®]LDS Sample buffer (4x) and NuPAGE[®]Reducing agent (10x) using the NUPAGE[®]Novex[®] Bis-Tris Mini Gels protocol, and boiled at 70 °C for 10 minutes.

Further, the 21OH-lysates were separated on a NuPAGE[®]10 % Bis-Tris SDS gel running at 180 V for 1 hour and blotted using the iBlot Gel Transfer Stacks PVDF mini Kit (program 3, 7 minutes, 20 Volt). The membrane was blocked with 5 % milk buffer overnight at 4 °C on a shaking platform and incubated with primary antibodies (patient or control plasma, purified

IgG or a commercial goat Anti-Human 21OH antibody at appropriate dilutions for 2 hours at RT on a shaking platform. After washing three times with PBS-T for 5 minutes, the membrane was incubated with secondary antibodies AP-conjugated goat Anti-human IgG (1:5000) or AP-conjugated donkey Anti-Goat IgG (1:5000) for 1 hour at RT on a shaking platform.

The membrane was washed as described above and antigen-antibody complexes were visualized by ImmobilonTM Western chemiluminescence AP substrate at Fluor chem[®]HD2. Alternatively, a colorimetric method was also used where the membrane was washed 3 more times in PBS-T for 5 minutes and 2 times 5 minutes in developing buffer. Finally, the NBT (100x) and BCIP (100x) substrate was added, and the membrane was developed for approximately 5 minutes until bonds were visible.

3.1.2 Purification of IgG antibodies

IgG from patient plasma was purified by a Protein A HP SpinTrap kit, using spin columns with Protein A SepharoseTM which have a strong affinity to IgG, especially human IgG₁. The purification was carried out exactly as described by the manufacturer.

Briefly, medium in the spin column was suspended and centrifuged at 70 x g for 30 seconds. The column was then equilibrated by adding Binding buffer followed by another centrifugation at 70 x g for 30 seconds. The columns were loaded two times with 600 μ l of serum and incubated 4 minutes with gentle mixing before centrifugation at 70 x g for 30 seconds. Subsequently, the columns were washed twice in Binding buffer and centrifuged at 70 x g for 30 seconds.

Finally, Elution buffer was added (400 μ l) to release IgG from the column and the spin columns was transferred to tubes with Neutralizing buffer and then centrifuged at 70 x g for 30 seconds two times to collect the IgG in two fractions. IgG antibody concentration was measured by UV-light absorption at 280 nm on a Nano[®]Drop ND-1000 Spectrophotometer and a correction factor at 0.72 was used to calculate the final IgG concentration (mg/ml).

Immune complexes of IgG autoantibodies and 21OH (130 μ g/ml) were prepared by incubating the two in a 2:1 molar ratio for 1 hour at RT. This corresponded to 250 μ g/ml of purified IgG and 2.5 μ g/ml 21OH.

3.2 Cell culture

3.2.1 Isolation of peripheral blood mononuclear cells (PBMC) for in vitro assays

PBMC was separated from venous blood by differential density centrifugation by Ficoll-Paque PREMIUM medium with a density of 1.077 g/ml. Anticoagulant-treated blood (heparin) was mixed with an equal volume of PBS, layered carefully over Ficoll-PaqueTMPLUS medium in a 4:3 ratio and centrifuged at 400 x g for 40 minutes. The upper layer was drawn off leaving the mononuclear cells on top. Carefully the mononuclear cell layer was transferred to a new centrifuge tube and 3 times the volume of PBS was added. The tube was centrifuged at 300 x g for 10 minutes, supernatant was decanted and cells suspended in 10 ml PBS.

Viable cells were counted using Trypan Blue Stain in a Bürker counting chamber. Simultaneously, the cell suspension was centrifuged at 300 x g for 10 minutes before being resuspended in growth medium. PBMC from AAD patients and controls were grown in serum-free X-VIVO 15 medium at 37 °C with 5 % CO_2 and 95 % humidity.

Freezing of cells was done directly after cell separation from venous blood in 10 % (v/v) DMSO and 90 % (v/v) AB serum, first at -80 °C for 24 hours, thereafter at -150 °C.

3.2.2 Ex-vivo ELISPOT

T-cell responses were measured by IFN- γ ELISPOT after pre-stimulation with 21OH alone or 21OH/IgG immune complexes. This method is very specific and sensitive and yields both qualitative and quantitative results.

Cryopreserved PBMC from AAD (stored at -150 °C) was thawed in a water bath preheated to 37 °C and suspended in preheated X-VIVO 15 medium and centrifuged at 300 x g for 10 minutes. The pellet was suspended in 10 ml X-VIVO 15 and viable cells were counted as described above. Simultaneously the complete cell suspension was centrifuged at 300 x g for 10 minutes and the pellet was suspended to contain 5 x 10^6 cells per ml. Cell suspensions containing 1 million PBMC's (200 µl) were rested for 12-24 hours in 37 °C and 5 % CO₂ before pre-stimulation. 50 µl of medium only, 210H (2.5 µg/ml) alone, IgG alone (250 µg/ml) or 210H/IgG immune complexes were added the next day, and the cells were pre-stimulated for 24 hours.

For experiments detecting the importance of $Fc\gamma$ -receptors Anti-Human CD32 and CD64 was incubated with PBMC for 1 hour to block these receptors prior to addition of immune complexes.

ELIspot^{PRO} kit for Human IFN- γ was used to perform the ELISPOT analyzes. ELISPOT wells were washed 4 times with PBS and then blocked with 10 % Human Sera Type AB in X-VIVO 15 for 30 minutes at RT to prevent unspecific binding to the membrane. Cell pellets from pre-stimulation were suspended twice in 1 ml X-VIVO 15 after centrifugation at 300 x g for 10 minutes and 200 x g for 8 minutes. Finally, the cell pellet was suspended in 600 µl X-VIVO 15 and transferred at 150 µl in triplicates or quadruplicates to the ELISPOT plate for 24 hours incubation in 37 °C and 5 % CO₂, with aluminum foil wrapped around it. Supernatants from selected pre-stimulation steps was saved for ELISA analyzes and frozen at -20 °C.

The ELISPOT wells were decanted and washed 5 times in 200 μ l PBS before it was incubated with secondary antibody 7-BG-ALP (1:200) in 0.5 % FCS for 2 hours at RT wrapped in aluminum foil. A second wash with PBS was performed, before incubation with BCIP/NBT-plus substrate for 5-15 minutes and a final wash in tap water to stop the chromogenic reaction. The plate was dried and spots were counted manually in a microscope or by the use of C.T.L ImmunoSPOT automated scanning software at The Gade Institute at UoB.

3.2.3 Cell isolation from PBMC

3.2.3.1 Magnetic cell sorting (MACS) of CD4+, CD8+ and CD14+

MACS is based on a method which magnetically labels co-receptors on cells with respective MicroBeads, which are conjugated to monoclonal mouse Anti-Human CD4, CD8 or CD14 antibodies. Purification of selected cells was performed according to the manufactures protocol.

PBMCs were counted as described previously (chapter 3.2.1), centrifuged at 300 x g for 10 minutes and supernatant was completely removed. The cell pellet was suspended in 80 μ l buffer per 10⁷ cells and 20 μ l MicroBeads per 10⁷ cells were added before mixing and incubation at 4-8 °C for 15 minutes. Next, cells were washed in 1-2 ml buffer per 10⁷ cells and centrifuged at 300 x g for 10 minutes and supernatant was again completely removed. The pellet was then suspended in 500 μ l buffer.

An MS column was placed on a magnetic board together with the MACS Separator magnet and equilibrated with 500 µl buffer before addition of the cell suspension. The unlabeled cells run through the column while target cells stay in the magnetic field because of the binding to magnetic MicroBeads. A three-step wash with 500 μ l buffer was performed, and the column was removed from the MACS Separator and placed on a suitable tube while 1 ml buffer was added to elute the positively selected cell fraction.

3.2.3.2 B-cell purification

B-cells express co-receptor CD19 [5] and were isolated from PBMC by superparamagnetic polysterene Dynabeads[®] coated with monoclonal CD19 antibodies. Dynabeads[®] CD19 pan B and DETACHaBEAD[®] CD19 protocols was followed exactly as described by the manufacturer.

Briefly, Dynabeads[®] were washed prior to usage and adequate amounts (25 μ l) were added to prepared PBMC (1 ml) and incubated on shaking platform for 20 minutes at 2-8 °C. A magnetic field was used to wash the bead-bound cells 4 times with buffer. Purified cells were then resuspended in culture medium following the DETACHaBEAD CD19 protocol to attach the magnetic beads and release bead-free CD19⁺ cells.

DETACHaBEAD CD19 contain Anti-Fab polyclonal antibodies specific for CD19 antibodies coated on Dynabeads and may detach Dynabeads from $CD19^+$ cells in use of a magnetic field. DETACHaBEAD (10 µl) was incubated with CD19-coated PBMC for 45 minutes on shaking platform in RT and washed 3 times in medium. Supernatants containing bead-free cells were pooled and collected for further examination.

3.2.4 Establishment of T-cell lines

In order to induce T-cell lines (TCLs) specific for 21OH, we first differentiated monocytes from PBMC into dendritic cells (DCs) to be used as antigen presenting cells (APC): A PBMC cell suspension was transferred to a 6-well plate (5 x 10^6 cells in 3 ml) and incubated at 37 °C in 5 % CO₂ for 75-90 minutes in X-Vivo 15 medium. The medium containing non-adherent cells was then aspirated and the remaining adherent cells were washed twice with preheated X-VIVO 15 medium.

The adherent cells were cultured with cytokines to promote differentiation of monocytes into DC. The cells were incubated with IL-4 (1000 U/ml) and GM-CSF (1000 U/ml) for 6-8 days and every third day half of the medium was replaced with medium containing fresh cytokines. After 6-7 days, the resulting immature DCs were resuspended by pipetting and washed twice

with X-VIVO 15. Cells were counted as described above and the pellet was suspended in X-VIVO 15 (450 μ l per well) and transferred to a 24-well plate.

The immature DC's were loaded with 21OH alone or 21OH/IgG immune complexes, and incubated for 1-3.5 hours before LPS (1 μ g/ml) was added for 24 hours to induce mature DC's presentation function. Autologous PBMC or purified CD4⁺- or CD8⁺ T-cells were then added at 2 x 10⁶ cells/ml to the mature DC's. Two days later IL-2 (10 IU/ml) and IL-7 (10 μ g/ml) were added (only IL-2 was used for CD4⁺ T-cells). The responding T-cells were then cultured for 10–18 days, with replacement of medium including cytokines and splitting as required.

3.2.4.1 ELISPOT analysis of T-cell lines

ELISPOT with the TCLs was performed to determine if cells stimulated several times with antigen and cultured for a longer period would give an enriched T-cell response compared to cells tested *ex-vivo*. After 10-18 days of culturing following priming with mature DCs, the TCLs were tested against 21OH protein, as well as against 21OH-derived peptides (displayed in appendix 6.2).

In addition to provide information about the enrichment of 21OH-specific T-cells, the use of peptides would also reveal any differential recognition of 21OH epitopes following priming against 21OH alone, or 21OH/IgG immune complexes. The ELISPOT assay was then set up exactly as described above (3.2.2), except that the pre-stimulation step was omitted. Instead, the autologous PBMC or BCLs were pulsed with peptides or protein for 1-12 hours, and used as APC. The APC (1-2 x 10^5) were then suspended into the ELISPOT wells along with 1-2 x 10^5 T-cells.

3.2.5 Cytokine enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to measure quantitative amounts IFN- γ secreted into culture by activated T-cells. 100 µl cell culture media was tested in sandwich ELISA, exactly as described by the manufacturer's protocol. All incubation steps were performed on a shaking platform in RT.

Briefly Capture Antibody (1:200) was diluted in Coating Buffer and incubated overnight at 4 $^{\circ}$ C on a 96 well plate from the kit. Subsequently, supernatants was discarded and plate was washed 4 times with Wash Buffer (400 µl) prior to incubation with 1X Assay Diluent (200 µl) for 1 hour to reduce background and block unspecific binding. The plate was washed another

4 times before prepared standards and patient plasma (100 μ l) was added and incubated for 2 hours. Next, the plate was washed 4 times and Detection Antibody solution was added and incubated for 1 hour before a new 4 step wash and addition of Avidin-HRP solution (1:1000) diluted in 1X Assay Diluent incubated for 30 minutes.

Plate was washed 5 more times for 30-60 seconds to minimize background and added mixed TMB Substrate Solution and incubated for approximately 15 minutes in the dark. At last Stop solution (2 N, H_2SO_4) ended the reaction and absorbance was measured at 450 nm within 30 minutes at Spectramax Plus. IFN- γ concentration (pg/ml) was calculated from a logarithmic standard curve.

3.3 Measurement of T-cell cytotoxicity

3.3.1 Culturing H295R human adrenocortical carcinoma cell line

NCI-H295R was cultured in prepared supplemented medium containing; Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with HEPES buffer (15 mM) added Na₂CO₃ (1.2 g/L), Nu-serum (2.5 %) and ITS+ (1 %), in culture condition 37 °C with 5 % CO₂. Cells were grown to about 90 % confluence and sub-cultivated at a 1:3-4 ratio.

3.3.2 Culturing lymphoblastoid B-cell lines

Lymphoblastoid B-cell lines (PGF and PRIESS) were cultured in RPMI 1640 medium containing fetal bovine serum (10 % v/v), L-glutamine (100x, 1 % v/v), Hepes buffer (1 M, 1 % v/v) and antibiotics (PEN-STREP, 10000 U/ml, 1 % v/v) at 37 °C with 5 % CO₂. The B-cell lines (BCLs) were handled and thawed in the same way as PBMC and PGF cells was used as antigen presenting cells or target cells in cytotoxicity assays. For cell-culture passage, the BCLs were maintained at 3-10 x 10^5 per ml and split at 1:3-4 every third day.

3.3.3 Detection of HLA genotype by immunofluorescence on NCI-H295R cells Immunofluorescence was performed to investigate if NCI-H295R cells express HLA class I, A2 molecules on their surface.

NCI-H295R cells were cultured on coverslips $(10^6 \text{ cells per/well in 24-well plate})$ for 24 hours. The coverslips were washed with PBS (1 ml) and fixated with 4 % paraformaldehyde in PBS for 10 minutes in RT. A new wash was performed with BSA (3 % v/w in PBS), before blocking with 10 % (v/v) FBS in PBS for 1 hour at RT. Wells were washed with 1 % (w/v) BSA in PBS and added Mouse Anti-Human HLA-A2 (1 and 2 µg) diluted in 1 % (w/v) BSA in PBS incubated for 1 hour at RT.

After washing, Alexa488-conjugated donkey Anti-Mouse was added at 1:200. Cells were washed 3 times in 1 % (w/v) BSA in PBS and coverslips were washed in water and excess liquid was removed before mounting coverslips with a drop of ProLong Gold with DAPI on a microscope slide. The preparation was allowed to harden for at least 24 hours in dark before analyzed on a confocal microscope, Zeiss LSM 510 META, at the MIC platform, UoB.

3.3.4 Identification of Fcy-receptors on APC by flow cytometry

Flow cytometry was used to detect expression of Fcγ-receptors (II: CD32 and I: CD64) on APC`s and HLA class I A2 on NCI-H295R and PRIESS cells.

Cells were cultured for approximately one week; they were counted and split during this period. Prior to the flow cytometry analysis cells were stimulated with IFN- γ (0.5 µg/ml) to turn up HLA-I and Fc-receptor expression. Subsequently, cells were counted as described above (chapter 3.2.1), centrifuged at 300 x g for 10 minutes, washed in PBS and resuspended in FC-buffer. Cell suspension was pipetted into tubes (100 µl, 1x10⁶ cells/ml) and APC`s was added Fc-blocker (1 µl), incubated for 5-10 minutes in RT to bind and occupy the Fc γ -receptor seats.

Further, primary antibodies, mouse Anti-Human CD32 and CD64 (1:100) and FITCconjugated mouse Anti-Human HLA-A2 (1:5), were added cells and mixed well before incubation on ice for 30 minutes. Cells were suspended in 2 ml FC-buffer and centrifuged at 400 x g for 5 minutes, supernatants were discarded and the cell pellet was resuspended in FCbuffer (100 μ l). For APC the secondary antibody, Alexa488-conjugated donkey Anti-Mouse IgG (1:200), was added and incubated on ice for 30 minutes covered with aluminum foil due to light sensitive secondary antibody. At last, cells were washed in FC-buffer (200 μ l) and stored in fridge covered with aluminum foil until analyzed on the AccuriC6 instrument.

3.3.5 Cytotoxicity of cultured CD8⁺ T-cells

The ability of cultured CD8⁺ T-cells to induce cytotoxicity was measured with the LDH Cytotoxicity Assay Kit. NCI-H295R cell-line or autologous PBMC's were used as target cells pulsed with 210H peptides.

Target cells were counted and transferred (100 μ l) to a 96 well plate and incubated overnight at 37 °C and 5 % CO₂ before peptide pools (10 μ g/ml) of 21OH were added (for PBMC). CD8⁺ T-cells was centrifuged at 300 x g for 10 minutes, suspended in X-VIVO 15 and counted as described above (chapter 3.2.1). Target cells, peptide pools and CD8⁺ T-cells at different ratios were incubated for 4 hours in 37 °C and 5 % CO_2 . Different ratios of $CD8^+$ Tlymphocytes against target cells were used to determine if there was a dose response in cytotoxic activity with more $CD8^+$ T-cells.

Standards were made before usage and pipetted into a new 96 well plate as duplicates. As positive controls, cells were added 0.2 % Triton X-100, and mixed for 10 minutes before centrifuged at 400 x g for 5 minutes. Supernatants were transferred to the new plate with standards and LDH Reaction solution was added and incubated for 30 minutes at RT. The absorbance was read at 490 nm on a spectrometer and the LDH activity (μ U) was calculated by the linear equation using the logarithmic and blank subtracted absorbance values. 0.2 % Triton X-100 was used a positive control for maximum cytotoxicity. Target cells alone were used as a control for spontaneous LDH release.

3.4 Statistical analyses

A two-tailed paired T-test was done to detect a significant difference between cells treated with various pre-stimuli where p values less than 0.05 was considered significant.

4. Results

In a previous study we have observed a considerable increased T-cell response against 21OH in Addison's patients, when stimulated in the presence of autologous plasma containing 21OH-autoantibodies (21OH-aab). As autoimmune Addison's disease is believed to be T-cell mediated, the question is which role 21OH-aab have in the mechanism behind the development of the disease.

4.1 Detection of 210H-autoantibodies in plasma of Addison's patients

Addison's patients are characterized by antibodies against 210H, where 210H is exclusively expressed in the adrenal cortex. Initially, we determined levels of 210H-aab in AAD patients and healthy controls by ELISA in order to identify patients with high levels of 210H-aab for further experimentation (Figure 4.1). An expected and significant (p < 0.0001) higher absorbance was measured in AAD patients compared to healthy controls, although large individual differences between patients were noted.



Figure 4.1: ELISA analysis detected high levels of 21OH-autoantibodies in plasma of Addison's patients. 21OH protein samples (5 μ g/ml) was coated on 96-well flat-bottomed plates and incubated with plasma from AAD patients (n=12) and healthy controls (n=12). After adding AP-conjugated Anti-Human IgG secondary antibody and chromogenic substrate, optical density was measured at 405 nm with two parallels per sample. The average of all samples in each group is displayed by the dark line, with error bars representing the standard deviations for each group. The dotted line is 3 standard deviations above the average of controls and indicates the cut-off value between samples negative and positive for 21OH-aab. The graph represents one independent experiment where a two-tailed unpaired T-test show a significant (p < 0.0001) difference between the two groups.

Western blot analyzes were done to confirm the presence of 21OH-aab in plasma of AAD patients and its binding capacity to 21OH. Different lysates containing 21OH (Table 4.A) were separated by SDS-PAGE and blotted on a PVDF membrane before incubation with plasma. There were detected bonds for 21OH at ~54 kDa for the positive control (a commercial Anti-Human IgG/21OH polyclonal antibody) and Addison patient plasma identifying the presence of 21OH-aab (Figure 4.2.A). No bonds were detected for healthy control plasma.

Total IgG was purified chromatographically by Protein A spin columns from patients and healthy control plasma with high levels of 21OH-aab where the yields are shown in Table 3. Plasma, purified total IgG and IgG depleted plasma were also analyzed to determine if plasma samples with variable contents of 21OH-aab would bind 21OH lysates (Table 4.B) and that purified IgG retained its 21OH binding capabilities (Figure 4.2.B). Plasma and purified IgG displayed strong bonds to 21OH lysates at ~54 kDa while IgG depleted plasma displayed weaker bonds.

IgG concentration, mg/ml					
Patient	Elution 1	Elution 2			
1	3.2	0.5			
7	4.2	0.3			
8	3.1	0.6			
9	3.7	0.7			
10	1.8	0.4			
24	4.8	-			
25	3.4	0.7			
26	4.0	0.6			
27	2.2	0.3			
28	2.2	0.5			
29	1.9	0.2			
30	2.0	0.2			
31	3.8	0.2			
32	4.4	0.5			
34	3.2	0.6			

Table	3:	Profit	from	IgG	purification	from	1.2 m	l plasma.
				-8~	F			



Figure 4.2: Western blot analysis confirmed the presence of 21OH-autoantibodies in AAD plasma and purified IgG. 210H lysates were separated on a PVDF membrane to determine the presence of 210H-aab in plasma and purified IgG. AP-conjugated goat Anti-Human IgG was used as secondary antibody and SeeBlue[®]Plus2 Prestained standard were used for both blots. The blots represent one individual experiment each. A: 210H (54 kDa) was detected right upon the third standard bond at 51 kDa (Glutamic Dehydrogenase) indicating the presence of 210H-aab. The positive control and patient 1 both detected bonds at ~54 kDa confirming the presence of 210H-aab. Plasma from healthy control did not contain 210H-aab. B: Identification of 210H-aab in plasma and purified IgG from patient 1. IgG depleted plasma also seemed to contain some residual

Table 4: 21OH-containing protein preparations used for Western blot analyzes

\boldsymbol{A}	Patient plasma
Sample 1	Purified microsomes from insect cells containing recombinant 21OH
Sample 2	NCI-H295R lysate (adrenocortical carcinoma cell line expressing 21OH)
Sample 3	Purified21OH from E. coli (lower molecular weight due to removal of
	some of the protein)
Sample 4	Purified microsomes from insect cells without recombinant 21OH
B	Purified IgG
B Sample 1	Purified IgG Purified microsomes from insect cells containing recombinant 210H
<i>B</i> Sample 1 Sample 2	Purified IgG Purified microsomes from insect cells containing recombinant 21OH 21OH from E. coli (lower molecular weight due to removal of some of the protein)
<i>B</i> Sample 1 Sample 2 Sample 3	Purified IgGPurified microsomes from insect cells containing recombinant 21OH21OH from E. coli (lower molecular weight due to removal of some of the protein)Purified microsomes from insect cells without recombinant 21OH

4.2 IFN-γ production enhanced by 210H-autoantibodies

Peripheral blood mononuclear cells (PBMC) were directly pre-stimulated with 21OH \pm IgG in immune complexes after thawing to investigate if IgG could increase the uptake of 21OH into APC and subsequently enhance the T-cell response as measured by IFN- γ . Eight independent

ex-vivo ELISPOT analyses showed an increased number of spots detected after stimulation of 21OH in presence of IgG. However, a two-tailed paired T-test showed no significance (n.s.) but only a relative trend between the groups; un-stimulated cells vs. pre-stimulated with immune complex (p = 0.056) and 21OH alone vs. immune complex (p = 0.063) (Figure 4.3 and Figure 4.4).



Figure 4.3: IFN-y production in response to 21OH \pm **IgG stimulation of PBMC.** PBMCs from AAD patients (n=8) were stimulated with 21OH \pm IgG for 24 hours in independent experiments and spots due to IFN- γ production was measured by ELISPOT. Each dot represents the average of one patient with 3-4 parallels and the line represents mean of all patients for each stimuli. Two-tailed paired T-test was performed, however no significance (n.s.) was detected between the groups; Blank vs. 210H/IgG (p = 0.056), 210H vs. 210H/IgG (p = 0.063), identifying only a trend of 210H-aab.



Figure 4.4: Independent representative *ex-vivo* ELISPOT's with enhanced T-cell response due to 21OH/IgG pre-stimulation. PBMC from individual patients pre-stimulated with $21OH \pm IgG$ for 24 hours showed an increased IFN- γ production and T-cell response against 21OH/IgG. Significance was tested by A: a two-tailed paired T-test (p = 0.041) between 21OH alone vs. 21OH/IgG with four parallels per sample. B: a paired two-tailed paired T-test (p = 0.095) between Blank vs. 21OH/IgG with three parallels per sample, n.s. C: two-tailed paired T-test with three parallels per sample, n.s.

Spots produced by activated T-cells were manually counted in a microscope for the *ex-vivo* ELISPOT. Typical appearances of the ELISPOT wells are displayed in Figure 4.5.



Figure 4.5: *Ex-vivo* ELISPOT illustrating detected IFN- γ spots after pre-stimulation with 21OH alone and 21OH/IgG immune complexes. Increased amounts of spots were detected for immune complexes (right image, 11 spots) compared to 21OH alone (left image, 4 spots) in patient 1 where each spot represent one IFN- γ producing cell.

4.3 Importance of Fcy-receptors in T-cell response

4.3.1 Blocking of FcyRII (CD32) and FcyRI (CD64) in PBMC

For selected patients, Fc γ -receptors (Fc γ R) on PBMC were blocked by Anti-Fc γ RI (CD64) and II (CD32) before cells were stimulated with 21OH ± IgG. IFN- γ production was measured by *ex-vivo* ELISPOT as described above and the blocking of Fc γ Rs lead to a decreased T-cell response for one of the patients (Figure 4.6).



Figure 4.6: The importance of Fcy-receptors on PBMC. Fcy-receptors on PBMC from patient 10 were blocked by Anti-CD32 and Anti-CD64 (Fcy-receptor II and I) for 1 hour before stimulation with immune complexes. Cells were stimulated for 24 hours until IFN- γ production was measured by *ex-vivo* ELISPOT. A decreased T-cell response was detected in the presence of blocking antibodies against the Fc γ –receptors. The decrease did not reach statistical significance by a two tailed paired T-test however; 210H+IgG vs. Anti-CD32 (p=0.353) and 210H+IgG vs. Anti-CD64 (p=0.519).

Ex-vivo ELISPOT with blocking antibodies against $Fc\gamma Rs$ was performed for two more patients. However, none of these two patients showed any IFN- γ response against either 21OH or 21OH/IgG immune complexes, even in the absence of $Fc\gamma R$ blockade (results not shown).

4.3.2 Antigen presenting cells and the uptake of immune complexes

4.3.2.1 Expression of Fcγ-receptors on APC by flow cytometry

Initially, the expression of $Fc\gamma Rs$ on different APC was determined by flow cytometry to confirm our hypothesis that the immune complexes are ingested by $Fc\gamma Rs$.

Priess cells are an immortalized B-cell line and it was of interest to investigate if they express $Fc\gamma Rs$ CD32 and CD64 on their surface where 21OH-aab may bind. Unstained cells were used as a negative control and a gate was set up for cells staining positive for $Fc\gamma Rs$. Only CD32 was detected in Priess cells (Figure 4.7).



Figure 4.7: Flow cytometry analysis for detection of Fcγ-receptors on Priess cells. The expression of Fcγ-receptors CD32 and CD64 in Priess cells was assessed by flow cytometry using indirect staining with unconjugated mouse Anti-CD32 and Anti-CD64 primary antibodies, and Alexa488-conjugated donkey Anti-Mouse as secondary antibody. Priess cells were treated with Fc-blocker to avoid unspecific binding to the Fc-receptors prior to staining. A: Gating of live cells using their forward and side scatter properties, allowing only live cells to be analyzed for fluorescence. B: Background (PRIESS cells with secondary antibodies). C: Quantification of Priess cells expressing CD32 treated with Anti-CD32 and Alexa488-conjugated secondary antibody. D: Quantification of Priess cells expressing CD64 treated with Anti-CD64 and Alexa488-conjugated secondary antibody. Except for A, the X-axis represents the size of the cells and the Y-axis the fluorescence intensity of Alexa488.

Monocytes were purified by CD14 Microbeads from PBMC prior to the flow cytometry experiment. Flow cytometry was performed after culturing for 24 hours with or without the presence of IFN- γ . The expression of Fc γ Rs was considerable increased due to IFN- γ

stimulation and unlike the first experiment CD64 was even higher expressed than CD32 (Figure 4.8).



Figure 4.8: Flow cytometry determined expression of $Fc\gamma$ -receptors on monocytes. The expression of $Fc\gamma$ -receptors CD32 and CD64 in monocytes was conducted by flow cytometry using Alexa488-conjugated Donkey Anti-Mouse as secondary antibody. Monocytes were incubated with Fc-blocker and primary antibody CD32/CD64 (1:100) prior to detection and selected cells were stimulated with IFN- γ for 24 hours in advance. A/E: Gating of live monocyte populations allowing only live cells to be analyzed for fluorescence. B/F: Background (monocytes and secondary antibody only). C/G: Quantification of monocytes expressing CD32 stained with Anti-CD32 and Alexa488-conjugated secondary antibody. D/H: Quantification of monocytes expressing CD64 stained with Anti-CD64 and Alexa488-conjugated secondary antibody. Except for A/E, the X-axis represents the size of the cells and the Y-axis the fluorescence intensity of Alexa488.

Primary B-cells may also have uptake of immune complexes on the basis of their expression of Fc γ R's. To analyze this, we purified B-cells from PBMC by CD19⁺ Dynabeads[®]. The purified B cells were then cultured for 24 hours with or without the presence of IFN- γ . The B-cells expressed CD32 at quite high levels, even in the absence of IFN- γ , which only increased the expression slightly. Interestingly, however, the B cells did not express CD64 regardless of the presence or absence of IFN- γ (Figure 4.9).



Figure 4.9: Flow cytometry analysis of B-cells and their expression of Fcy-receptors. Purified B-cells was treated with Fc-blocker and Anti-CD32/64 prior to incubation with Alexa488-conjugated seconday antibody for fluorescence detection in flow cytometry. A/E: Gating of live B cell populations. B/F: Background (B-cells stained with secondary antibody only) C/G: Expression of CD32 receptor on B-cells. D/H: Expression of CD64 receptors on B-cells. The X-axis represents the size of the cells and the Y-axis the fluorescence intensity of Alexa488.

4.3.2.2 Ex-vivo ELISPOT measuring T-cell response in different populations of APC

After detection of $Fc\gamma Rs$ on the various cell types it was of interest to look at the T-cell responses with monocytes and B cells as APC. The effect of $Fc\gamma Rs$ in a T-cell response was determined for Priess cells and primary monocytes and B-cells.

Priess cells were incubated with $21OH \pm IgG$ to allow for uptake of the immune complex, and equal amounts of Priess cells and PBMC were cultured together for 24 hours in an ELISPOT assay. A significantly increased T-cell response was observed in the presence of 21OH/IgG immune complexes compared to 21OH alone (Figure 4.10).



Figure 4.10: Effect of immune complex-stimulated Priess cells in an *ex-vivo* **ELISPOT.** Priess cells were stimulated for 1 hour with IgG alone, 21OH alone, or 21OH/IgG immune complex prior to PBMC addition from patient 4. Increased T-cell response was significantly (p = 0.044) determined by a two-tailed paired T-test between the two groups with four parallels for each stimulation.

Purified monocytes (CD14⁺) were treated with IFN- γ (1 µg/ml) and cultured for 24 hours until stimulation with immune complex and Anti-CD32/64 to block respective Fc γ Rs. Autologous CD4⁺ T-cells were then isolated and added to the monocytes three hours after the addition of immune complexes. A considerable increased T-cell response was observed for patient 34 (Figure 4.11.A) stimulated with immune complex compared with 21OH alone, with a subsequently decreased response after Fc γ R blockage. For patient 7, no specific T-cell response against 21OH was observed, regardless of the presence of IgG (Figure 4.11.B).



Figure 4.11: Fc γ -receptor dependent increase in CD4⁺ T-cell responses against 21OH/IgG immune complexes using monocytes as APC. Purified monocytes were stimulated with IFN- γ for 24 hours to upregulate the expression of Fc γ -receptors, pulsed with 21OH or 21OH/IgG in the absence or presence of Anti-CD32/64, and used as APC for purified autologous CD4⁺ T-cells. A: Patient 34 showed an increased response to the immune complex and a correlated decrease due to blocking of Fc γ -receptors. However n.s. was determined between Blank and 21OH/IgG after a two-tailed paired T-test (p = 0.422) with three parallels per sample. B: For patient 7 no cellular responses above background against either 21OH or the immune complex were detected. Instead, a rather high response against IgG alone was noted.

A similar protocol as for monocytes was performed for B-cells with IFN- γ stimulation prior to addition of Anti-CD32/64 and immune complex before incubated with autologous CD4⁺ T-cells. Cells from one of the same patients as in the monocyte experiment (patient 7) were used. Unfortunately, again no specific responses against 21OH or the immune complex were detected for this patient (Figure 4.12).



Figure 4.12: No Fc γ -receptor dependent increase in CD4⁺ T-cell responses against 21OH/IgG immune complexes using B-cells as APC. B-cells from patient 7 were stimulated with IFN- γ for 24 hours, pulsed with 21OH and 21OH/IgG immune complex and used as APC with purified autologous CD4⁺ T-cells. Again, an apparent response against IgG alone was noted, but no specific responses against either 21OH or 21OH/IgG. Two-tailed paired T-test was n.s.

4.4 T-cell line and recognition of 210H peptides

4.4.1 Effect of stimulating PBMC with peptides of 210H

As we now had shown that immune complexes containing 21OH could be ingested into an APC to enhance T-cell immune responses, we next wanted to investigate whether the 21OHderived peptides presented by the APC is altered in the presence of immune complexes. Initially we used a panel of 21OH-derived peptides to detect peptides capable of invoking IFN- γ responses. *Ex-vivo* Elispot with PBMC was conducted as before and stimulated with single peptides or peptide pools of 21OH for 24 hours. For the analyzed patients the peptide pools p17-23, p31-38 and the peptide p34 gave a detectable T-cell response (Figure 4.13).



Figure 4.13: PBMC stimulated with 21OH-peptides for detection of possible T-cell activation by *ex-vivo* **ELISPOT.** PBMC was stimulated with peptides of 21OH for 24 hours and IFN- γ spots were counted by *ex-vivo* ELISPOT. A: Patient 25 was stimulated with p34 and the pool p31-38 (except p34) and three parallels per sample were used. A specific response was observed against both p34 and the pool p31-38. B: Patient 26 was stimulated with peptides covering almost the whole 21OH-peptide sequence, but p17-23 was the only peptide pool which gave a specific response. Three parallels per stimulation were analyzed.

In order to confirm the *ex-vivo* ELISPOT analysis an IFN- γ ELISA was conducted with supernatants from the stimulated PBMC from patient 26. ELISA gave similar results as ELISPOT with p17-23 being the only peptide pool inducing IFN- γ production in this patient (

Figure 4.14).



Figure 4.14: IFN- γ ELISA of supernatants from stimulation of immune complex and 21OH-peptides from the *ex-vivo* ELISPOT. Supernatants from *ex-vivo* ELISPOT (Figure 4.13) was incubated on a 96 well plate coated with Anti-IFN- γ capture antibody and detection of IFN- γ was performed by IFN- γ ELISA A notable increased level of IFN- γ was detected for peptide pool p17-23 which was over 6 folds above un-stimulated cells. IFN- γ (pg/ml) was calculated from a logarithmic standard curve.

4.4.2 T-cell lines induced by mature dendritic cells pulsed with 210H alone or in immune complex with IgG

In order to investigate if autoantibodies against 21OH could influence the processing and presentation of 21OH-derived peptides, we established short term T-cell lines using mature dendritic cells (DC) pulsed with 21OH alone, or 21OH/IgG immune complexes.

The resulting T-cell lines were then tested in a re-stimulation step with 21OH-derived peptides. A considerable increase in IFN- γ producing cells was detected for p34 and p17-23 for T-cell lines established using immune complex pulsed mature DC, indicating that 21OH-aab might be important for the processing and presentation of these peptides (Figure 4.15 A). ELISA was also performed using the supernatants from the re-stimulated T-cell lines. These results also show an increase in IFN- γ responses for T-cells stimulated by DC pulsed with 21OH and IgG autoantibodies (Figure 4.15 B).



Figure 4.15: Only 21OH-specific T-cell lines induced in the presence of 21OH-autoantibodies recognize peptides p34 and p17-23. Adherent PBMC were differentiated into mature dendritic cells (DC) and pulsed with 21OH \pm IgG. Autologous PBMC were then added in order to establish 21OH-specific short term T-cell lines. After 7 days these T-cell lines were tested against 21OH-derived peptides in an ELISPOT assay, using autologous PBMC as APC. A: Peptides spanning the whole 21OH-peptide sequence were tested but only p34 and p17-23 gave a raised T-cell response in the T-cell lines using ELISPOT. B: IFN- γ ELISA was performed by supernatants from the ELISPOT cultures, and showed the same results: Only T-cell lines raised against the 21OH/IgG immune complex could recognize peptides p34 and p17-23.

4.4.3 CD4⁺ T-cell lines induced by mature dendritic cells pulsed with 210H alone or in immune complex with IgG

An analogous experiment as outlined above (chapter 4.4.2) was performed, except that instead of using PBMC as the source of T-cells, purified CD4⁺ T-cells were used. Even though a broad T-cell response was detected, no increased T-cell responses were detected against any



peptides with CD4⁺ T-cell lines raised against 210H/IgG immune complexes compared to 210H alone (Figure 4.16).

Figure 4.16: No increase in T-cell responses of CD4⁺ T-cell lines was raised against 21OH/IgG immune complexes compared to 21OH alone. Adherent PBMC from patient 30 were differentiated into mature dendritic cells (DC) and pulsed with 21OH \pm IgG. Autologous CD4⁺ T-cells were then added in order to establish 21OH-specific short term T-cell lines. After 9 days, these T-cell lines were tested against 21OH-derived peptides in an Elispot assay, using autologous PBMC as APC. Two parallels per sample were used.

4.5 Measurement of T-cell cytotoxicity induced by CD8⁺ T-cells

After measurement of cytokine production in response to stimulation with 21OH, it would be interesting to look at another T-cell response, such as cytotoxicity. We therefore assessed the ability of cytotoxic T-cells raised against 21OH or 21OH/IgG immune complexes to kill target cells expressing 21OH endogenously, or pulsed with 21OH-derived peptides.

4.5.1 Detection of HLA-A2 expression in NCI-H295R

As effector and target cells must express the same HLA molecule in order to be used together in cytotoxicity assays, we performed immunofluorescence to determine the expression of HLA-A2 in NCI-H295R cells.

NCI-H295R cells were grown on coverslips and stimulated with IFN- γ (1 µg/ml) ahead of fixating of cells and staining for HLA-A2 expression. Cells stimulated with IFN- γ had a higher expression of HLA-A2 (C) compared to cells grown under basal conditions (B). Cells incubated with secondary antibody only (A) did not show any specific staining (Figure 4.17).



Figure 4.17: Detection of class I HLA-A2 in NCI-H295R target cells by immunofluorescence. NCI-H295R cells were grown on coverslips, fixed with 4 % formaldehyde and stained with mouse Anti-Human HLA-A2 (2 μ g and 1 μ g), followed by Alexa488-conjugated donkey Anti-Mouse. Cells treated with IFN- γ had a higher expression of HLA-A2 compared to basal conditions. A: Background, only cells and DAPI with 2 μ g primary antibody. B: Basal Anti-HLA-A2 with 2 μ g primary antibody. C: Anti-HLA-A2 + IFN-y (1 μ g/ml) with 2 μ g primary antibody. Blue = DAPI (nucleus), green = Alexa488 (HLA-A2).

Flow cytometry was also performed for detection of HLA-A2 in NCI-H295R cells for a more quantitative analysis. Priess cells also express HLA-A2 and were used as positive control (Figure 4.19). As expected the NCI-H295R cells expressed the HLA-A2 molecule and cells stimulated with IFN- γ in advance showed an increase in HLA-A2 expression (Figure 4.18).



Figure 4.18: Detection of HLA-A2 subclass on NCI-H295R target cells by flow cytometry. NCI-H295R cells were stained with FITC-conjugated mouse Anti-HLA-A2 antibodies for flow cytometry. A gate was set for unstained cells to detect HLA-A2 expressing cells. The X-axis represents the granulation of the cells and the Y-axis the fluorescence intensity of FITC.



Figure 4.19: Detection of HLA-A2 in Priess cells used as positive control in flow cytometry. Priess cells were stained with FITC-conjugated Mouse Anti-HLA-A2. The X-axis represents granulation of the cells and the Y-axis the fluorescence intensity of FITC.

4.5.2 Cytotoxicity induced by 210H-specific CD8⁺ T-cells

Prior to measuring the cytotoxicity produced by CD8⁺effector T-cells, the optimal triton concentration used as positive control and the number of target cells; NCI-H295R was determined. NCI-H295R cells were cultured to an adequate cell number and two levels of cell density were added various concentrations of Triton X-100 to find the best and most sensitive condition using LDH release as a marker for cytotoxicity.

The LDH release was detected for the cell density at $5*10^4$ cells with 0.1 % Triton. Despite of this the $2*10^4$ cell density was chosen because of the difficulty in work up of a sufficient number of CD8⁺ T-cells for the highest cell density of target cells (Figure 4.20).



Figure 4.20: Modification of cytotoxicity experiment measuring released LDH from NCI-H295R target cells. Two cell density populations of NCI-H295R were cultured and treated with various concentrations of Triton X-100 in order to modify the cytotoxicity method. Release of LDH into cell culture media was measured at a spectrometer at 490 nm. X-axis represents raised Triton concentrations and Y-axis the concentration of LDH released into the medium.

Again, we established short term T-cell lines using mature DCs pulsed with 21OH alone, or 21OH/IgG immune complexes. This time, however, purified CD8⁺ cells were used as the source of T-cells. The resulting T-cell lines were then tested in a LDH cytotoxicity assay with NCI-H295R cells as target cells. After modifications NCI-H295R cells ($2*10^4$ cells) were stimulated with IFN- γ (0.5 µg/ml) and then incubated with the cultured CD8⁺ T-cell lines for 4 hours. Supernatants were transferred to a Nunc-96 well plate with standards and the LDH content was measured by adding substrate and reading the absorbance by spectrometry at 490 nm (Figure 4.21).



Figure 4.21: Cytotoxic effect of CD8⁺ T-cells against NCI-H295R target cells. NCI-H295R cells were incubated with IFN- γ (0.5 µg/ml) to increase the HLA-I expression and used as target cells for CD8⁺ T-cells specific for 210H. The X-axis represents ratio between effector and target cells and Y-axis concentration of released LDH into cell culture medium. A: Patient 27 was pre-stimulated with IgG alone and IgG/210H. B: CD8⁺ T-cells from patient 28, stimulated with mature DCs pulsed with 210H/IgG autoantibody showed a slightly higher rise in cytotoxicity than those raised against 210H alone.

The cytotoxicity study was also performed with 21OH-peptide pulsed PBMC as target cells for CD8⁺ T-cell lines. CD8⁺ T-cell lines were established as described above, using mature DC pulsed with 21OH alone or 21OH/IgG immune complexes. Then, an autologous PBMC suspension pulsed with peptide pools of 21OH-derived peptides were used as target cells for the CD8⁺ T-cell lines in a 4 hour cytotoxicity assay as described above for the NCI-H295R cells (Figure 4.22).



Figure 4.22: Increased cytotoxicity of CD8⁺ T-cell lines against 21OH/IgG immune complexes compared to 21OH alone. Mature DC were pulsed with 21OH \pm IgG and used to establish CD8⁺ T-cell lines. Autologous PBMC were used as target cells after pulsing with 21OH-derived peptide pools (at 10 µg/ml) in a 4 hour LDH cytotoxicity assay with different ratios of CD8+ T-cells. The X-axis represents the stimulation of peptides in different effector:target cell ratios and Y-axis is LDH concentration released to cell culture medium calculated from the linear equation from the standard curve.

IFN- γ ELISA was also performed from the supernatants from the cytotoxicity study and the results correlated only partially with the data from the cytotoxicity assay (Figure 4.22) (results not shown).

5. Discussion

Addison's disease is an organ specific autoimmune disease thought to develop because of a cell mediated immune response against the adrenal cortex. Although autoantibodies against 21OH and the adrenal cortex are highly characteristic for the disease, their role in the pathogenesis is largely unknown. A previous study showed a possible involvement of 21OH-autoantibodies (21OH-aab) in the enhancement of T-cell responses against 21OH [40].

This project aimed to further investigate if 21OH-aab in Addison's patients enhance the uptake of 21OH into antigen presenting cells (APC) trough Fc γ -receptors (Fc γ Rs) and subsequently increase the activation of autoreactive T-cells. *Ex-vivo* T-cell responses from PBMC were determined after pre-stimulation with 21OH ± 21OH-aab. T-cell responses were also investigated in the presence of blocking antibodies against the Fc γ Rs and with purified subsets of APC. Furthermore, T-cell lines were established using mature DC pulsed with 21OH alone or in 21OH/IgG immune complexes. These T-cell lines were then compared by testing their reactivity against a panel of 21OH-derived peptides.

The main discovery was the clear enhancement of *ex-vivo* T-cell responses when stimulated with 21OH in the presence of autoantibodies. This enhancement seemed to be Fc γ R dependent. Furthermore, T-cell lines generated by mature DC pulsed with 21OH/IgG complexes showed an enhanced IFN- γ response against 21OH or 21OH-derived peptides, compared to T-cell lines generated by mature DC pulsed with 21OH.

5.1 Detection of 210H-autoantibodies

Addison's patients are characterized by the presence of serum antibodies against the selfantigen 21OH, exclusively expressed in the adrenal cortex. We therefore started with the detection of such autoantibodies in our patient cohort, using an indirect ELISA based on purified 21OH recombinant protein as described [51]. This assay clearly distinguished between plasma samples from patients and healthy controls (Figure 4.1), but also revealed quite heterogeneous levels of autoantibodies between patients. We therefore selected plasma samples that contained high levels of autoantibodies for IgG purification (Table 3).

Furthermore, Western blot analysis confirmed the presence of 21OH-aab in AAD plasma and its binding capacity to the 21OH enzyme. Various protein preparations containing 21OH, either recombinant or naturally expressed (Figure 4.2.A) were all recognized by patient plasma and a polyclonal control antibody against 21OH, but not by plasma from healthy

controls. No antibodies were detected against the negative control, sf9 insect cell lysates, which is an insect cell line used for production of recombinant 210H [51].

Subsequently, plasma with high levels of 21OH-aab, as detected by ELISA and Western blot, were used for the purification of IgG. Another Western blot analysis was performed to validate that 21OH binding capabilities were contained in the purified IgG fractions. Plasma and purified IgG showed the expected bonds around 54 kDa identifying 21OH-aab (Figure 4.2.B). Despite weak bonds observed for plasma without IgG, which demonstrates an incomplete depletion of IgG in plasma by the purification method used, stronger bonds were detected for purified IgG confirming a sufficient level of purified 21OH-specific IgG.

Actin or a similar control could have been used as a control for comparing the strength of the different bands from different samples (e.g. purified IgG vs. whole plasma vs. IgG depleted plasma). On the other hand the main purpose of this experiment was to determine the actual presence and binding capacity of 210H-aab to 210H in the purified IgG fractions. Also, equal amounts of 210H protein were loaded on the SDS-PAGE gels for each sample.

5.2 IFN-γ production enhanced by 210H-autoantibodies

Bratland et al [40] have previously reported a possible role for 21OH-aab in activating 21OHspecific autoreactive T-cells in AAD. It was therefore of great interest to take this a step further with a more sensitive method – *ex*-vivo ELISPOT. Cytokine production are often measured by ELISA due to cytokine release into cell culture medium [53] requiring an in vitro stimulation which will not give a direct analysis of PBMC [54]. ELISPOT is then 200 times more sensitive and have been used to determine cytokine profiles in e.g. T-cell subsets [55]. The ELISPOT assay used has previously been shown to clearly distinguish between AAD patients and healthy controls (unpublished, Bratland et al.).

In the present study, we also used more stringent criteria with a defined serum-free medium, which means that the only IgG molecules present were those purified from patient plasma. In the presence of 21OH-aab associated with 21OH in an immune complex, a significantly increased IFN- γ production was detected among PBMC (Figure 4.4.A), indicating that 21OH-aab have a significant role in activating 21OH-specific autoreactive T-cells.

Eight *ex-vivo* ELISPOT experiments were performed where seven AAD patients showed a specific T-cell response against 21OH in complexes with 21OH-aab (Figure 4.3). Even

though *ex-vivo* ELISPOT is among the most sensitive methods for these kinds of experiments, it may not be sensitive enough to detect frequencies lower than 1 cell per million PBMC.

Also, we used a very heterogeneous group of patients for these studies, with disease durations ranging from two months to over 40 years. At such long disease duration, remaining adrenocortical tissue is probably non-existent, and 21OH-specific autoreactive T-cells may be extremely infrequent. However, a subset of patients showed a clear response to 21OH and intriguingly these responses were further increased with presence of 21OH-aab.

5.3 Importance of Fcy-receptors in T-cell response

Fc γ Rs expressed on APC are thought to have a role in uptake of immune complexes by binding to the Fc-part of IgG [56, 57]. Interestingly, blockage of these receptors on PBMC prior to pre-stimulation with immune complexes led to a decreased IFN- γ production (Figure 4.6). Fc γ Rs mediate a 50-100 times more efficient antigen uptake [58] in contrast to antigen internalization, due to their low antigen requirement [59]. Thus, the amount of antigen required for a full T-cell activation may be considerably less when present as an immune complex. This could initiate a possible T-cell response mechanism in AAD due to low levels of 210H released out of the adrenal cortex to local lymph nodes to stimulate an immune response against 210H. 210H-specific autoantibodies produced by the resulting immune response might then migrate to the adrenal cortex and lead to a self-amplifying circuit and enhancement of the anti-210H T-cell response.

In order to decide which APC populations were involved in the enhanced T-cell response against immune complexes, the expression of the Fc γ R II (CD32) and I (CD64) on different APC populations was determined by flow cytometry. Priess cells, a B-cell line immortalized by Epstein-Barr virus infection, were shown to express CD32 receptors, but not CD64 (Figure 4.7).The Priess cells could also induce IFN- γ production by HLA matched PBMC, but only when pulsed with 210H as immune complex. No specific IFN- γ production was observed when 210H was added alone (Figure 4.10). This suggests that uptake of 210H is only possible via Fc γ Rs in Priess B-cells, as B cells are usually poor phagocytes [8].

The expression of CD32 and CD64 in monocytes were very low (Figure 4.8.C, D), but increased considerably after IFN- γ stimulation (Figure 4.8.G and H) indicating that IFN- γ is able to stimulate cells to up-regulate the expression of Fc γ Rs, which have been confirmed earlier [17, 60, 61]. Earlier studies have also seen expression of CD32 and CD64 in isolated monocytes where CD64 expression was significantly up-regulated in both monocytes and DC

due to IFN- γ stimulation for 24 hours [57]. IFN- γ stimulated monocytes also enhanced T-cell responses against 21OH when present as immune complex, but these responses decreased when blocking antibodies for the Fc γ Rs where present. The decrease was most abundant for the CD64 receptor but the CD32 receptor also seemed involved (Figure 4.12).

Previous studies [56] on B-cells have already shown expression of CD32 which correlates with our findings of expression patterns (Figure 4.9). B-cells were also tested in an *ex-vivo* ELISPOT to determine their antigen presenting capabilities of 21OH immune complexes in the absence and presence of blocking Fc γ Rs. Unfortunately, for the patient tested in this particular experiment, no detectable IFN- γ response against either 21OH alone or 21OH/IgG could be measured (Figure 4.12). Instead, there seemed to be an IFN- γ response against IgG alone, which was also the case with monocytes for this particular patient (Figure 4.11). This finding is peculiar, but may reflect T-cell responses against idiotypic determinants in the antigen-binding sites of IgG antibodies. Such T-cells have been described in other autoimmune diseases such as multiple sclerosis [62].

A decreased cell response due to blockade of $Fc\gamma Rs$ on B-cells have also been observed in a mouse model of autoimmune diabetes where the Anti-islet IgG autoantibodies may induce a $CD4^+$ T-cell mediated response eventually leading to disease [63].

To sum up, although based on few experiments, we can confirm a role in the induction of Tcell responses against 21OH/IgG immune complexes by monocytes. The uptake of the immune complexes may occur through both CD32 and CD64. However, a similar role of primary B-cells cannot be excluded due to their strong expression of CD32 receptors, which can be correlated to the experiments with the Priess B cells and also to other earlier studies [56]. These experiments therefore need to be repeated with patients that have a specific IFN- γ response to 21OH (either isolated or as immune complex) to determine the role of primary B cells.

To ensure the purities of the isolated APC subsets, flow cytometry could have been done ahead of the *ex-vivo* ELISPOT in use of Anti-CD19 for B-cells and Anti-CD14 for monocytes even though the purification methods we have used should give adequate purity.

5.4 T-cell line and recognition of 210H peptides

As we now had established that immune complexes indeed have a role in enhancing T-cell responses to 210H, we wanted to investigate whether the state of 210H, e.g. alone or as part

of immune complexes, influence the epitopes of 210H presented by APC. Again using *exvivo* ELISPOT, we initially performed a screen with PBMC from patients with known HLA types against a panel of peptides of the 210H sequence. The peptides were pooled with seven and eight peptides in each pool. As p34 (210H aa430-447) is a known epitope for 210H and have been identified as a great inducer of IFN- γ production [64] it was used alone to investigate its stimulating capabilities in our patients. As expected, p34 was one of the activating peptides of 210H together with the pools p31-38 (-34) and p17-23 (Figure 4.13). In order to confirm the *ex-vivo* ELISPOT analyzes an IFN- γ ELISA was conducted with supernatants from the stimulated PBMC cultures. Even in use of two different methods in measuring cytokine production the same peptides of 210H proved to elicit specific IFN- γ production (

Figure 4.14).

In order to investigate if the stimulating peptides described above represented naturally processed and presented peptides from 21OH, and if 21OH-containing immune complexes could influence the presentation of the peptides, we established T-cell lines using mature DC pulsed with 210H alone or 210H in immune complex. The resulting lines were then tested against a panel of peptides comprising the whole 21OH sequence. As expected, p34 gave an increased T-cell response together with p17-23 (Figure 4.15.A), but only for the T-cell line primed with the immune complex. In addition, this T-cell line produced considerably more IFN-y to 210H immune complex, than the T-cell lines raised against 210H alone. This could indicate that the 21OH-aab facilitate the natural processing and presentation of immunogenic 210H peptides when 210H is phagocytized as an immune complex. IFN-y ELISA was also conducted for this experiment showing the same trend with no response to peptides for T-cell lines raised against 21OH alone, but high responses for T-cell lines raised against immune complexes (Figure 4.15.B). However, the trend for each peptide was different between the two methods indicating that p17-23 activated T-cells produce more IFN-y per cell than for cells stimulated with p34. This may reflect phenotypical differences between the responding T-cells (e.g. $CD4^+$ vs. $CD8^+$).

Several studies that have looked into T-cell lines have determined an increased T-cell response against immune complex compared to stimulation with antigen alone, indicating that antigens are ingested into APC in an antibody dependent manner [65-67].

Purified CD4⁺ T-cells were also used in a similar experiment as above, in order to establish CD4⁺ T-cell lines. However, no differences between the two T-cell lines could be detected, even though a broad and robust response against many peptides was noted (Figure 4.16).We speculate that this could be due to a technical error by not allowing an adequate incubation time with the substrate for the final development step in the ELISPOT assay. However, it could also be a natural variation reflecting the heterogeneous pattern observed previously for the AAD patients.

5.5 Measurement of T-cell cytotoxicity by CD8+ T-cells

After measurement of cytokine production it would be interesting to look at another T-cell response parameter, such as cytotoxicity, and whether the state of 21OH when given to APC, e.g. alone or as part of immune complexes, could influence the action of 21OH-specific cytotoxic CD8⁺ T-cells. 21OH-specific CD8⁺ T-cell lines were established as above, using mature DC pulsed with 21OH alone or 21OH as part of immune complexes with 21OH-aab`s. As target cells we used the adrenocortical carcinoma cell line NCI-H295R, which expresses 21OH endogenously, or autologous PBMC pulsed with 21OH peptides. We knew from previously that the NCI-H295R cells carry the HLA A*0201 genotype which encodes the HLA A2 molecule (Professor SR Bornstein, University of Dresden, personal communication). This genotype is very common in the Western world, and is carried by 26.5 % of the Norwegian AAD patients [52].

Since the NCI-H295R cells are cancer cells, we first wanted to make sure that their expression of HLA A2 at the protein level was normal. Immunofluorescence showed a normal expression of HLA-A2 in NCI-H295R cells (Figure 4.17). Furthermore, a higher expression of HLA-A2 was seen after IFN- γ stimulation, meaning that the gene expression of HLA class I could be influenced by cytokine stimuli (Figure 4.17 C). This difference in staining intensity was evident even though less primary antibody (1 µg) were used for IFN- γ treated cells than cells cultured under basal conditions (2 µg).

Flow cytometry was also conducted for NCI-H295R cells with conjugated antibody to confirm the immunofluorescence analyses, and revealed the same pattern with rather high basal levels which could be driven even higher in the presence of IFN- γ (Figure 4.18). As Priess cells are known to express HLA-A2 they were used as positive control (Figure 4.19). These analyses confirm that NCI-H295R is suitable as target cells for HLA A2 restricted cytotoxic CD8⁺ T-cells.

LDH was used as marker for cytotoxicity due to its release into cell culture medium by dying cells. This is a fast and simple assay, and a harmless alternative to more conventional cytotoxicity assays based on radioactive isotopes, such as the chromium release assay [68] which has been a standard assay for CTL-measurements [15]. A modification of the cytotoxicity analysis was done were two levels of NCI-H295R cell density was treated with various concentrations of Triton X-100 to find the best and most sensitive condition. The highest cell death was detected for the cell density at $5*10^4$ cells with 0.1 % Triton. Despite this the $2*10^4$ cell density was chosen because of the difficulty in work up enough CD8⁺ T-cells for the highest cell density of target cells (Figure 4.20).

Patients with HLA-A2 genotype were chosen and CD8⁺ T-cell lines were established. These were incubated with NCI-H295R target cells (2*10⁴) in different ratios to determine a possible increase due to more cytotoxic T-cells. T-cell lines from both patients investigated increased LDH concentrations with higher CD8⁺ T-cell ratios; however the response was generally very low, making it hard to determine if the immune complexes containing 21OH played a significant role in inducing cytotoxic 21OH-specific CD8⁺ T-cells. One of the T-cell lines from patient 27 was by mistake raised against IgG alone instead of 21OH, but as this T-cell line differed only marginally from 21OH/IgG induced T-cell lines in its cytotoxic potential, we may conclude that no specific cytotoxicity was induced in these experiments (Figure 4.21). Other studies have used higher ratios of cytotoxic T-cells, up to 50:1 [69] and 100:1 [70] (compared to 10:1 in the present study) and this should be tested out in future studies. A more sensitive flow cytometry based method has also been established in our laboratory. This method uses differential fluorescent staining of live and dead cells ([10]), but due to time limitations we were not able to include this method in the present study.

CD8⁺ T-cell lines were also raised from another patient against 21OH alone, or 21OH/IgG. All peptides of 21OH gave an increase in LDH activity, but a differential pattern was evident when comparing T-cell lines raised against 21OH alone vs. 21OH/IgG (Figure 4.22). Taken together, these data indicate that also CD8⁺ cytotoxic T-cells may be influenced by the state of which 21OH (alone or as part of immune complexes) is ingested by APC. A similar finding has previously been reported for the autoimmune disease primary biliary cirrhosis [66].

5.6 Future perspectives

AAD is a rare disease with a poorly understood pathogenesis. In order to devise novel treatment options and possibly prevent the disease, a much more detailed understanding of the

molecular mechanisms is needed. Compared to related but more common autoimmune endocrine diseases like type 1 diabetes, very little research on cellular autoimmunity have been performed on AAD.

As each experiment in this thesis was only performed on few patients, and the individual differences between patients are clear in some instances, the most interesting experiments should be reproduced. In particular IFN- γ stimulated B-cells with blockade of Fc γ Rs need to be repeated with other AAD patients to draw a respective conclusion. Even though monocytes gave an increased IFN- γ response against 210H in presence of 210H-aab, these studies should also be repeated in a larger patient cohort.

Due to the unsuccessful LDH measurements with a too low cellular response, perhaps because of the low target and effector cell ratio, the new flow cytometry method mentioned above should be used. Hopefully, this method should give a more specific determination between live and dead target cells. Purified CD8⁺ T-cells should be cultured in order to generate a maximum expansion of 21OH-specific cells, allowing for a higher ratio of effector:target cells. This could be accomplished by pulsing with a higher IL-2 dose than we have used in our studies, in order to increase the CD8⁺ proliferation [71].

For an even more specific experimental set-up 21OH-specific autoantibodies might have been isolated from autologous plasma of patients, [66] instead of using total IgG which contain several antibodies other than 21OH-specific IgG. Due to time limitations, however, we were not able to isolate such purified 21OH-aab for the present thesis.

5.7 Conclusion

21OH in immune complex with IgG autoantibodies showed a clearly enhanced T-cell response compared to 21OH alone in Addison's patients. Monocytes were involved in such an increased T-cell response with a clear effect in the uptake of immune complex through their Fc γ -receptors. However, a similar role for B-cells cannot be excluded based on our current data. Furthermore, the autoantibodies also seem to be able to modulate which peptides of 21OH that are processed and presented to T-cells by APC.

To conclude, 21OH-autoantibodies may have an important role in the pathogenesis of AAD.

6. Appendix

6.1 210H sequence

>sp |P08686 |CP21A_HUMAN Steroid 21-hydroxylase OS=Homo sapiens GN=CYP21A2 PE=1 SV=1

MLLLGLLLLPLLAGARLLWNWWKLRSLHLPPLAPGFLHLLQPDLPIYLLGLTQKFGPI YRLHLGLQDVVVLNSKRTIEEAMVKKWADFAGRPEPLTYKLVSKNYPDLSLGDYSL LWKAHKKLTRSALLLGIRDSMEPVVEQLTQEFCERMRAQPGTPVAIEEEFSLLTCSIIC YLTFGDKIKDDNLMPAYYKCIQEVLKTWSHWSIQIVDVIPFLRFFPNPGLRRLKQAIE KRDHIVEMQLRQHKESLVAGQWRDMMDYMLQGVAQPSMEEGSGQLLEGHVHMA AVDLLIGGTETTANTLSWAVVFLLHHPEIQQRLQEELDHELGPGASSSRVPYKDRARL PLLNATIAEVLRLRPVVPLALPHRTTRPSSISGYDIPEGTVIIPNLQGAHLDETVWERPH EFWPDRFLEPGKNSRALAFGCGARVCLGEPLARLELFVVLTRLLQAFTLLPSGDALPS LQPLPHCSVILKMQPFQVRLQPRGMGAHSPGQNQ

6.2 210H peptide sequence Provided by Professor Vincenzo Cerundolo at University of Oxford. Peptide pools used in experiments are displayed in different colors under.

1	MLLLGLLLLPLLAGARLL	
2	GARLLWNWWKLRSLHLPP	
3	LHLPPLAPGFLHLLQPDL	
4	LQPDLPIYLLGLTQKFGP	
5	QKFGPIYRLHLGLQDVVV	
6	QDVVVLNSKRTIEEAMVK	
7	EAMVKKWADFAGRPEPLT	
8	PEPLTYKLVSKNYPDLSL	
9	PDLSLGDYSLLWKAHKKL	
10	AHKKLTRSALLLGIRDSM	
11	IRDSMEPVVEQLTQEFCE	
12	QEFCERMRAQPGTPVAIE	
13	PVAIEEEFSLLTCSIICY	
14	SIICYLTFGDKIKDDNLM	
15	DDNLMPAYYKCIQEVLKT	
16	EVLKTWSHWSIQIVDVIP	
17	VDVIPFLRFFPNPGLRRL	
18	GLRRLKQAIEKRDHIVEM	
19	HIVEMQLRQHKESLVAGQ	
20	LVAGQWRDMMDYMLQGVA	
21	LQGVAQPSMEEGSGQLLE	
22	GQLLEGHVHMAAVDLLIG	
23	DLLIGGTETTANTLSWAV	
24	LSWAVVFLLHHPEIQQRL	
25	IQQRLQEELDHELGPGAS	
26	GPGASSSRVPYKDRARLP	
27	RARLPLLNATIAEVLRLR	
28	VLRLRPVVPLALPHRTTR	
29	HRTTRPSSISGYDIPEGT	
30	IPEGTVIIPNLQGAHLDE	
31	AHLDETVWERPHEFWPDR	
32	FWPDRFLEPGKNSRALAF	
33	RALAFGCGARVCLGEPLA	
34	GEPLARLELFVVLTRLLQ	
35	TRLLQAFTLLPSGDALPS	
36		
	DALPSLQPLPHCSVILKM	
37	DALPSLQPLPHCSVILKM VILKMQPFQVRLQPRGMG	

7. References

- 1. Pietropaolo, M., et al., *Primer: immunity and autoimmunity*. Diabetes, 2008. **57**(11): p. 2872-82.
- 2. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts.* N Engl J Med, 2000. **343**(1): p. 37-49.
- 3. Medzhitov, R. and C. Janeway, Jr., *Innate immunity*. N Engl J Med, 2000. **343**(5): p. 338-44.
- 4. Kruisbeek, A.M., Introduction: regulation of T cell development by the thymic microenvironment. Semin Immunol, 1999. **11**(1): p. 1-2.
- 5. Harwood, N.E. and F.D. Batista, *Early events in B cell activation*. Annu Rev Immunol, 2010. **28**: p. 185-210.
- 6. Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system*. Science, 2002. **296**(5566): p. 298-300.
- 7. Abbas, A.K., Lichtman, Andrew H and S. Pillai, *Cellular and molecular immunology*2010: Saunders Elsevier.
- 8. Clark, M.R., et al., *Molecular mechanisms of B cell antigen receptor trafficking*. Ann N Y Acad Sci, 2003. **987**: p. 26-37.
- 9. Klein, J. and A. Sato, *The HLA system. First of two parts.* N Engl J Med, 2000. **343**(10): p. 702-9.
- 10. Mbitikon-Kobo, F.M., et al., *Ex vivo measurement of the cytotoxic capacity of human primary antigen-specific CD8 T cells.* J Immunol Methods, 2011.
- 11. Delves, P.J. and I.M. Roitt, *The immune system. Second of two parts.* N Engl J Med, 2000. **343**(2): p. 108-17.
- 12. Love, P.E. and S.M. Hayes, *ITAM-mediated signaling by the T-cell antigen receptor*. Cold Spring Harb Perspect Biol, 2010. **2**(6): p. a002485.
- 13. Lea, T., *Immunologi og immunologiske teknikker*2002, Bergen: Fagbokforlaget.
- 14. Van Parijs, L. and A.K. Abbas, *Homeostasis and self-tolerance in the immune system: turning lymphocytes off.* Science, 1998. **280**(5361): p. 243-8.
- 15. Schmittel, A., U. Keilholz, and C. Scheibenbogen, *Evaluation of the interferon*gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. J Immunol Methods, 1997. **210**(2): p. 167-74.
- 16. Hickling, J.K., *Measuring human T-lymphocyte function*. Expert reviews in molecular medicine, 1998. **1998**: p. 1-20.
- 17. Gattoni, A., et al., *Interferon-gamma: biologic functions and HCV therapy (type I/II)* (1 of 2 parts). Clin Ter, 2006. **157**(4): p. 377-86.
- 18. Mosmann, T.R. and S. Sad, *The expanding universe of T-cell subsets: Th1, Th2 and more*. Immunol Today, 1996. **17**(3): p. 138-46.
- 19. Zhu, J. and W.E. Paul, *CD4 T cells: fates, functions, and faults.* Blood, 2008. **112**(5): p. 1557-69.
- 20. Garrett-Sinha, L.A., et al., *PU.1 and Spi-B are required for normal B cell receptormediated signal transduction.* Immunity, 1999. **10**(4): p. 399-408.
- 21. Haas, A., K. Zimmermann, and A. Oxenius, *Antigen-dependent and -independent mechanisms of T and B cell hyperactivation during chronic HIV-1 infection.* J Virol, 2011. **85**(23): p. 12102-13.
- Porter, R.R., *Structural studies of immunoglobulins*. Science, 1973. 180(4087): p. 713-6.
- Kamradt, T. and N.A. Mitchison, *Tolerance and autoimmunity*. N Engl J Med, 2001. 344(9): p. 655-64.

- 24. Fierabracci, A., *Recent insights into the role and molecular mechanisms of the autoimmune regulator (AIRE) gene in autoimmunity.* Autoimmun Rev, 2011. **10**(3): p. 137-43.
- 25. Peterson, P., T. Org, and A. Rebane, *Transcriptional regulation by AIRE: molecular mechanisms of central tolerance*. Nat Rev Immunol, 2008. **8**(12): p. 948-57.
- 26. Fierabracci, A., Unravelling autoimmune pathogenesis by screening random peptide libraries with human sera. Immunol Lett, 2009. **124**(1): p. 35-43.
- 27. Marrack, P., J. Kappler, and B.L. Kotzin, *Autoimmune disease: why and where it occurs*. Nat Med, 2001. **7**(8): p. 899-905.
- 28. Lovas, K. and E.S. Husebye, Addison's disease. Lancet, 2005. 365(9476): p. 2058-61.
- 29. Boe, A.S., et al., Autoantibodies against 21-hydroxylase and side-chain cleavage enzyme in autoimmune Addison's disease are mainly immunoglobulin G1. Eur J Endocrinol, 2004. **150**(1): p. 49-56.
- 30. Turnbull, A.V. and C.L. Rivier, *Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action.* Physiol Rev, 1999. **79**(1): p. 1-71.
- 31. Arlt, W. and B. Allolio, Adrenal insufficiency. Lancet, 2003. 361(9372): p. 1881-93.
- 32. Bratland, E. and E.S. Husebye, *Cellular immunity and immunopathology in autoimmune Addison's disease*. Mol Cell Endocrinol, 2011. **336**(1-2): p. 180-90.
- 33. Martin Martorell, P., B.O. Roep, and J.W. Smit, *Autoimmunity in Addison's disease*. Neth J Med, 2002. **60**(7): p. 269-75.
- 34. Betterle, C., et al., Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev, 2002. **23**(3): p. 327-64.
- 35. Winqvist, O., F.A. Karlsson, and O. Kampe, 21-Hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet, 1992. **339**(8809): p. 1559-62.
- 36. Wolff, A.S., et al., Autoimmune polyendocrine syndrome type 1 in Norway: phenotypic variation, autoantibodies, and novel mutations in the autoimmune regulator gene. J Clin Endocrinol Metab, 2007. **92**(2): p. 595-603.
- 37. Erichsen, M.M., et al., *Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry.* J Clin Endocrinol Metab, 2009. **94**(12): p. 4882-90.
- 38. Betterle, C. and L. Morlin, *Autoimmune Addison's disease*. Endocr Dev, 2011. **20**: p. 161-72.
- 39. Rosenthal, F.D., M.K. Davies, and A.C. Burden, *Malignant disease presenting as Addison's disease*. Br Med J, 1978. **1**(6127): p. 1591-2.
- 40. Bratland, E., et al., *T cell responses to steroid cytochrome P450 21-hydroxylase in patients with autoimmune primary adrenal insufficiency*. J Clin Endocrinol Metab, 2009. **94**(12): p. 5117-24.
- 41. Undlien, D.E., et al., *No independent associations of LMP2 and LMP7 polymorphisms with susceptibility to develop IDDM*. Diabetes, 1997. **46**(2): p. 307-12.
- 42. Novak, E.J., et al., *Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4+ T cell epitopes from complex antigens.* J Immunol, 2001. **166**(11): p. 6665-70.
- 43. Uibo, R., et al., *Autoantibodies to cytochrome P450 enzymes P450scc, P450c17, and P450c21 in autoimmune polyglandular disease types I and II and in isolated Addison's disease.* J Clin Endocrinol Metab, 1994. **78**(2): p. 323-8.
- 44. Furmaniak, J., et al., *Immunoprecipitation of human adrenal microsomal antigen*. FEBS Lett, 1988. **231**(1): p. 25-8.
- 45. Husebye, E.S. and K. Lovas, *Immunology of Addison's disease and premature ovarian failure*. Endocrinol Metab Clin North Am, 2009. **38**(2): p. 389-405, ix.

- 46. Higashi, Y., et al., *Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene.* Proc Natl Acad Sci U S A, 1986. **83**(9): p. 2841-5.
- 47. Coco, G., et al., *Estimated risk for developing autoimmune Addison's disease in patients with adrenal cortex autoantibodies.* J Clin Endocrinol Metab, 2006. **91**(5): p. 1637-45.
- 48. van de Winkel, J.G. and P.J. Capel, *Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications.* Immunol Today, 1993. **14**(5): p. 215-21.
- 49. Nimmerjahn, F. and J.V. Ravetch, *Fcgamma receptors as regulators of immune responses*. Nat Rev Immunol, 2008. **8**(1): p. 34-47.
- 50. Falorni, A., et al., *Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands.* J Immunol Methods, 1995. **186**(1): p. 89-99.
- 51. Bratland, E., et al., *The purification and application of biologically active recombinant steroid cytochrome P450 21-hydroxylase: the major autoantigen in autoimmune Addison's disease.* J Autoimmun, 2009. **33**(1): p. 58-67.
- 52. Skinningsrud, B., et al., *Multiple loci in the HLA complex are associated with Addison's disease*. J Clin Endocrinol Metab, 2011. **96**(10): p. E1703-8.
- Chang, T.W., et al., Use of monoclonal antibodies as sensitive and specific probes for biologically active human gamma-interferon. Proc Natl Acad Sci U S A, 1984. 81(16): p. 5219-22.
- 54. Schmittel, A., et al., *Application of the IFN-gamma ELISPOT assay to quantify T cell responses against proteins.* J Immunol Methods, 2001. **247**(1-2): p. 17-24.
- 55. Miyahira, Y., et al., *Quantification of antigen specific CD8+ T cells using an ELISPOT assay.* J Immunol Methods, 1995. **181**(1): p. 45-54.
- 56. Rabinovitch, N. and E.W. Gelfand, *Expression of functional activating and inhibitory Fcgamma receptors on human B cells.* Int Arch Allergy Immunol, 2004. **133**(3): p. 285-94.
- 57. Fanger, N.A., et al., *Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells.* J Immunol, 1996. **157**(2): p. 541-8.
- 58. Inoue, Y., et al., Activating Fc gamma receptors participate in the development of autoimmune diabetes in NOD mice. J Immunol, 2007. **179**(2): p. 764-74.
- 59. Gosselin, E.J., et al., *Enhanced antigen presentation using human Fc gamma receptor* (monocyte/macrophage)-specific immunogens. J Immunol, 1992. **149**(11): p. 3477-81.
- 60. Basham, T.Y. and T.C. Merigan, *Recombinant interferon-gamma increases HLA-DR* synthesis and expression. J Immunol, 1983. **130**(4): p. 1492-4.
- 61. Steeg, P.S., H.M. Johnson, and J.J. Oppenheim, *Regulation of murine macrophage Ia antigen expression by an immune interferon-like lymphokine: inhibitory effect of endotoxin.* J Immunol, 1982. **129**(6): p. 2402-6.
- 62. Holmoy, T., B. Vandvik, and F. Vartdal, *T cells from multiple sclerosis patients recognize immunoglobulin G from cerebrospinal fluid*. Mult Scler, 2003. **9**(3): p. 228-34.
- 63. Silva, D.G., et al., Anti-islet autoantibodies trigger autoimmune diabetes in the presence of an increased frequency of islet-reactive CD4 T cells. Diabetes, 2011. **60**(8): p. 2102-11.
- 64. Rottembourg, D., et al., 21-Hydroxylase epitopes are targeted by CD8 T cells in autoimmune Addison's disease. J Autoimmun, 2010. **35**(4): p. 309-15.
- 65. Allen, J.S., et al., *Plasmacytoid dendritic cells are proportionally expanded at diagnosis of type 1 diabetes and enhance islet autoantigen presentation to T-cells through immune complex capture.* Diabetes, 2009. **58**(1): p. 138-45.

- 66. Kita, H., et al., *Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells.* J Exp Med, 2002. **195**(1): p. 113-23.
- 67. Benitez-Ribas, D., et al., *Plasmacytoid dendritic cells of melanoma patients present exogenous proteins to CD4+ T cells after Fc gamma RII-mediated uptake.* J Exp Med, 2006. **203**(7): p. 1629-35.
- 68. Roder, J.C., et al., *A new immunodeficiency disorder in humans involving NK cells*. Nature, 1980. **284**(5756): p. 553-5.
- 69. Longhi, M.S., et al., Cytochrome P450IID6-specific CD8 T cell immune responses mirror disease activity in autoimmune hepatitis type 2. Hepatology, 2007. **46**(2): p. 472-84.
- 70. Skowera, A., et al., *CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope.* J Clin Invest, 2008. 118(10): p. 3390-402.
- 71. Salio, M., et al., *Mature dendritic cells prime functionally superior melan-A-specific CD8+ lymphocytes as compared with nonprofessional APC.* J Immunol, 2001. **167**(3): p. 1188-97.